RISK PREDICTION AND AN INJECTABLE COLLAGEN MATERIAL FOR INTERVERTEBRAL DISC DEGENERATION

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DEDICATION

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Clap for the heavyweight champ, **ME**. But I couldn't do it all alone, **WE.**

~Nicki Minaj

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RISK PREDICTION AND AN INJECTABLE COLLAGEN MATERIAL FOR INTERVERTEBRAL DISC DEGENERATION

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ABSTRACT

This research primarily focuses on early prediction and treatment for intervertebral disc degeneration (IVDD). In Phase 1, machine learning algorithms were evaluated to predict the risk of intervertebral disc degeneration in patients. This was done by using factors associated with IVDD and taken from patient medical history. Several classification algorithms were utilized to develop predictive models. Results demonstrated that machine learning algorithms could be used to predict IVDD risk and also the potential for developing an app from these predictive models.

Phase 2 focused on the development of a collagen-based, gold nanoparticle material for intervertebral disc regeneration. Gold nanoparticles were conjugated to viscoelastic collagen using a natural crosslinker, genipin. This material was then characterized to evaluate its ability to serve as a treatment for chronic back pain caused by IVDD. Results demonstrated successful attachment of the gold nanoparticles to the collagen using the genipin crosslinker. Overall, the characterization studies of the collagen composite were successful and demonstrated potential for further application in IVDD treatment.

Chapter One

Significance of Research

1. Introduction

The broad focus of this research project centers around the field of precision medicine. Precision medicine is an area garnering much attention because of the potential benefits to patients and the combination of different fields of technology, science, statistics, data science, and medicine for these patients. The NIH Precision Medicine Initiative defines precision medicine as an emerging approach to evaluate and utilize an individual's variability in environment, lifestyle, and genes, to develop disease preventions and treatments [1]. This method is different than past approaches in that it considers the differences of individuals for specific disease treatment and prevention strategies, instead of developing strategies based on the average person or a "one size fits all". By studying the differences in individual patients, doctors, and researchers can more accurately predict the risk of patients for specific diseases, as well as utilizing this information to determine what treatment is the best option.

Specifically, this dissertation research primarily focuses on early prediction and treatment for intervertebral disc degeneration (IVD). A disease known to be a common cause of chronic low back pain [2]. Lower back pain is the second most common cause of disability and doctor visits in the U.S [3]. This condition contributes $20–$100 billion in direct health care spending, with the total economic cost equaling $100-$200 billion annually [3].

This research is an interdisciplinary project between the fields of Data Analytics and Biomedical Engineering. By merging these two disciplines of engineering, medical decisions and treatment can be customized or tailored based on the patient's medical history and genetics, making life easier and less of an economic burden by those affected. This project is divided into two phases of research: prediction and treatment. The first phase will utilize predictive analytics and machine learning to better determine the risk of patients for disc degeneration. Phase 2 investigates the use of an injectable material as a template for intervertebral disc degeneration.

1.1 Phase 1

There is a need for prediction of disc degeneration in its earlier stages before surgery is required or disability occurs. This project evaluates the use of machine learning algorithms to predict the risk of intervertebral disc degeneration in patients using factors associated with IVD. This can lead to a reduction in the number of patients receiving spinal fusion surgery by proactively making lifestyle changes (weight loss, change occupation or eating habits) or receiving less intense treatment options.

1.2 Phase 2

The second area of my research focuses on the development of an injectable material as a template for intervertebral disc regeneration. By identifying risk factors associated with disc degeneration, these predictive factors can also be utilized to help design biomaterials tailored towards patients. Many of the current treatments are limited to only alleviating the symptoms temporarily, but do not restore the structure of the disc. If pain persists, major surgery such as spinal fusion may be required. It is hypothesized that this novel viscoelastic material to regenerate the nucleus pulposus will be developed

to modulate inflammation, serve as a chemoattractant, increase water content, and improve the overall biocompatibility.

The remainder of this dissertation is structured as follows: Chapter 2 provides a background on the intervertebral disc and disc degeneration, discusses data analytics in healthcare, and provides insight on past and current research on treatment for IVDD. Chapter 3 investigates Phase 1 and provides details of the investigative study for developing predictive models for intervertebral disc degeneration risk. Chapter 4 presents the details of the experimental study for Phase 2, which consists of developing and characterizing an injectable collagen material for disc degeneration. Chapter 5 discusses a study utilizing classification algorithms to predict the length of stay for animals in shelters to reduce euthanization. Finally, Chapter 6 will discuss possible future directions.

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Chapter 2

Literature Review

1. Intervertebral Disc

1.1 The Intervertebral Disc

1.1.1 Location and Function of the Disc

The intervertebral disc is a vital component of the spinal cord, having a largely mechanical role and serving as a shock absorber from the impact of daily activities [1]. The disc separates the vertebral bodies of the spine and accounts for 20-30% of the spine length, with the size of the disc increasing from the cervical to the lumbar region [2]. The intervertebral disc (IVD) provides protection for the spine and functions like a ligament by keeping the vertebrae together [3]. The IVD also provides limited flexibility to the body trunk and allows the spine to twist, bend, and rotate; serving as a "pivot point" [3]. The disc provides mechanical stability during axial compression and motion and prevents the spine from undergoing excessive motion. There are 23 discs that separate the vertebrae in the spine, with six discs in the cervical region, twelve discs in the thoracic, and five discs in the lumbar region [4]. The intervertebral discs increase in size from the cervical to the lumbar region and are approximately 7-10 mm in thickness [5]. The discs in the lumbar region are approximately 4 cm in diameter and around 1 cm in height [5]. There are 3 main components that make up the structure of the intervertebral disc: the gel-like nucleus pulposus (NP), the fibrous annulus fibrosus (AF), and the cartilage endplate [6].

1.1.2 Components of the Disc

Nucleus Pulposus

The nucleus pulposus is the hydrophilic, gelatinous center of the IVD and functions to resist larger compressive loads [7]. It is high in water content and the pressurized swelling allows the NP to resist compressive loading and redistribute those forces evenly within the spine [8]. The major extracellular matrix components of the nucleus pulposus include proteoglycans embedded within the collagen fibrils, with aggrecan being the most abundant proteoglycan [9,10]. The proteoglycans that make up the extracellular matrix (ECM) of the NP are negatively charged molecules that aid in increasing water content, allowing for retention of water and maintaining the swelling of the NP [11]. Proteoglycans are glycoproteins composed of at least one negatively charged glycosaminoglycans (GAG) such as chondroitin sulfate, which is covalently attached to a protein core such as aggrecan [12]. The high concentration of the negatively charged glycosaminoglycans present increases the osmotic pressure of the NP, providing its hydrostatic pressure [13].

Collagen type II is the prominent collagen type present in the NP, is structured sporadically, and aids in entrapping/embedding proteoglycans [14]. The loose network of type II collagen highly present in the NP begins to decrease in concentration closer to the annulus fibrosus, where type 1 collagen is more prevalent [15]. The notochordal cells are the early cells of the NP and are derived from the embryonic notochord [16]. Notochordal cells are present at birth but eventually disappear during early childhood [17]. The role of notochordal cells is not well established, but several theories suggest they form the NP directly and differentiate into chondrocytes, or they regulate cell movement and

proteoglycan synthesis [18]. These cells are eventually replaced by the sparse population of chondrocyte-like cells, which are what remain through maturity [17]. The disappearance of the notochordal cells is thought to be associated with early degeneration of the disc [19]. When maturity is reached, the cell density of the NP is around 4000 cells/mm3 [20].

Annulus Fibrosus

The annulus fibrosus is the structure that surrounds the nucleus pulpous and is separated from it by what is called the "transition zone" [21]. The AF serves to encase and restrain the nucleus pulposus during loading [22]. Proteoglycans, collagen type 1, and elastin fibers are the main components of the AF, with proteoglycans only making up a small percentage of the AF and collagen making up 2/3 of the composition [23]. Collagen type 1 becomes more prevalent and higher in concentration moving towards the outer AF. The collagen has an oriented lamellar (15-25 concentric rings of collagen) structure that is organized and densely packed [24]. Fibroblast-like cells are the dominant cell present in the AF, and the cellular density is roughly 9000 cells/mm3 [25].

Cartilage Endplate

The third region of the disc is the cartilage endplate, which is composed of hyaline cartilage and fibrocartilage [26]. The endplate has a horizontal thin layer of hyaline cartilage that is > 1mm, which joins the vertebrae and the disc [27]. Proteoglycans and collagen fibers are the main compositions of the CEP extracellular matrix [27]. The cartilage endplate surrounds the disc anteriorly and superiorly and separates the IVD from the spinal vertebrae, serving to equalize loading between the vertebra and IVD [28]. The IVD is mostly avascular and aneural in a healthy adult disc

[29]. Blood vessels surround the cartilage endplate and AF to provide the nucleus pulposus with limited nutrients such as glucose and oxygen and to transport and remove waste such as lactic acid [30].

1.2 Intervertebral Disc Degeneration

Degenerative disc disease is marked by the degeneration of the disc, causing or leading to chronic back pain. Degeneration of the disc has several changes to the disc environment, including cellular, biological and mechanical alterations. It is important to note that disc degeneration is a complex process, and the structural and compositional alterations that occur during aging and degeneration are still not well established. The pathogenesis of disc degeneration is complex, and researchers are tackling understanding the mechanism of IVDD.

1.2.1 Biological Changes

In the earliest stages, the water content of the nucleus pulposus is around 80-90% but decreases to around 70% in adulthood [31]. The negatively charged aggrecan molecules, especially the GAG, generate high osmotic pressures that aid in the hydration of the NP. A significant change identified in disc degeneration is the loss and degradation of aggrecan and GAG [32]. A shift in GAG chain composition from chondroitin sulfate to keratan sulfate occurs as well as the rapid accumulation of aggrecan fragments [33]. This leads to a decrease in osmotic pressure and loss of hydration as well as altered biomechanics [33, 34]. An increase in fibronectin is also observed in degenerated discs [35]. Studies have demonstrated that in vitro, fibronectin has been shown to downregulate aggrecan synthesis and upregulate MMPs [35]. Change in collagen type and distribution also occurs as degeneration progresses. The production of type II collagen present in the

NP decreases, and a shift into the synthesis of collagen type I occurs [36]. As degeneration progresses, this change in collagen synthesis leads to an increase in stiffness, annulus lamellar disorganization, and the demarcation or transition zone between the AF and NP becomes less defined [36].

While the outer area of the annulus fibrosus has vascularization, the inner region of the intervertebral disc is avascular [37]. Moving closer to the center of the disc, the concentration of glucose, oxygen, and other vital nutrients decreases [38]. The cells must have a viable level of nutrients to remain active, and any factors that affect the concentration of nutrients in the area could have negative effects. Factors that can impair the nutrient supply include endplate calcification, endplate damage, microvascular diseases such as sickle cell anemia, atherosclerosis, and smoking [39]. Calcification of the cartilage endplate causes a lack of blood supply to the nucleus pulposus by blocking and inhibiting diffusion of the nutrient supply from the capillaries that run through the endplates to the nucleus pulposus [40]. Calcification of the endplate has also been shown to limit the availability of growth factors to the disc, causing an increase in the rate of cell senescence in the disc [40]. Xiao et al. (2020) suggest degeneration of the cartilage endplate is observed before intervertebral disc degeneration occurs [41]. Lack of nutrient supply can also cause a reduction in cell availability [42].

Research has shown the association between cell senescence of the disc and the development of degenerative disc disease [43]. Cell senescence involves the irreversible cell cycle arrest or the process in which cells cease to divide and undergo alterations in phenotypes [44]. Moreover, the senescent cells have a reduced ability to function and production of the ECM decreases. Size increase, flattening and vacuolization, and cell

clustering are morphological changes displayed by in vitro senescent cells [44]. There are various stresses and mechanisms that can be a cause of cellular senescence, including external stimuli or telomere uncapping. It is still not fully understood the exact cause of senescence in IVD cells as it is thought to be cause dependent, though aging has shown to accelerate senescence of stem cells [45].

Studies have also demonstrated the presence of TNF-α in degenerated discs [46]. This inflammatory mediator is thought to be involved in the process of cellular senescence by promoting senescence marker p53 and p16 and ECM destruction by upregulating MMP-3 and MMP-13 [47]. It has also been found that during degeneration, there exists a shift in the balance between matrix anabolic and catabolic pathways in the ECM towards catabolism [48]. An imbalance exists between low rates of production and high rates in the breakdown of the ECM [48]. Oxidative stress is also thought to influence disc degeneration, and NP cells are a major source of reactive oxygen species (ROS) in the disc [49]. Oxidative stress involves the imbalance between the production and elimination of ROS [50]. Essentially, there is an overproduction of ROS in the cells and the antioxidative systems are unable to neutralize them. This imbalance causes damage to lipids, DNA, proteins and triggers the release of inflammatory signals leading to chronic inflammation [51]. The harsh environment stemming from lack of nutrient supply to disc cells and suppression of waste removal due to endplate calcification is suggested to be a trigger for excessive ROS production in the disc [52]. Research has shown a correlation between high ROS levels in the disc and increased progression of disc degeneration and activation of signal pathways leading to cell senescence [52]. Studies also show apoptosis, autophagy, and necrosis of IVD cells [53].

1.2.2 Mechanical Changes

These changes discussed above, such as alteration of the ECM composition, loss of hydration, and lack of nutrient supply hinder the disc's ability to resist compression and respond to increased loading, causing alterations to the biomechanics of the disc. Stokes et al. (2004) discuss the two contrasting mechanical conditions of overloading and immobilization [54]. The author compares mechanical overload causing disc injury versus hypomobility of the disc leading to adaptive changes causing tissue weakening and pain [54]. Hing et al. (1983) describes that a degenerated disc in the early stages initially has increased flexibility and results in hypermobility [55]. This increased range of motion, however, eventually leads to a limited range of motion and then finally in the later stages of disc degeneration, tissue stiffness, and hypomobility [55]. Another hypothesis suggests that the nucleus pulposus is unable to function as needed, leading to increased pressure and loadbearing from the nucleus pulposus onto the annulus fibrosus tissues causing injury [56]. Stress peaks appear in the posterior AF, which may be the cause of the damage inflicted on the AF and the loss of pressurization of the NP [57]. Studies have also demonstrated that when degeneration of the NP occurs, an increase in shear modulus is observed, and there is a decrease in relative energy dissipation, effective aggregate modulus, and swelling pressure [57]. One of the most notable changes in disc degeneration is the loss of disc height [58]. There are numerous degenerative causes that can be associated with loss of disc height, such as lack of NP pressurization or dehydration and damage to the cartilage endplate and AF.

It has also been observed that mechanical loading influences disc metabolism, specifically compressive loading influencing cell turnover and viability [59]. Some

mechanical stimulation is needed for matrix synthesis; however, cell viability and matrix synthesis are both sensitive to compressive stress and excessive loading can injure local tissue [60]. Those that undergo underloading, overloading or static loading for prolonged periods are suggested to be more susceptible to disc degeneration [61].

1.3 Back Pain

Intervertebral disc degeneration has proven to be a common cause of chronic lower back pain [62]. Lower back pain is a leading cause of disability, doctor's visits, and lost time at work and has a lifetime incident of around 80% [63]. This means that at some point in their lives, a large percentage of the population has an episode of back pain. Lower back pain contributes $20–$100 billion in direct health care spending, and the total economic cost equals $100-$200 billion annually [64, 65]. Approximately 11.9% of the population worldwide is estimated to be affected at any time by lower back pain, and 2-10% of that low back pain will become chronic [66]. It is estimated that the prevalence of chronic back pain is 23% [67]. Discogenic back pain is a common cause of chronic low back pain and has an incidence of 39% [68]. Discogenic pain is a condition where the source of back pain comes from the intervertebral discs. Back pain of the lumbar spine can be classified into two areas; leg dominant and back dominant [69].

1.3.1 Back Dominant Pain

Back Dominant Pain is mechanical in nature, with the source of pain usually coming from a structure within the spine [69]. Pain is triggered or aggravated by certain movements or changes to the spinal position. The exact mechanism leading to painful discs and why some discs are painful, and others are not is still not fully understood.

1.3.2 Pathway of Discogenic Pain

As composition and structure alterations occur with degeneration, the biomechanics of the disc shift as well. The shift of load bearing from the NP to the AF has a cascade effect, leading to injury of the annulus fibrosus. The AF serves to restrain or contain the swelling pressure of the NP and connect the adjacent vertebrae. When compressive loading is placed on the AF, annular fissures or tears in the AF occur, triggering the wound healing response of the body [70]. Tears and fissures of the AF lead to pathological changes such as decreased cellularity, inflammation, ECM degeneration, innervation and vascularization, and granulation tissue formation [71].

Growth factors such as the multifunctional transforming growth factor-beta (TGF-β) are important for promoting tissue repair and wound healing for the AF. Studies have shown that while TGF-β may promote collagen type II and proteoglycan synthesis, it may also promote abnormal changes to the disc, such as abnormal cell differentiation and matrix fibrosis [72, 73]. TGF-β also aids in the attraction of tissue repair and inflammatory cells to the wounded AF region [74, 75].

When the AF is injured, the body will recruit immune cells such as macrophages, T cells, and neutrophils to the wounded area, and these cells may also infiltrate or migrate to the NP at the center of the disc [76]. This can lead to an increase of immune cells secreting inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin eight (IL-8), and interleukin 1 (IL-1) in the NP [77, 78]. Studies have also found there is an increase in MMP production and a decrease in tissue inhibitors of matrix metalloproteinases, leading to degradation of the ECM of the disc's NP [79]. As the body tries to heal the injured AF, new blood vessel formation and nerve growth into

the nucleus pulposus occurs [80]. This becomes a problem as a healthy disc is mostly avascular and poorly innervated. Studies have demonstrated that in painful discs, neurovascular invasion from the AF into the NP via annular fissures is observed [81]. However, in nonpainful discs, the innervation and vascularization are restricted to the outer AF. Blood vessels are present in the outermost layers of the annulus fibrosus in a healthy IVD [82].

In a healthy and sparsely innervated IVD, the nerve fibers (sensory and postganglionic sympathetic) are located peripheral to the annulus fibrosus [83]. However, in a degenerated disc, hyperinnervation into the NP is observed along with the granulation tissue [84]. Though the pathology of discogenic pain is still not fully understand, current theory suggests an association between back dominant pain and innervation and vascularization. Kao et al. (2014) found that the AF injury leads to the increased expression of neutrophins, which have been shown in previous studies to regulate the density and distribution of nerve fibers [83]. Nerve growth factors and inflammatory mediators such as TNF-α and IL-1β have been shown to increase neutrophin expression, leading to an increase in nerve growth and distribution [85, 86]. The increase in neutrophin production may also increase chemical factors such as histamine, bradykinin, and vasodilators that may irritate the nociceptive receptors on nerve fibers that have infiltrated to the center of the disc, causing activation of pain pathways [87, 88]. Peng et al. (2013) classified discogenic pain into two types: annular disruption-induced low back pain (IAD) and internal endplate disruption-induced low back pain (IED) [89].

1.3.3 Other Forms of Back Dominant Pain

Back Dominant Pain: Facetogenic

Facetogenic pain can be caused by degeneration and loss of the cartilage of the facet joints. This deterioration of cartilage is known as facet's disease or facet joint syndrome [90]. Arthritis, degeneration, or trauma lead to the deterioration of the cartilage, resulting in inflammation and pain [91].

Back Dominant Pain: Vertebrogenic Pain

The cartilage endplate must have enough strength to withstand mechanical load while remaining porous enough to allow chemical transport. With aging, the composition of the cartilage endplate changes. A decrease in proteoglycans, water, and type 1 collagen occurs, resulting in calcification and thinning of the cartilage endplate [92, 93]. This increases the endplate's susceptibility to damage, especially under tensile forces. This damage and disruption to the cartilage endplate cause circumferential fissures to appear, resulting in innervation into the endplate [94]. While the pathology of vertebrogenic pain is still not fully understood, some researchers believe that the nociceptive receptors on these nerve fibers become stimulated chemically and mechanically, causing pain [94].

1.3.4 Leg Dominant Pain

Leg dominant pain involves compression of the spinal root that radiates pain to the lower extremities [95]. Common conditions that are associated with leg dominant pain are disc herniation and lumbar stenosis. Radicular pain is the term associated with leg dominant pain, where the pain is triggered by mechanical compression, inflammation, or injury to the spinal nerve root [96]. Radiculopathy or sciatica is a common symptom of radicular pain, where pain radiates down the sciatic nerve to the thigh, calf, and foot.

Leg Dominant Pain: Disc Herniation

In disc herniation, degenerated fragments of the nucleus pulposus may break off and escape the confinement of the annulus fibrosus [97]. When this occurs, these broken pieces compress on the spinal nerve root. Researchers suggest that pressure on nerve root alone cannot be the only cause of pain [98]. A common theory is that for symptomatic individuals, molecules coming from the inflammatory cascade (ex. arachidonic acid through to interleukins and MMPs) causes the nerves to become more sensitized to pressure [99].

Leg Dominant Pain: Spinal Stenosis

In lumbar spinal stenosis, a narrowing of the space within the spinal canal or the space narrowing around the nerve roots is observed. Narrowing of space can occur in the area under the facet joints or in the neural foramina [100]. This narrowing of the spinal canal is associated with disc degeneration and arthritis of the facet joints. Stenosis acquired from degeneration is the most observed type of stenosis [100]. Pain stems from compression on spinal nerves as well as from bone spurs that may also be present.

2. Prediction

2.1 Data Analytics

In the business world, companies are also taking advantage of vast amounts of data collected for better business decisions and as a competitive advantage [101]. Data has grown, computers have become more powerful, and algorithms are being developed to allow deeper analysis of data, which has led to the emergence of the field of data science [101]. Data science is a field of study utilizing qualitative and quantitative techniques to better understand and gather information from large volumes of data to

make predictions [102]. Extracting this information from large data sets requires the data to undergo cleansing, preparation, and finally analysis. In data analysis, data will be examined and arranged to uncover meaningful information leading to an outcome or conclusion based on a prior hypothesis. Data analytics is a subset of data analysis as well as a field of Business Intelligence (BI) [102]. It involves discovering patterns and obtaining information from raw data using an algorithmic or mechanical process [103]. Data analytics can be divided into three areas: descriptive analytics, predictive analytics, and prescriptive analytics [104].

2.2 Predictive Analytics

Machine Learning involves programming computers on training experiences to learn and improve on its own [105]. These computer systems should learn from and process large amounts of data to make predictive outcomes [106]. The system developed should analyze big data and quickly deliver accurate and repeatable results, as well as be able to adapt to new data independently [106]. Systems are trained by learning from examples of desired or known input-output data to make accurate predictions [107]. Solving these problems utilizing a system involves the use of algorithms, which are a sequence of instructions that when carried out, transform the input to the output. Predictive analytics combines techniques from data mining, statistics, machine learning, and computer science [108]. These various areas are combined to predict unknown future events from patterns by observing the relationship between the attribute variables (input) and target variables (output) [109]. Machine learning algorithms are utilized to detect these patterns and develop models to predict future outcomes [110].

As technology becomes more advanced, there is a dramatic increase in the amount of clinical data made available electronically as the U. S healthcare system adopts and adapts from written files to electronic healthcare records [111]. As this occurs, there is a growing trend of utilizing predictive systems in health care [112]. More specifically, using analytics to predict risk for diseases and adverse events such as infection and drug events [113]. Amin et al. (2019) identified which features are significant in improving the accuracy of predictive models to determine risk for heart disease [114]. Zheng et al. (2017) utilized machine learning algorithms to more accurately and efficiently predict which patients have Type 2 Diabetes Mellitus from electronic health records [115]. Austin et al. (2013) investigated which classification techniques would provide the best performing model in the prediction and classification of heart failure subtypes [116].

3. Treatment

3.1 Current Treatments

Current noninvasive treatments for back pain include rest, epidural steroid injections, physical therapy, and nonsteroidal anti-inflammatory drugs [117]. While these methods and medications may treat the symptoms of back pain, the underlying problem of degeneration remains. If the pain becomes severe and debilitating, major surgeries such as spinal fusion may be required. However, spinal fusion relieves pain but doesn't restore the function of the disc [118].

In spinal fusion, the disks or vertebrae are fused permanently using bone grafting materials, allografts, autografts, or a synthetic material substitute [119]. Metal screws and plates are also used to fuse the bones. Though this surgery is being performed, there are numerous problems that can occur after surgery, such as the persistence of back pain

[120]. Another problem is the weakening and wear of other disks and vertebrae that are around or next to the fusion. Studies conducted by Park et al. (2004) demonstrated the accelerated development of adjacent segment disease and disc degeneration in the areas adjacent to the spinal fusion [121]. Studies have also noted changes to the biochemistry of the adjacent segments, including an increase in morbidity, pseudarthrosis, and failed back syndrome [121, 122]. There are also the risk factors that arise from any major surgery, such as infection and damage to spinal nerves and tissue surrounding the area. These numerous risks have led researchers to explore less invasive treatments.

3.2 Future Treatments

3.2.1 Disc Regeneration

Current research in the areas of anti-inflammatory drugs, gene therapy, and biological therapies are being investigated for the treatment of IVD degeneration [123]. Several branches of biological therapy, protein-based and cell-based, may provide a new type of treatment in the future. In protein-based therapy, the research aims to increase the production of the ECM and proteoglycans, reduce inflammation, and improve mechanical stability and water retention in the degenerated disc [124]. The use of anti-catabolic and pro-anabolic proteins, as well as growth and differentiation factors, have been investigated through intradiscal injections [125].

Cell-based therapy evaluates the application of various stem cells such as mesenchymal stem cells, chondrocytes, nucleus pulposus derived stem cells, and human umbilical cord-derived stem cells for regeneration [126]. Mesenchymal stem cells are the most widely investigated due to previous studies demonstrating their synergism in vivo with nucleus pulposus cells leading to an increase in extracellular matrix production

[126]. Mesenchymal stem cells have also demonstrated the ability to differentiate into other cell types [127]. Current studies are investigating growing, embedding, and encapsulating stem cells into a material to prevent their migration to non-targeted areas. Gan et al. (2017) evaluated cell-based regeneration through the encapsulation of nucleus pulposus derived cells in an interpenetrating network hydrogel [128]. Reported results demonstrated the ability of the hydrogel to rehydrate the nucleus pulposus and maintain cell survival long term. Tsaryk et al (2015) analyzed a hydrogel composed of low molecular weight hyaluronic acid and polymerized collagen for nucleus pulposus regeneration [129]. Gelatin microspheres containing mesenchymal stem cells, nasal chondrocytes, and TGF-β3 were embedded within the hydrogel. Results demonstrated the hydrogel supported the growth and chondrogenic differentiation of the MSCs, ease of injection of the hydrogel, and biocompatibility.

3.2.2 Disc Replacement

Researchers are currently exploring the use of artificial disk replacements as a viable method in treating chronic pain in the cervical, thoracic, and lumbar regions of the spine. This can involve either the complete replacement of the intervertebral disc or the replacement of the nucleus pulposus. Prosthetics, metal alloys, hydrogels, polymers, and synthetic materials are considered as viable replacement materials [130]. The use of artificial disks has had little success due to the complex nature of the IVD's structure and function [131]. Prosthetics have not had complete success due to the challenges of developing a sufficient size prosthetic, trouble matching the mechanical properties of the intervertebral disc, and the inability to mimic the range of motion of a normal spine [131].

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Chapter Three

Developing Predictive Models and a User App for Risk of Intervertebral Disc Degeneration

Section 1: Predictive Models

Abstract

Background: Intervertebral disc degeneration (IVD) has been associated with causing chronic back pain. Lower back pain is a common cause of doctor's visits and disability and affects 11.9% of the worldwide population. The total direct healthcare spending caused by lower back pain is $20-100 billion. The goal of this study is to utilize classification algorithms to predict the patient risk for intervertebral disc degeneration.

Results: The classification algorithms utilized in this study include logistic regression, decision tree, artificial neural network, boosting, bagging, and random forest. The SMOTE method was performed on the data set and then inputted into clustering and classification algorithms to develop the predictive models. Results demonstrated that the performance of the models improved after k means clustering. All models showed an increase in performance across all five-performance metrics. The bagging algorithm developed the best performing model. Results also demonstrated an increase in the performance of the models when the SMOTE method was applied, as well as when ensemble methods were utilized for classification algorithms.

Conclusion: The finding from this study can be utilized to develop a predictive tool for the early detection of disc degeneration. Future studies include acquiring more data from healthcare facilities across the United States to further develop these predictive models.

Keywords: Intervertebral disc degeneration; classification algorithms; SMOTE; clustering

1. Introduction

Intervertebral Disc (IVD) is a major component of the spine serving as a shock absorber from daily activities [1]. The IVD separates the vertebrae of the spine and serves as a pivot point to allow twisting, bending, and rotating of the spine [2]. Intervertebral discs also account for 20-30 of the spinal length. Intervertebral disc degeneration (IVDD) is marked by degenerative biological, cellular, chemical, and biomechanical changes to the disc, leading to chronic back pain. Disc degeneration is a common cause of chronic lower back pain and is a common reason for doctor's visits, disability, and lost work time [3]. $20-100 billion in direct healthcare spending can be contributed to lower back pain [4]. With 11.9% of the population estimated to be affected by lower back pain, the total economic burden equals $100-200 billion annually [5,6].

Predictive analytics combines techniques from areas such as data mining, statistics, and computer science to predict unknown future events from patterns by observing the relationship between the attribute variables (input) and target variables (output) [7, 8]. Machine learning algorithms are utilized to detect these patterns and develop models to predict future outcomes [9].

There has been a dramatic increase in the amount of clinical data made available electronically as the U. S healthcare system adopts and adapts from written files to electronic healthcare records [10]. The increase in available electronic data has led to a growing trend of utilizing predictive systems in health care [11, 12]. More specifically, machine learning algorithms are utilized to predict risk for diseases and adverse events such as infection and drug events [13]. Amin et al. (2019) evaluated predictive models for heart disease risk and identified which features are significant in improving the accuracy of these models [14]. Zheng et al. (2017) investigated the use of machine learning algorithms to improve accuracy and efficiency when predicting which patients have Type 2 Diabetes Mellitus from electronic health records [15]. Austin et al. (2013) investigated which classification techniques would provide the best performing model in the prediction and classification of heart failure subtypes [16].

Research has shown that there is a need for earlier prediction of intervertebral disc degeneration [17]. The goal of this study is to provide a tool to aid in earlier detection of disc degeneration using predictive analytics. Models will be developed to predict the risk for intervertebral disc degeneration as early as a patient's first doctor's visit for back pain. The models will result in early monitoring, and pro-active treatment such as lifestyle changes (change in occupation, exercise, less invasive methods) before degeneration becomes severe enough to require major surgery. This goal will be accomplished by first observing and acquiring data from a patient's medical history to identify factors associated with the disease. For this study, factors associated with disc degeneration are the input variables and risk for disc degeneration is the output variable. Next, machine learning will be used by inputting the data from these factors into

classification algorithms to develop a predictive model. The system will then be tested to determine the performance of the predictive model. Finally, a comparison between the predictive models from each classification algorithm will be conducted to determine which model will most accurately predict the correct outcome for patients. We are the first, to our knowledge, to apply machine learning algorithms to predict the risk of patients for intervertebral disc degeneration. Figure 1 provides a diagram of the steps for developing predictive models.

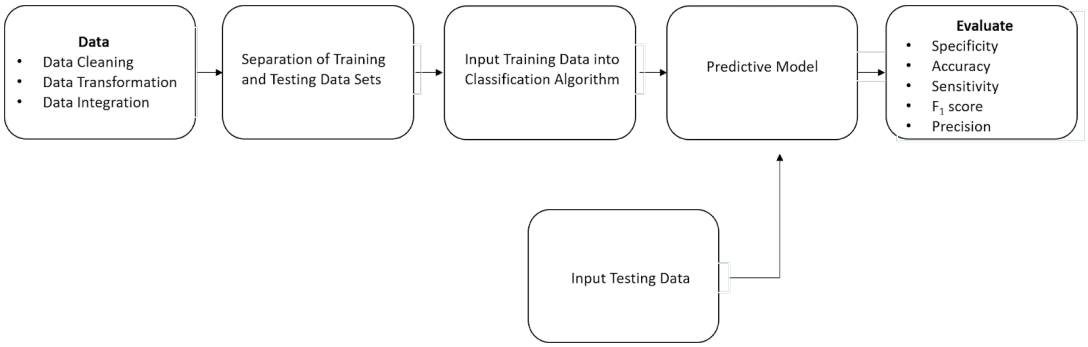

Figure 1: Diagram of steps for developing predictive models

2. Materials and Methods

2.1 Risk Factor

In machine learning and predictive analytics, it is important to determine factors that influence or are associated with the outcome that is to be predicted. These input variables are called predictive factors. A literature review was conducted to determine factors associated with disc degeneration and Table 1 provides the findings from the search.

Table 1: Risk Factors for Disc Degeneration

Number	Factor/Attribute Variable
1	Smoking[18]
2	Diabetes/Diabetes Mellitus[19]
3	Atherosclerosis[20]
4	Sickle Cell Anemia[21]
5	Caisson's Disease[22]
6	Gaucher's Disease[23]
7	Genetics/Hereditary[24]
8	Obesity[25]
9	Occupation[26]
10	Age[27]

2.2 Data

2.2.1 Data Acquisition

Once the literature search was complete, next came the process of data retrieval for the predictive factors listed in Table 1. Approval was obtained from the Institutional Review Board (IRB) to gain access to de-identified patient medical history data from the Missouri Orthopaedic Institute (MOI). A data analyst from the University of Missouri's

School of Medicine was then consulted to procure the de-identified patient medical history from the MOI. A search was also conducted to determine the health codes (ICD-9 and EC) of the associated risk factors which are listed in Table 1. Over 2 million de-identified data points were received from the MOI. Two data sets were procured which consisted of data points on patients with and without disc degeneration. The factors that were able to be collected for each patient are listed in Table 2. Once the data was acquired, the data set underwent transformation, integration, and data cleaning.

Table 2: Health Care Codes for Risk Factors

Factors	**Healthcare Codes**
Diabetes	ICD9-250
Obesity	ICD9-278
Weight	EC-279
Height	EC-178
Smoking/Nicotine Exposure	ICD9-305.1 and V15.82
Other Infection	ICD9-999.3
Atherosclerosis	ICD9-440.20
Sickle Cell Disease	ICD9-282.6
Occupation	Data directly given
Spinal Cord Injury	ICD9-952.2
Age	Data directly given
Sex	Data directly given

2.2.2 Data Cleaning Methods

Data cleaning methods were utilized to detect discrepancies in the data set, such as missing values, erroneous data, and inconsistencies. In order to help ensure that the data being utilized is unbiased, data cleaning methods are an important step in the data analysis process. It is important to identify and clean erroneous data before inputting it into the algorithms as it can significantly impact the output results.

The following is a list of commonly used data cleaning techniques in the literature. [28]

Substitution with a Unique Value: Replace erroneous data with values that are outside of the range that the input variables can accept (ex. negative number).

Substitution with Median: Replace missing or incorrect data with the median value for that input variable.

Discard Variable and Substitute with a Median: If a predictor variable has a significant number of missing values, the values are removed from the data set, and the missing or erroneous data that remains for other features are replaced with the median.

Discard Variable and Substitute with a Unique Value: Remove from the data set input variables with a significant number of missing values and the features that remain with missing or erroneous data are coded as -1.

Remove Incomplete Rows Entirely: Incomplete rows are removed from the data set.

2.2.3 Data Preprocessing

Once the data was received, the data set underwent data transformation to match the healthcare codes with the specific predictor variable (ex. ICD9-282.6 represents diabetes) for each patient. A pivot table was constructed to match the patient number with corresponding healthcare codes and to summarize the number of times the patient

appeared for each factor or healthcare code. When the number was greater than or equal to 1, a 1 was inputted for each factor for that patient. When the number was less than 1, a 0 was inputted. This represented whether that patient did or did not have that factor. Then the data sets for patients with and without disc degeneration were integrated. The MeSH age range definitions were utilized to categorize the age range of patients into classes [29]. Age of patients was categorized as an infant (1 year or less), child (2-12), adolescent (13-18), young adult (19-24), adult (25-44), middle-aged (45-64), senior (65-79), and elder (80-90). Next, we evaluated what the patient wrote in for occupation and assigned it to 1 of 19 categories. Table 3 describes these 19 categories and their impact on the spine. Restoration Chiropractic was consulted to determine the impact of each occupation category on the spine. The impact was classified as low, medium, or high. For infants and children, the impact on the spine was categorized as low. Finally, the output variable was the risk for disc degeneration. If the patient had disc degeneration, the risk was categorized as high and low for patients without disc degeneration.

Table 3: Description of Occupation Categories

Occupation Categories	**Description**	**Impact Category**
Armed Forces	Army, navy, etc.	High
Automotive	Mechanic, auto shop	High
Construction	Construction, landscaping, etc.	High
Disabled	Disabled	Medium
Education	Daycare, professor, public school, etc.	Medium

Farmer	Farmer	High
Food Services	Restaurant, grocery store, etc.	High
Government	Government building, etc.	Medium High: mail carrier
Hospital	Nurse, hospital, etc.	High
Human Services	Banking, physical therapy, cleaning service, etc.	Medium High: carrier, cleaning service
Laboratory	Laboratory	Medium
Manufacturing	Manufacturing company, etc	High
Retired	Retired	Medium
Self-Employed	Self-employed	Low
Sports	Coach, dance, etc.	Medium High: Dance
Student	Student	Medium
Technology	IT service, technology company, etc.	Low
Trucking	Trucking companies	High
Unemployed	Unemployed	Medium

After receiving the de-identified patient data from the MOI Institute, the data points were integrated, leading to a sample size of over 52,000 patient records. Next, the process of data transformation and data cleaning was performed. After evaluation of these data points, 14,644 data records were found to have missing values for factors such as age, height, and gender. After applying data cleaning techniques, the final data set includes 38,118 data points. In this cleaned data set, there were 357 patients with IVDD and 37,761 patients with no intervertebral disc degeneration. For the patients with IVDD, 175 were males and 182 were females. It was also discovered that 320 patients diagnosed with IVDD were 30 years or older and 180 patients with IVDD that were 50 years and older. Table 4 describes the final input variables and variable type.

Table 4: Factor Description

Variable	Description	Variable Type
Diabetes	Patients with and w/o Diabetes	Categorical
Sex	Patient Sex (Female and Male)	Categorical
Impact	Impact of Occupation on the Spine. (low, medium, high)	Categorical
Atherosclerosis	Patients with and w/o Atherosclerosis	Categorical
Spinal Cord Injury	Patients with and w/o Spinal Cord Injury	Categorical
Age Group	Age of patient (infant, child, adolescent, young adult, adult, middle aged, senior, elder)	Categorical
Occupation	Occupation of Patient	Categorical
Height	Height of Patient	Numerical
Other Infection	Infection following an injection or spinal fusion	Categorical
Obesity	Patients diagnosed with obesity	Categorical
Sickle Cell Anemia	Patients with and w/o sickle cell anemia	Categorical

Spinal Fusion	Patients with and w/o spinal fusion	Categorical
Smoking	Patients who smoke	Categorical
Risk	Patients with and w/o disc degeneration	Categorical

2.3 Exploratory Data

In Figure 2, the distribution of the age groups for the data set of patients with and without disc degeneration is displayed. At 35.6%, the middle-aged category had the highest number of patients, followed by the adult category at 26.9% and the senior category at 17.7%. The least amount of data points came from the infant category at 1%. Figure 3 shows the gender distribution for the entire data set of patients with and without disc degeneration. 54.4% of the patients are female and 45.6% are male.

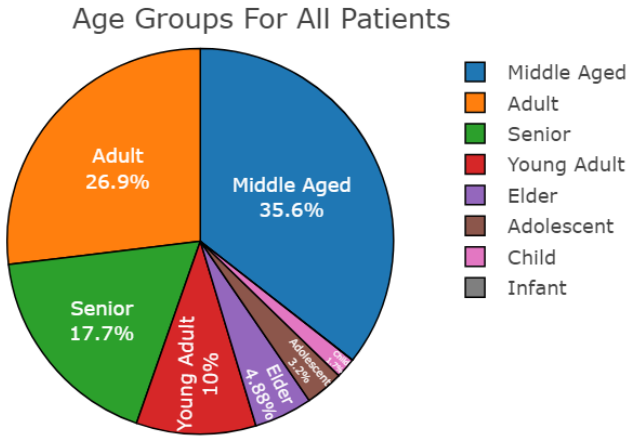

Figure 2: Distribution of Age Groups for All Patients

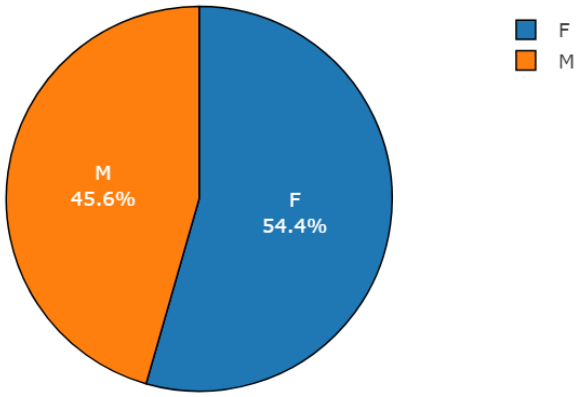

Figure 3: Distribution of Sex Groups for All Patients

From Figure 4, it is evident that the middle-aged category at 42% has the highest number of patients at high risk, followed by adults at 31.1% and the senior age category at 17.9%. This is also similar to the results from Figure 2. Figure 5 shows a comparison between occupation and risk for patients with disc degeneration. This graph displays the distribution of high risk for each occupation for patients with disc degeneration. Observing the results, patients that were unemployed had the highest risk for disc degeneration at 59 patients. The human services category had the next highest number of patients at high risk at 57, followed by retired patients at 35 and patients involved in education at 33. Figure 6 evaluates which impact category (low, medium, and high) had the highest number of patients that are at high risk for disc degeneration. Patients with occupations that were medium impact on the spine had the highest number of patients at high risk, with high impact on the spine following next.

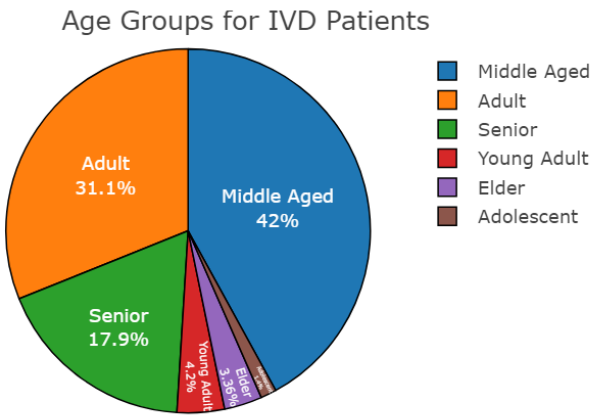

Figure 4: Distribution of Age Groups for IVD Patients

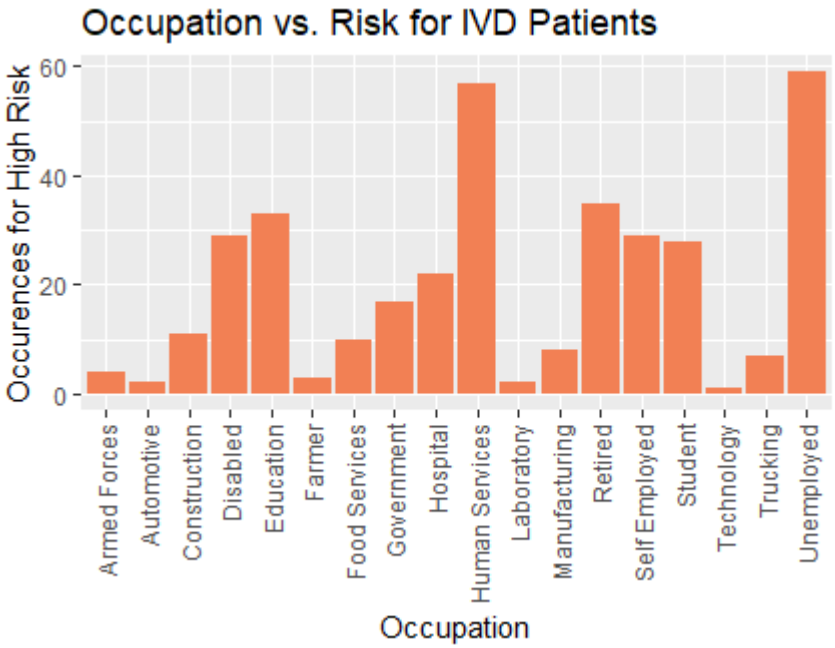

Figure 5: Comparison of Occupation for High-Risk Patients

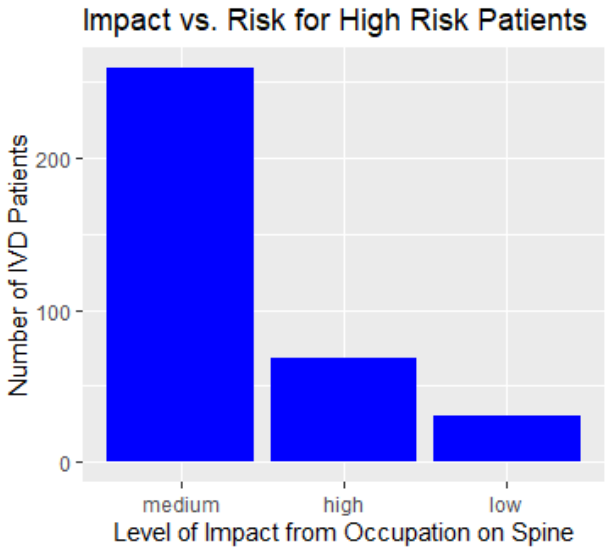

Figure 6: Comparison of Occupation on Spine Impact for High-Risk Patients

2.4 Overview of Machine Learning Algorithms

Data Mining tasks that are widely used are supervised learning, unsupervised learning, and reinforcement learning [30]. In unsupervised learning, unlabeled datasets are used to train systems to find hidden patterns within the data [31]. An example of unsupervised learning is clustering. Reinforcement learning involves training a system through direct interaction with the environment and utilizing trial and error [31]. Supervised learning involves training the system on a labeled dataset, and the output is classified as a discrete label (classification) or as a continuous numerical value (regression) [32]. In supervised learning, an algorithm needs to learn and approximate the mapping function from the input to the output variable.

Classification algorithms are used when the predicted output is discrete and has a finite set of possible outcomes [33]. Specifically, classification algorithms involve developing a learning function that will map data to a predefined category or class [34].

The relationship between the attribute or input variables (x) and the target or class value (y) can be expressed as the function y=F(x) [35].

For this study, the attribute variables were the predetermined factors associated with disc degeneration such as smoking, occupation, and age. The output value or predefined class was the risk of the patient for disc degeneration (low/no risk and high risk). Prediction of the output values involves two phases where the original data set was divided into training and testing data. The first phase includes inputting training samples into a supervised learning algorithm to develop a prediction model. The second phase uses the testing data set to evaluate this predictive model by comparing the predicted outcome to the actual outcome. In this study, several types of classification algorithms were evaluated in order to determine the best model for the application of early prediction of disc degeneration.

2.4.1 Clustering

K means clustering is a type of unsupervised learning algorithm where an unlabeled data set is utilized [36]. Data samples are grouped in k clusters based on the features that are shared. A data sample is inputted into the model, and the output is the cluster the new data point belongs within [36]. K is a hyperparameter that specifies the number of clusters the algorithm is to yield. The algorithm will choose “k” number of sample dots to represent the initial cluster focal points [36]. The rest of the sample dots are gathered to their focal points based on the criterion of minimum distance and an initial classification is reached [37]. The algorithm will continue to modify or calculate each cluster focal point until a final classification is determined. K means clustering has been demonstrated to be efficient, brief, and have celerity [37].

2.4.2 Decision Tree

Decision tree algorithms are tree-like models, where branches are used to demonstrate all the possible outcomes from a decision [38]. The goal of decision trees is to develop a model to predict a class for a given sample based on learning decision rules determined from using a training data set [39]. The root node is located at the top of the tree and represents all data records in the training data set. The root node consists of the attribute that best classifies the training data. Moving down the tree, the root node will be "split" into more sub-nodes. These sub-nodes can be categorized as a decision node or leaf node. A decision node will be split again using another attribute (ex. smoking, diabetes) and the categorical value associated with the attribute. A leaf or terminal node cannot be split further and will consist of a final class or discrete label. We have chosen to evaluate the decision tree algorithm for our study because it is easy to interpret, provides a view of all possible outcomes from a decision, and the feature selection of the root node is automatically done by the algorithm [40].

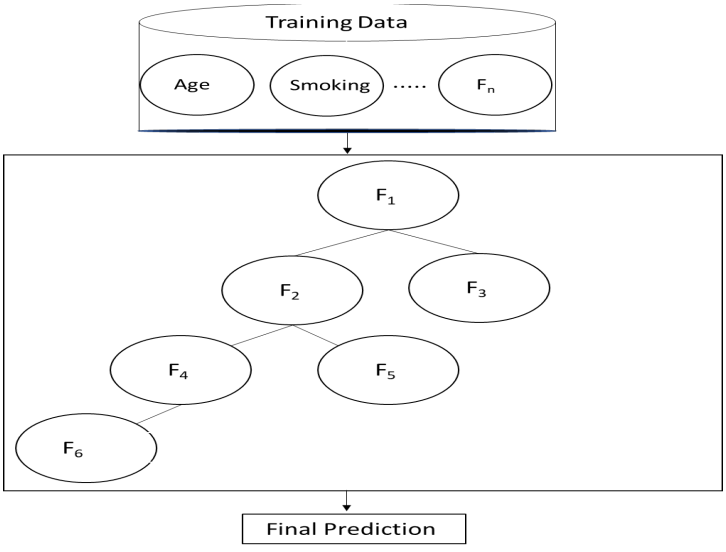

Figure 7: Pictorial Representation of the Decision Tree Algorithm

2.4.3 Logistic Regression

Logistic regression is a classification technique utilized for understanding the probability of an event occurring [41]. This method evaluates and measures the relationship between the attributes and the discrete label. A linear function will be developed based on a set of predictor or attribute variables [42]. The probability of success (p) of a dependent variable will be equal to 1, while the probability that the dependent variable is 0 is 1-p. This probability will be converted to a class label using a threshold value. When p is greater than the threshold value, a final classification can be determined. We have chosen to utilize the logistic regression algorithm because it is easy to implement and interpret, fast to learn, widely used, and can be used as a performance baseline for more complex algorithms [43].

$$f(low, i) = \beta_{0,\text{low}} + \beta_{\text{age,low}} x_{\text{age,i}} + \beta_{\text{impact,low}} x_{\text{impact,i}} + \cdots \quad (1)$$

$$f(high, i) = \beta_{0,\text{high}} + \beta_{\text{age,high}} x_{\text{age,i}} + \beta_{\text{impact,high}} x_{\text{impact,i}} + \cdots \quad (2)$$

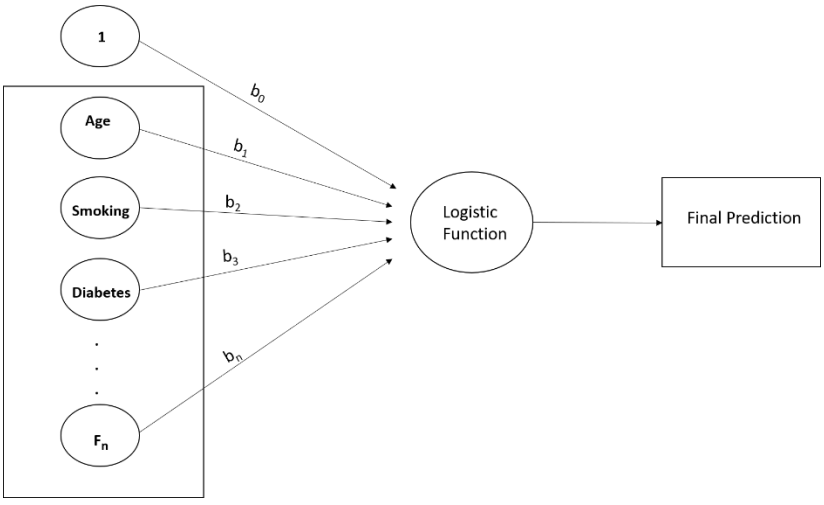

Figure 8: Pictorial Representation of the Logistic Regression Algorithm

2.4.4 Artificial Neural Network

The artificial neural network algorithm is designed to replicate the way humans learn and is inspired by the workings of the body's nervous system [44]. The artificial neural network itself consists of 3 layers; the input layer, hidden layer, and output layer [45]. The input layer is where the training data is fed through the artificial neurons. The hidden layer is the intermediate stage where the relationship between the input (factors associated with disc degeneration) and output (risk for disk degeneration) is learned. The artificial neuron will decide to fire or pass the single output value to other neurons of the next layer to which it is connected. This is determined based on the threshold value. Finally, the output layer extracts the final output from the last hidden layer. After the network is developed from the training data, a comparison between the predicted and actual output is done. We have chosen to utilize the artificial neural network algorithm because of its ability to learn hidden relationships within the data and to adapt to changes in input [44]. We have also chosen the artificial neural network algorithm because of its ability to learn and model linear and non-linear relationships [46].

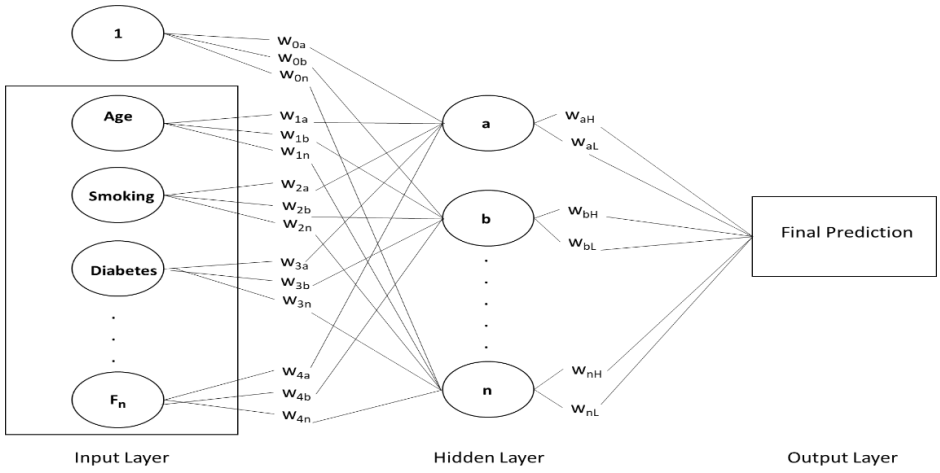

Figure 9: Pictorial Representation of the Artificial Neural Network Algorithm

2.4.5 Ensemble Methodology

Ensemble learning is an approach used to improve the robustness over a single predictive model [47]. The main goal of ensemble methodology is to combine the results of multiple predictive models, creating a new predictive model that is less likely to misclassify a new sample [48]. For this study, we utilized the ensemble method on classification algorithms to develop integrated predictive models.

2.4.6 Bagging

In bagging classification, the bootstrap aggregation procedure is applied to decision trees. When a sample is selected for a training data set from the initial set of samples, the sample is placed back in the original data set and may be chosen again [49]. This makes it possible for the same sample to be selected multiple times in a training data set or not appear at all. This is called “sampling with replacement” and involves sampling and replacing data while testing multiple models [50]. Once the training and testing data sets are determined, multiple decision trees are run in parallel to develop multiple models. A hypothesis or set of rules will be developed from each model, stating conditions for when the risk is either low/no risk or high for intervertebral disc degeneration. Each hypothesis has the same weight, and majority voting will aggregate them into a single hypothesis that determines the final classification. We have chosen to evaluate the bagging algorithm for our study because it has been shown to increase the stability of a model by reducing variance and overfitting [51].

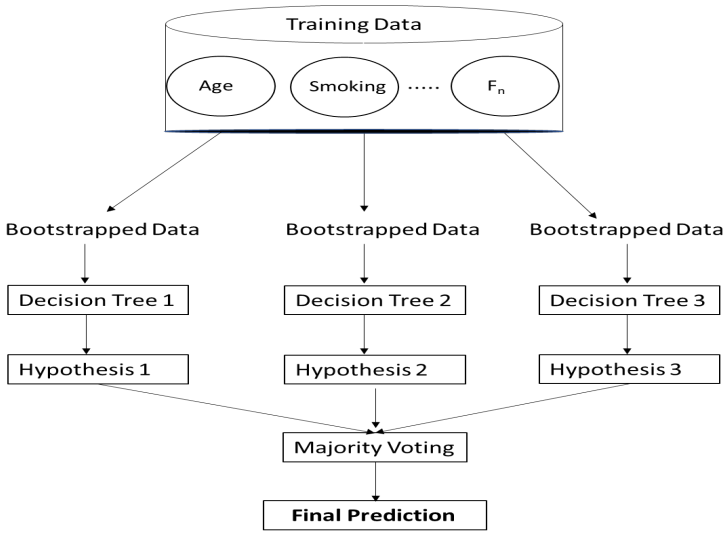

Figure 10: Pictorial Representation of the Bagging Algorithm

2.4.7 Boosting

Boosting can be performed on various classification algorithms such as decision trees, logistic regression, and neural networks [52]. In boosting, the models run in a sequence where each run of a model dictates the attributes of the next model. Bootstrap aggregation is also used in boosting to determine sample data sets. The boosting method utilizes the incorrect learning from "weak classifiers" to develop a predictive model. An algorithm is run with samples being assigned uniformed initial weights. By assigning equal weight to each sample, a "weak classifier" can be identified [53]. A weak classifier is a sample that is incorrectly classified. Reweighing will then occur based on which samples were not correctly classified by the model. With a penalty being assigned to weak classifiers. Samples that aren't correctly classified and are not learned by the weak classifier will increase in weight each time a model is run [54]. While a decrease in the weight of samples that are correctly learned is done until a weighted vote of weak classifiers determines the final classification or risk. We have decided to utilize the

boosting algorithm for our study due to its ability to penalize incorrect learning, and it can be used on multiple types of classification algorithms [54].

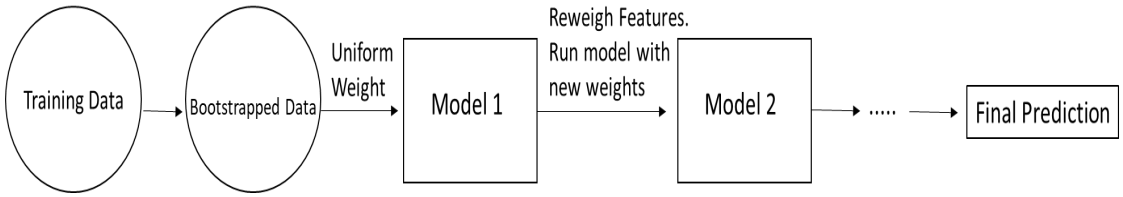

Figure 11: Pictorial Representation of the Boosting Algorithm

2.4.8 Random Forest

Random Forest is another machine learning algorithm that falls under the ensemble method and can be used in both classification and regression tasks. The random forest algorithm generates multiple decision trees and combines the output of these decision trees to make classifications [55]. This algorithm has been shown to overcome the problem of overfitting observed in individual decision trees [56]. Random forests have two main parameters: ntree and mtry [16]. The parameter ntree is defined as the number of decision trees to train in parallel, and mtry is defined as the number of independent variables or attributes to choose to split each node. In random forests, different subsets of attributes are chosen for each decision tree model. For classification, majority voting of all decision trees will determine the final prediction of risk for disc degeneration. The random forest algorithm has been previously shown to run efficiently on big data [57]. Using the random forest algorithm also allows for further randomness of the trees by the random selection of attributes [57].

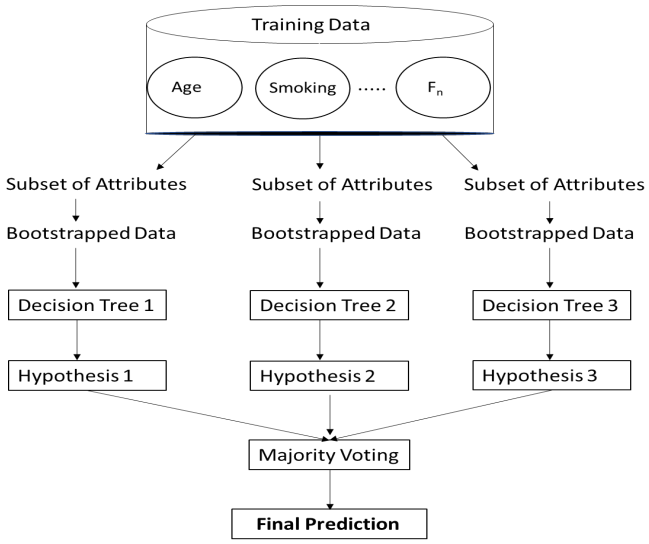

Figure 12: Pictorial Representation of the Random Forest Algorithm

2.5 Performance Metrics

Five performance measures were utilized to evaluate the ability of machine learning algorithms in developing the best predictive model for the intended application. The measures considered are precision, F1 score, and sensitivity/recall, specificity, and balanced accuracy to determine the best model given the inputted data samples [58]. Table 5 defines the terms used for all possible outcomes.

Table 5: Confusion Matrix

		Predicted	
		Negative	**Positive**
Actual	**Negative**	True Negative	False Positive
	Positive	False Negative	True Positive

Sensitivity or recall is a performance metric that evaluates the number of correctly predicted true positives and incorrectly predicted negatives by the model (Equation 3). Recall is utilized to determine the model's ability to correctly predict when a patient is at

high risk for disc degeneration. In medical terms, out of all the patients that are at high risk for disc degeneration, how many was the model able to predict to be high risk. Recall is utilized when the focus is on minimizing false negatives (type II error). Specificity is a performance metric that evaluates the number of correctly predicted true negatives and incorrectly predicted positives by the model (Equation 4). In medical terms, specificity is utilized to determine the model's ability to correctly predict when a patient doesn't have disc degeneration. Specificity is utilized when the focus is on minimizing false positives (type I error).

Precision evaluates the number of correct true positive predictions by the model while still evaluating the incorrectly predicted positive when it should have been negative (Equation 5). Precision measures the percentage of the predicted results that are relevant or were correctly predicted to be positive. In medical terms, out of the patients predicted to be high risk for disc degeneration, how many were actually high risk. Having high precision demonstrates that there is a low rate of false positives or type I error. F1 score (shown in Equation 6) observes type I and type II errors simultaneously and evaluates the ability of the model to avoid false positives and false negatives. F1 score is considered the harmonic mean of precision and recall, as it takes both into consideration. This performance metric evaluates the robustness (low number of missed classifications), as well as the number of data points that are classified correctly by the model. Balanced accuracy is utilized when an imbalanced data set exists (Equation 7). Balanced accuracy considers both specificity and sensitivity.

$$\text{Sensitivity/Recall} = \frac{True\ Positive}{True\ Positive + False\ Negative} \quad (3)$$

$$\text{Specificity} = \frac{True\ Negative}{True\ Negative + False\ Positive} \quad (4)$$

$$\text{Precision} = \frac{True\ Positive}{True\ Positive + False\ Positive} \quad (5)$$

$$\text{F1 Score} = \frac{2(True\ Positive)}{2(True\ Positive) + False\ Positive + False\ Negative} \quad (6)$$

$$\text{Balanced Accuracy} = \frac{Sensitivity + Specificity}{2} \quad (7)$$

2.6 Machine Learning Parameters

The cleaned and integrated patient data points were randomly separated into two datasets: training and testing data. These split datasets were utilized to train the algorithm and then to test the model developed by the algorithm. Eighty-five percent of the data was utilized to train the algorithm, while the other 15% was utilized to test the model. Synthetic Minority Oversampling Technique (SMOTE) was utilized due to the imbalance between patients with disc degeneration (minority) and patients without disc degeneration (majority). A dataset is considered imbalanced if both classification categories are not represented equally [59]. The SMOTE technique involves under-sampling of the majority and oversampling of the minority. For this study, the percent over was set to 12,000 and the percent under was set to 100. The balanced data set consisted of 36,784 data points that were high-risk patients and 36,480 data points that were low-risk patients. K means clustering was set to 25 centers with max iterations set to 100. For the logistic regression algorithm, there are no associated parameters. To tune the parameters of the random forest, gradient boosting, and artificial neural network, a grid search method was utilized. This allows an in-depth search to be run by the user during training so that the best

parameter for a specific set is chosen. For the random forest algorithm, the default setting of 100 trees was utilized. For the ANN, a feed-forward method was used to develop the predictive model. In a feed-forward algorithm, there consists of an input layer, a hidden layer, and output layer, as well as backpropagation learning. The predictive factors and output variable represent the input and output layers. An optimal point is achieved for the number of nodes in the hidden layer when between 1 and the number of input variables. For this application, the nodes vary from 1 to 14. The values of the learning rates utilized to train the ANN are 0.01, 0.05, and 0.10.

3. Results

Table 6 provides the results of the machine learning algorithms before k means clustering was utilized. Examining the results, the best performing predictive model was developed by the random forest algorithm followed by the bagging algorithm and the gradient boosting algorithm. The predictive model developed by the random forest algorithm had the best scores for all five-performance metrics at 89% and higher. The lowest metric for the bagging algorithm was precision at 89%. The predictive model developed by the decision tree algorithm performed the worst at around 63-67% for the five-performance metrics, with precision being the lowest at 63%.

Table 7 summarizes the results of the machine learning algorithms when k means clustering was utilized and added as another factor or column in the data set. Observing the results from Table 7, there is an increase in performance for all predictive models when k means clustering was utilized and added to the data set. The bagging predictive model performed the best on the given data set with a balanced accuracy of 94%, precision at 92%, F1 score at 94%, specificity at 92%, and sensitivity/recall at 97%. The

model developed by the random forest algorithm came in second with all performance metrics 90% or higher. Predictive models developed by the decision tree algorithm and logistic regression algorithm performed the worst. Between the two, the logistic regression algorithm was found to be best when evaluating sensitivity/recall (Type 1 error), while the decision tree algorithm had a higher percentage for specificity, F1 score, precision, and balanced accuracy. For the application of risk prediction for disc degeneration, type II error would be the biggest problem or error to evaluate, as it would be worse to predict a false negative (predicted low risk when actually high risk) for a patient. For the bagging algorithm, the specificity was at 92%.

Table 6: Consolidated Results (Without Clustering)

	Sensitivity/Recall	**Specificity**	**F1 Score**	**Precision**	**Balanced Accuracy**
LR	0.6889	0.6772	0.6803	0.6719	0.6831
DT	0.6796	0.6565	0.6584	0.6386	0.668
GBM	0.8118	0.7868	0.7957	0.7803	0.7993
RF	0.9520	0.9033	0.9246	0.8987	0.9277
ANN	0.6919	0.6728	0.6759	0.6607	0.6823
Bagging	0.9377	0.8753	0.9009	0.867	0.9065

Table 7: Consolidated Results (With Clustering)

	Sensitivity/Recall	Specificity	F1 Score	Precision	Balanced Accuracy
LR	0.7332	0.7376	0.7383	0.7433	0.7354
DT	0.6869	0.7701	0.7454	0.8148	0.7285
GBM	0.862	0.8241	0.8385	0.8162	0.843
RF	0.9559	0.9052	0.9274	0.9005	0.9306
ANN	0.7197	0.8188	0.7812	0.8541	0.7692
Bagging	0.9705	0.927	0.9467	0.9241	0.9487

4. Discussion

Observing the results in both Table 6 and Table 7, algorithms within the ensemble methodology family developed the best performing predictive model for this application. Ensemble methods can be thought of as combining the predictions or knowledge of several experts to create a composite model with better accuracy. Researchers have shown that a composite model delivers better results in prediction than a single model [60, 61]. Results from this study support this claim, as the predictive models developed by the ensemble methods utilized (random forest, bagging, and gradient boosting) performed the best before and after k means clustering was applied. All three of these ensemble algorithms were utilized on decision trees. It is also important to note that the best performing models were when ensemble methods were utilized on decision trees. Alone, the classifier developed by the decision tree algorithm was one of the worst

performing models, however, when combining a majority vote through ensemble methodology, an increase in performance and prediction was observed.

The data set acquired from the MOI included information on patients with and without disc degeneration, however, this data set was unbalanced. The data set was skewed to more example data points on patients without disc degeneration (majority) than those with disc degeneration (minority). Imbalanced data sets can cause a problem in the learning process of the algorithm, such as poor prediction of the minority class and classifying new samples to the majority class [62].

In initial studies before the SMOTE technique was applied, the random training and testing sets of the unbalanced data were inputted into the classification algorithm. Results showed zero for both the actual and the predicted in the confusion matrix for the high-risk patients, but the overall accuracy of the model was still high. This can mean there is a problem in the distribution of the data set as either there were not enough data points for the algorithm to train or not enough data points to test the model. For this study, this is an issue as we are most interested in the minority class.

Techniques of re-sampling, such as random oversampling (replicating the minority data points) or undersampling (removing data points from the majority) are utilized when dealing with an unbalanced data set [62]. An important limitation of undersampling is that vital information may be lost when removing data points from the majority and limitations of oversampling include over-fitting of the model [63]. SMOTE is a technique that takes advantage of both under and oversampling and has been shown to improve the prediction of the minority class without forfeiting the accuracy of the entire data set [63, 64]. Chawla et al. (2003) combined the SMOTE technique and

boosting to improve the prediction of the minority class [65]. Batista et al. (2004) utilized a combination of SMOTE and data cleaning methods to evaluate model performance and compare over and undersampling [66]. Results demonstrated an improvement in prediction for the minority class and an increased accuracy of the model when utilizing random oversampling. The results from this study agree with the use of the SMOTE method for imbalanced data sets. After the SMOTE technique was utilized to balance the data set, an improvement of the prediction of the minority class was observed.

Cluster-then-predict model is a growing technique used to increase the performance of the model by applying k means to discover clusters within the given data set. These clusters can then be utilized as a new factor or input variable into the classification algorithm. Cluster-then-predict combines the methods of supervised and unsupervised learning to improve the performance of classification models [67]. Trivedi et al. (2015) discussed observing increased prediction accuracy when clustering is used in combination with a supervised algorithm such as bagging [68]. It was also observed that this method improved the prediction of the random forest algorithm. Alyousef et al. (2018) investigated the use of clustering in conjunction with decision trees to improve prediction as well as understand the differences between the discovered groups [69]. Results demonstrated an increase in performance accuracy and prediction when supervised and unsupervised learning was combined. Comparing Tables 6 & 7, results support the use of k-means to cluster the data and then adding these clusters in the data set to improve model performance. An increase is observed for all performance metrics across all classification models developed.

One limitation of this study is the need for more data on patients with disc degeneration. The SMOTE technique was applied to the data set to fix the problem of an unbalanced data set. Collecting more data on IVDD patients would provide more information for the algorithms to learn and predict. It would also be beneficial to collect data from across the United States. Right now, the data acquired only investigates high-risk patients in Missouri, however, IVDD patients may look different based on region, socioeconomic status, access to healthcare, and what the type of occupations look like in the state. It is important to gather more information to truly develop a representative model.

Another limitation of this study is the lack of data on patients where disc degeneration is hereditary or genetic. Studies have shown that there is a correlation between genetics and intervertebral disc degeneration [70]. Dowdell et al. (2017) suggests that genetics may play a larger role in disc degeneration than lack of nutrition and mechanical damage [71]. Polymorphism of the extracellular matrix (aggrecan, collagen), anticatabolic and catabolic mediators (tissue inhibitors of matrix metalloproteinase, matrix metalloproteinase (MMP)), and other genetic polymorphisms such as the Vitamin-D receptor, have all been found to contribute to IVDD [71]. Having more information about this factor for patients can aid in developing the predictive model for the risk of disc degeneration.

5. Conclusion

The overall objective of this study was to develop a predictive model for the risk of disc degeneration. A literature review was conducted to determine factors associated with the disease. Once the factors were determined, data was acquired from the Missouri

Orthopaedic Institute for patients with and without disc degeneration. Data was transformed, integrated, and cleaned, and a classification of high risk (patients with degenerative disc disease) and low risk (patients without degenerative disc disease) was given. The SMOTE method was performed on the data set and then inputted into clustering and classification algorithms to develop the predictive models.

Results demonstrated that the performance of the models improved after k means clustering. All models showed an increase in performance across all five-performance metrics. The bagging algorithm developed the best performing model when k means clustering was utilized, while the models developed by decision tree and logistic regression algorithms performed the worst. Results also demonstrated an increase in the performance of the models when the SMOTE method was applied, as well as when ensemble methods were utilized for classification algorithms. As more information on patients with disc degeneration is gathered, the findings from this study will lead to a tool that serves as an aide in earlier prediction and detection for intervertebral disc degeneration. These findings also support utilizing machine learning algorithms for risk prediction of disc degeneration.

Section 2: User App

This section is about developing a user-friendly app for patients to utilize on the first onset of back pain. Doctors can utilize this app to identify patients that are at risk for disc degeneration. The predictive model developed by the logistic regression algorithm in section I was utilized with the Shiny R program to provide the user with an app that is interactive with the information that they input. First, the user will select what factors they have from the provided list of variables previously described in Table 4.

Descriptions are provided to the user on how age groups, occupation, and spinal impact are categorized. For the occupation and spinal impact factors, the description paragraphs give examples for the user to know which occupation and impact level to select. The user will also have to provide their height in inches. Finally, once the user has inputted their answer for each factor, the "Click for Risk Prediction" should be pressed. The output will be a table that provides a summary of the user's inputs and the risk prediction of the model based on of the user's inputs. The prediction from the model will either say "low" for low risk or "high" for high risk. Figures 13-14 provide an image of the app.

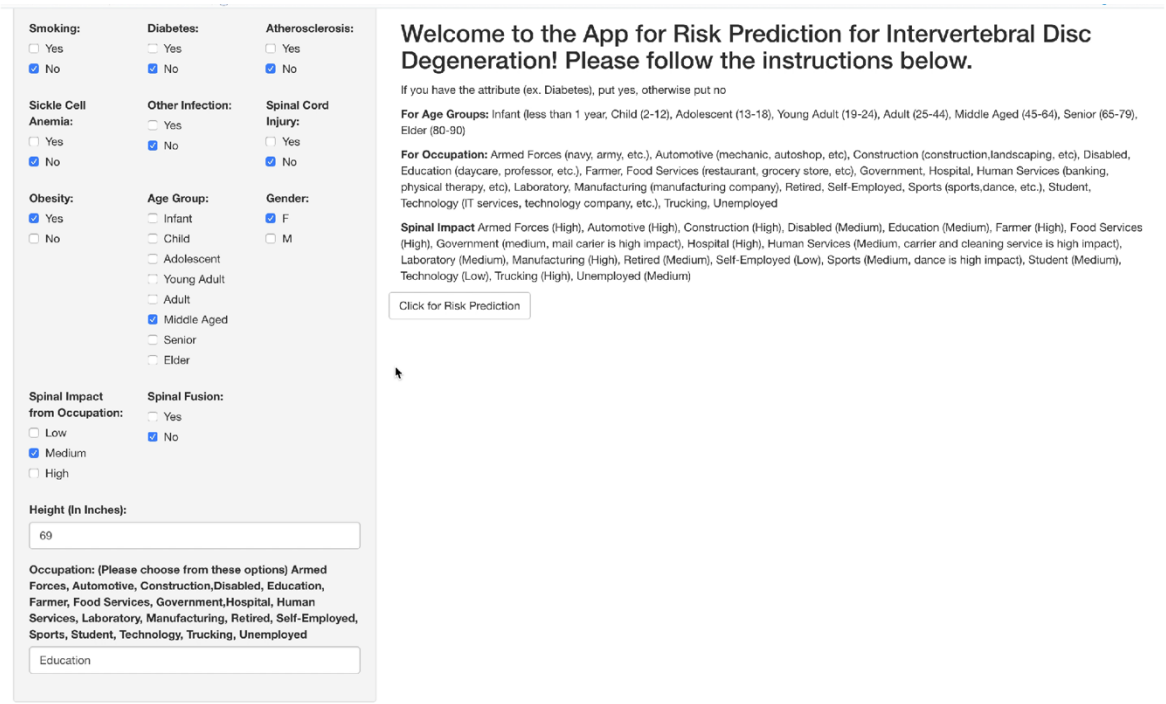

Figure 13: Example Case for IVDD Risk Prediction App

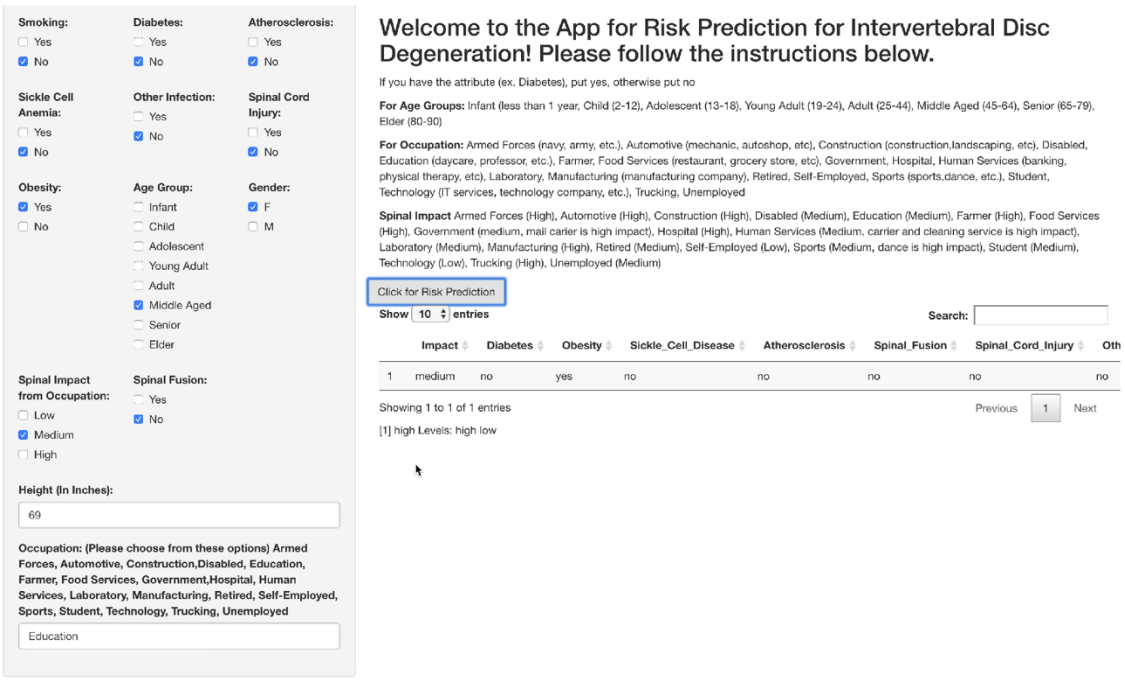

Figure 14: Example Case of Final Prediction for App

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Chapter Four

Developing and Characterizing a AuNP-Genipin-Viscoelastic Collagen Material for Intervertebral Disc Degeneration

Section I: Viscoelastic Collagen Studies with Genipin and Gold Nanoparticles

1.Introduction

Intervertebral disc degeneration is a known common cause of lower back pain [1]. Lower back pain is a leading cause of doctor's visits, disability, and missed work. Lower back pain also has a lifetime incidence of 80%, which means that at some point in their lives, a large percentage of the population is having to live with back pain [2]. The total economic burden caused by lower back pain is between $100-200 billion annually, and with lower back pain being the second most common cause of doctor visits, it contributes to $20–100 billion in direct health care spending [3, 4].

1.1 Intervertebral Disc

The intervertebral disc (IVD) has a largely mechanical role in the spine and operates as a shock absorber for impact from daily activities [5]. The vertebral bodies of the spine are separated by the IVDs, with the discs accounting for 20-30% of the spinal length [6]. The intervertebral disc also allows for limited flexibility of the spine, allowing it to rotate, twist, and bend [7]. Three main components make up the structure of the

IVD: the gel-like nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilage endplate (CEP) [8].

The nucleus pulposus is the gelatinous, hydrophilic center of the intervertebral disc. The NP has a high-water content and its pressurized swelling allows for resistance against compressive loads [9]. The major components of the extracellular matrix of the NP include proteoglycans embedded in Type II collagen fibrils, with aggrecan being the most abundant proteoglycan [10, 11]. Degeneration, which can be caused by aging or severe injury, is marked by several changes to the disc environment including biological, cellular, and mechanical alterations. For the nucleus pulposus, these degenerative changes include loss and degradation of aggrecan and GAG and loss of water content leading to decreased disc height [12, 13]. Other degenerative alterations include changes to collagen type, causing increased stiffness and loss of definition of the demarcation or transition zone [14]. Degenerative disc disease is the term used to describe the degeneration of the disc leading to or causing chronic back pain.

Current noninvasive treatment includes rest, epidural steroid injections, and nonsteroidal anti-inflammatory drugs [15]. However, these methods and medications treat the symptoms, while the underlying problem of degeneration remains. In spinal fusion, the disks or vertebrae are fused permanently using a bone grafting material [16]. The use of allografts, autografts, or a synthetic material substitute can be utilized as well as metal screws and plates to fuse the bones [16]. Though this surgery is common, numerous problems can occur after the surgery is implemented. Back pain may persist for patients even after the spinal fusion has been performed [17]. Another problem is the weakening and wear of other disks and vertebrae that are around or next to the fusion.

Studies conducted by Park et al. (2004) showed the accelerated development of adjacent segment disease and disc degeneration in the areas adjacent to the spinal fusion [18]. There are also the risk factors that come from any major surgery such as infection and damage to spinal nerves and tissue surrounding the area. These numerous risks have led researchers to explore less invasive treatments.

Current treatments being investigated for possible use include biological therapies, anti-inflammatory drugs, and gene therapy [19]. Biological therapies include both cellular and protein-based treatments. In protein-based therapy, research in this area aims to reduce inflammation, improve mechanical stability, and water retention in the degenerated disc, and increase the production of the ECM and proteoglycans [20].

1.2 Collagen

Collagen is a structural protein that is prevalent in the extracellular matrix (ECM) of numerous connective tissues throughout the body. Collagen is also one of the main components of the ECM of the disc. It is currently used in the field of tissue engineering and regenerative medicine as a structural scaffold due to its biocompatibility, mechanical structure, and biodegradability [21]. Collagen has been shown to promote cellular migration and adhesion through signaling pathways, focal adhesion, and cellular attraction to the large surface area [22]. Prior studies have also investigated the use of collagen as a biomaterial for disc regeneration [23, 24]. Previous studies have indicated that the decrease in water content and increase in fiber content is an important factor in the biological and mechanical changes in the nucleus pulposus [25, 26]. In our study, a non-polymerized or non-fiber forming, viscoelastic collagen template was developed.

The viscoelastic collagen will display the viscous and elastic properties of the disc as well as mimic the gel-like properties and environment of the nucleus pulposus.

1.3 Genipin

Limitations for the potential use of collagen include limited mechanical strength and rapid biodegradation as seen with naturally derived tissues [27]. To overcome these limitations, chemical crosslinking agents are utilized to improve mechanical strength and stability and provide more control of the degradation rate of the collagen [28]. Crosslinkers such as EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide), glutaraldehyde, and formaldehyde are currently used, however, their potential high cytotoxicity and induction of the inflammatory response can affect the overall biocompatibility of the collagen [28, 29, 30]. Genipin is a naturally derived crosslinking agent isolated from the Gardenia Jasminoides fruit [31, 32]. Genipin has demonstrated low toxicity, biochemical durability, and mechanical strength comparable to glutaraldehyde, while not requiring numerous washes steps to remove the toxic byproduct of synthetic crosslinkers such as glutaraldehyde and EDC [33, 34]. It has also been shown to have good resistance to *in vitro* enzymatic degradation, comparable to synthetic crosslinkers. Previous studies have also utilized genipin for its pharmacological properties such as anti-inflammatory, antiangiogenic, antioxidant, and antithrombotic effects [35, 36, 37, 38, 39]. These types of crosslinkers may also serve as a catalyst for the attachment of nanomaterials. The genipin crosslinker was used as a link to attach gold nanoparticles to the viscoelastic collagen.

1.4 Gold Nanoparticles

Nanoparticles in the field of regenerative medicine and tissue engineering are utilized to control properties of tissue scaffolds such as mechanical strength and durability, as well as controlling the release of bioactive agents for drug delivery [40]. Metallic nanoparticles, specifically gold nanoparticles (AuNPs), have been utilized in previous studies to conjugate ligands and proteins to the gold surface through the use of amine, thiol, and phosphines groups due to their high affinity to the gold [40]. Gold nanoparticles have shown potential to be utilized in numerous biomedical applications such as drug delivery, sensors, and cancer research for their biocompatibility. Previous research indicates the use of gold nanoparticles as a chemoattractant to increase cell migration and attachment due to their large surface energy and surface topography which promote cellular attraction and attachment [41].

Oxidative stress has been shown to influence disc degeneration. The harsh environment stemming from the lack of nutrient supply to disc cells and the suppression of waste removal due to endplate calcification is suggested to be a trigger for excessive ROS (reactive oxygen species) production in the disc [42]. The degradation of viable tissue due to the presence of inflammatory mediators has also been observed to be a trigger and an effect of disc degeneration [43]. Gold nanoparticles have been used in therapeutic treatments for chronic inflammation and rheumatoid arthritis in part due to their anti-oxidative properties [44, 45]. Studies have demonstrated the ability of AuNPs to reduce oxidative stress levels by inhibiting reactive oxygen species formation and by also scavenging free radicals [46]. Previous studies have also overcome the limitation of rapid degradation of collagen and other tissue-engineered scaffolds by blocking the

collagenase enzyme from the carboxylic binding sites through the attachment of AuNPs functionalized with cysteamine [47].

We are developing a collagen-based nanoparticle material to promote regeneration of the intervertebral disc. Exploring the combination of these three components (viscoelastic collagen, genipin, gold nanoparticles) can lead to advancing knowledge in the area of tissue templates for tissue regeneration. Specifically, it can lead to a radical new treatment to mimic the environment of the nucleus pulposus and serve as a template for regeneration and cell adhesion. The purpose of this study is to evaluate the attachment of the gold nanoparticles to the VE collagen and to study its biocompatibility and material/mechanical properties.

2. Materials and Methods

2.1 Viscoelastic Collagen Crosslinked with Genipin and AuNPs

2.1.1 Viscoelastic collagen

Type 1 bovine collagen (Sigma Aldrich) was utilized to develop the viscoelastic collagen. The powdered collagen was first dissolved in acetic acid to achieve a concentration of 1.5 mg/ml. Next, the collagen was mixed with 1N NaOH (Thermal Fischer) to achieve a pH of 9. The acylation process was started by adding glutaric anhydride (Sigma Aldrich) followed by succinic dichloride (Sigma Aldrich). Through the course of the acylation procedure, a pH of 9 was maintained. The acylation process was then stopped by decreasing the pH of the collagen to 4.3 by adding HCl (Sigma Aldrich).

The collagen precipitate was then collected, washed, and re-suspended in sodium phosphate monobasic buffer (Sigma Aldrich) and left to sit overnight.

2.1.2 Genipin, VE collagen, and AuNP Crosslinking

For the crosslinking process, 1N NaOH was added to bring the pH of the viscoelastic collagen to 7. Twenty and 100nm size gold nanoparticles were functionalized using cysteamine (Thermal Fischer) and water while genipin (Sigma Aldrich) was dissolved in dimethyl sulfoxide and water. Next, the genipin solution and functionalized gold nanoparticles were added to the viscoelastic collagen and incubated for 1 hour at 36.8º Celsius. Then, the crosslinked gold nanoparticle viscoelastic collagen was placed on a rocker for 24 hours. Finally, the gold nanoparticle viscoelastic collagen underwent washing three times with double distilled water. Table 1 provides a description of the VE samples.

Table 1: Description of VE collagen samples with 12mM of genipin

Viscoelastic Collagen	Description
1x, 20	• 1x concentration of 20 nm size AuNPs
1x, 100	• 1x concentration of 100 nm size AuNPs
4x, 20	• 4x concentration of 20 nm size AuNPs
4x, 100	• 4x concentration of 100 nm size AuNPs

Crosslinked	• Genipin with no AuNPs
Natural	• No genipin or AuNPs

2.2 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy was conducted to assess the structure of the non-polymerized collagen. The VE collagen samples with no AuNPs or genipin crosslinker were assessed. Samples were negatively stained with nano tungsten to provide contrast between the sample and background. The viscoelastic collagen samples were then placed on a carbon-coated copper grid. The JEOL-JEM 1400 microscope was used at 10 kx, 25 kx, and 50 kx. Voltage was set to 120 kV. The emission current was 72 uA and a pinta holder was utilized.

2.3 Scanning Transmission Electron Microscopy (STEM)

The FEI Tecnai F30 G2 Twin Transmission Electron Microscope was utilized in TEM mode and STEM mode to evaluate the structure of the collagen and the attachment of the 20 and 100 nm gold nanoparticles. VE collagen with 4x concentration of 20 and 100 nm AuNPs were negatively stained with uranyl acetate and placed on the carbon-coated copper grid utilizing the smearing method. Voltage was set to 300 kV, emission current to 96 uA, and spot size to 6. The holder was a single tilt TEM holder with the alpha tilt at 15º.

2.4 Energy Dispersive Spectroscopy (EDS)

The Bruker Energy Dispersive X-ray spectrometer QUANTAX 400-STEM was utilized to determine whether gold was present on the VE collagen sample. VE collagen with 20 and 100nm AuNPs at 4x concentration were negatively stained with uranyl acetate and the smearing method was used to place the sample on a carbon-coated copper grid. 500 eV/KeV was utilized.

2.5 Fourier-Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy was utilized to determine collagen fibril formation of the VE collagen. An ATR-FTIR (Nicolet 6700, Thermo Scientific) ATR-FTIR was utilized. The viscoelastic collagen samples were stored in a -20º refrigerator overnight and lyophilized using a Labconco FreeZone-1.

2.6 Neutron Activation Analysis (NAA)

Neutron Activation Analysis was utilized to assess the amount of gold present on the VE collagen materials. The viscoelastic collagen materials were stored in a -20º refrigerator overnight and lyophilized using a Labconco FreeZone-1. Samples were then weighed and secured in high-density polyethylene vials for analysis. For two minutes, the samples were irradiated and then allowed to decay for seven hours. A Canberra High Purity Germanium detector was used to measure gamma radiation for ten minutes.

2.7 Cell Viability (WST-1)

L929 murine fibroblast cells (ATCC) were cultured in Eagle's Minimum Essential Media supplemented with 10% (v/v) horse serum and 200 U/mL of Penicillin Streptomycin at 36.8 ºCelsius and 5% CO_2.

A Water-Soluble Tetrazolium Salt Assay was conducted to determine the cell viability for each of the various compositions of VE collagen. The viscoelastic collagen material was stored in a -20º refrigerator, lyophilized using a Labconco FreeZone-1, weighed, and then lyophilized again for 2 hours for sterilization. The Natural VE collagen material was autoclaved for 45 minutes in order to sterilize due to it being in liquid form. The collagen samples were then incubated for 24 hours with supplemented cell media (horse serum and Penn strep). Cell media was then removed and L929 murine fibroblast cells were seeded at a concentration of 6.0×10^4 cells/mL. Assays were run with cells in contact with the VE collagen materials for 5 and 7 days. On days 5 and 7, the WST reagent was added and allowed to incubate for 4 hours. After 4 hours, media from each well was removed to a new well plate, and absorbance was read at 450 nm with a reference reading at 655nm using a spectrofluorometer plate reader (Cytation 5, BioTek). The baseline for 100% cell viability consisted of the positive control of fibroblast cells with no VE collagen materials.

2.8 Quasi-Static Force Test

Quasi-Static Force Testing was conducted to evaluate the stiffness of the VE collagen with gold nanoparticles and genipin crosslinker. A TA-HDI Texture Analyser (Stable Micro Systems) was utilized to perform unconfined compression testing on the

different viscoelastic collagen compositions. Samples were placed between two impermeable plates and applied displacement control loading at a rate of 0.25 mm/sec. The plates were set at $1/10^{th}$ of a mm, allowing further compression and movement at a slower rate. The samples were compressed to a final height of 0.1 mm and force was measured with a 5 kg load cell.

2.9 Reactive Oxygen Species Assay

The reactive oxygen species (ROS) assay was conducted to evaluate the production of oxidative chemicals such as hydrogen peroxide produced by cells when in the presence of the collagen material. VE collagen samples (except natural VE collagen) were sterilized with neutral pH peracetic acid and then lyophilized. Natural VE collagen was autoclaved for 45 minutes to sterilize in its liquid form. Murine fibroblast cells were seeded at a concentration of $4.42 \text{ X } 10^4$ cells/mL and incubated for 24 hours before the DCFH-DA stock was added. Next, the fibroblast cells were incubated for 12 hours in the presence of each of the collagen compositions with genipin and gold nanoparticles, after which the assay was terminated. The fluorescence was then measured at 530 nm emission and 480 nm excitation.

2.10 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to determine the denaturation temperature of the viscoelastic collagen templates. Viscoelastic collagen template samples of 2 – 5 mg were placed in Tzero plates/hermetic lids and sealed using the Tzero press (TA Instruments, New Castle, DE). Two microliters of double distilled water were used as the reference pan. Each of the samples underwent modulated differential

scanning calorimetry using the Q2000 DSC (TA Instruments) to ramp the temperature from 5°C to 120°C at a rate of 5°C per minute. The mean denaturation temperature (°C) is reported below in Figure 4. Four trials/repetitions of the samples were performed.

2.11 Analysis

All statistical analysis will be carried out using GraphPad Prism version 4.0 (GraphPad Software, Inc.). A one-way ANOVA analysis of variance with a 95% confidence interval and Tukey's post-test was performed. The variance between controls and VE collagen:AuNP samples are expected to be observed. A small effect size will be used to determine whether large variances between the samples exist. In order to do so, a small sample size was utilized.

3. Results

3.1 Electron Microscopy

TEM was utilized to determine the non-polymerization of the viscoelastic collagen. The morphological structure of the natural viscoelastic collagen was observed in Figures 1-3. The results did not demonstrate or show the characteristic banding of polymerized collagen. The lack of a banding structure on the individual collagen fibrils confirms that while the collagen did develop into a fibril structure, the viscoelastic collagen did not polymerize. TEM and STEM were also utilized to determine the attachment of the gold nanoparticles to the viscoelastic collagen for both 20 and 100 nm size. In Figure 3, small particles are observed on the viscoelastic collagen for both collagen samples where 20 and 100 nm gold nanoparticles were attached.

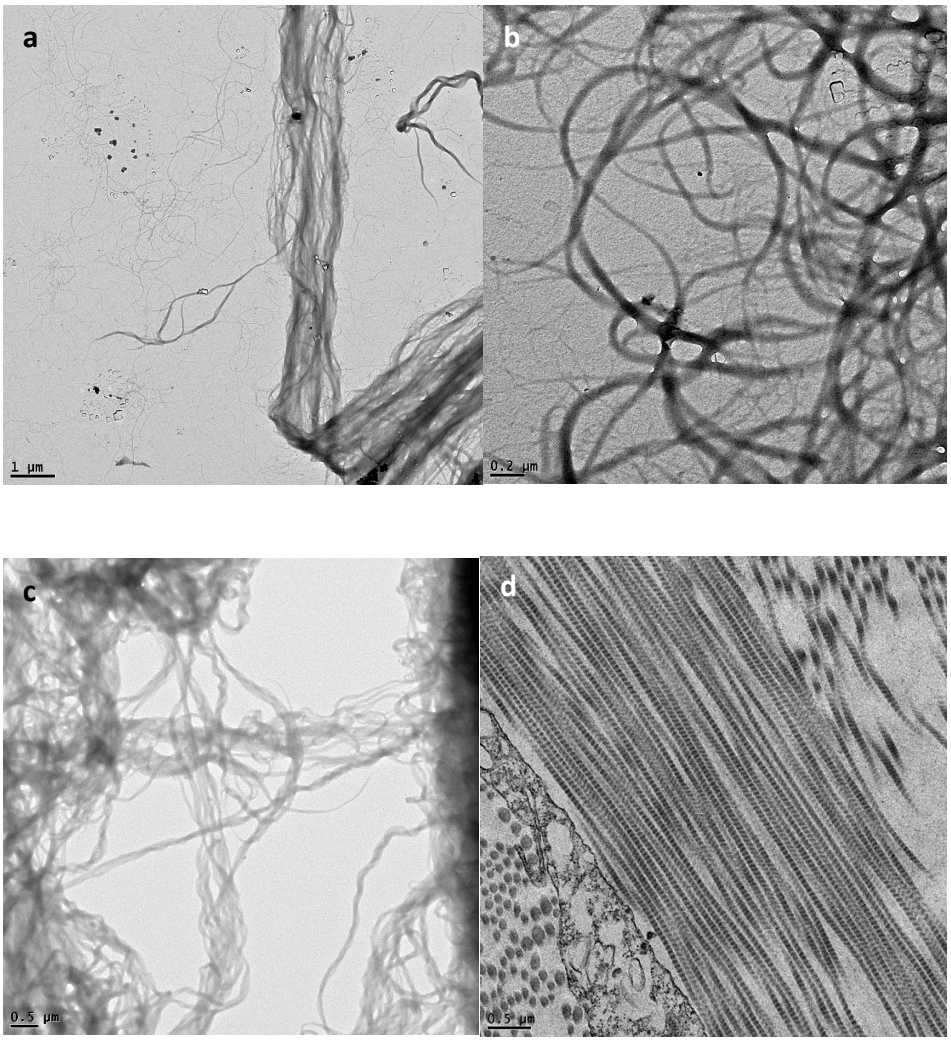


Figure 1: TEM images of viscoelastic collagen shown in 1a, 1b, and 1c while polymerized collagen is shown in 1d

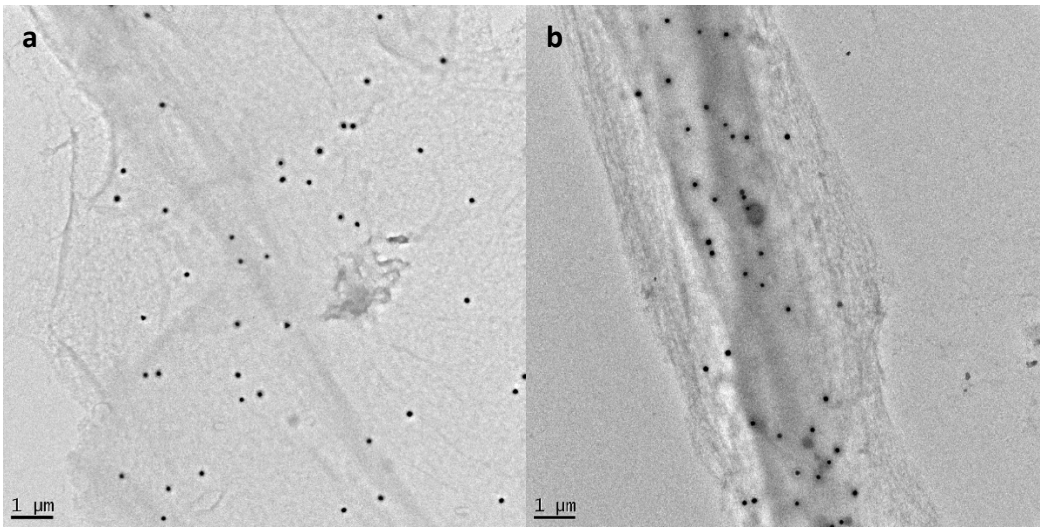


Figure 2: TEM images of (a) 4x concentration of 20 nm AuNPs (b) 4x concentration of 100 nm AuNPs

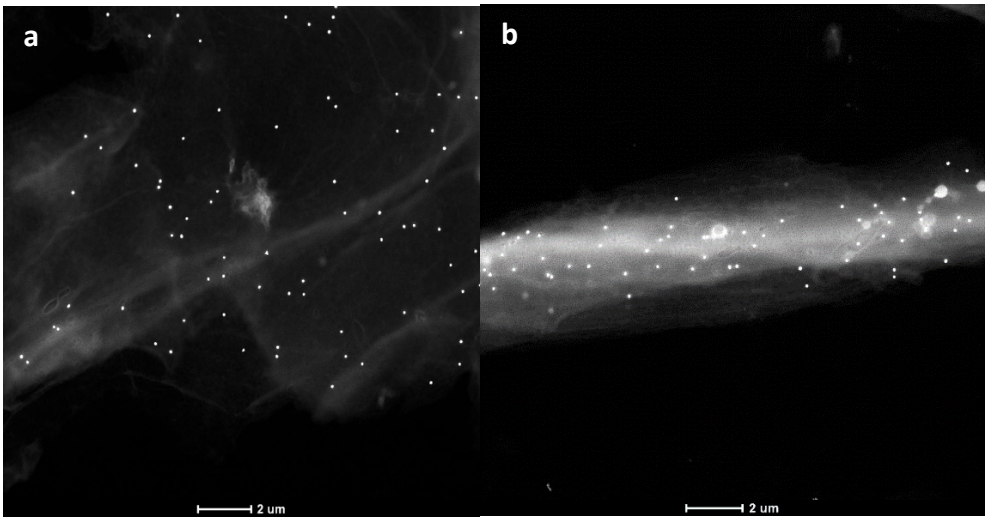
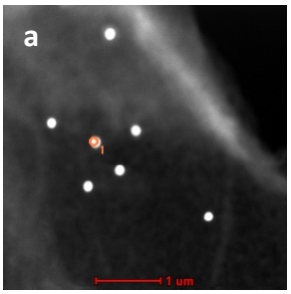
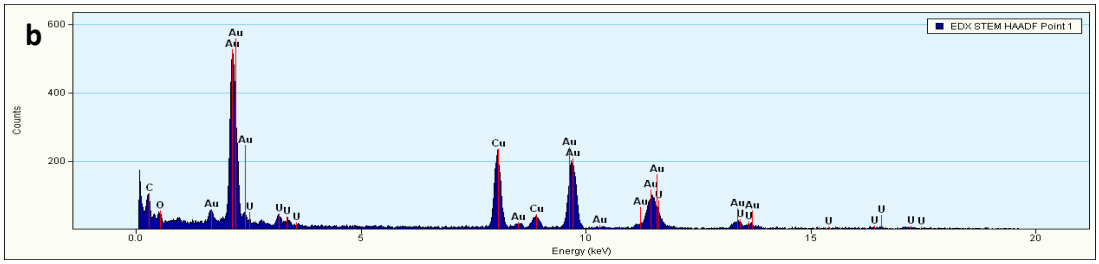


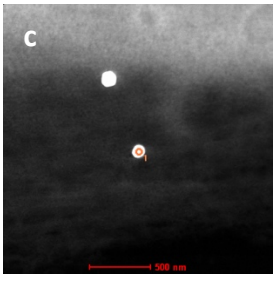
Figure 3: STEM images of (a) 4x concentration at 20nm and (b) 4x concentration at 100nm

3.2 Energy Dispersive Spectroscopy

Gold nanoparticle attachment to the viscoelastic collagen was confirmed using Energy Dispersive Spectroscopy. Figure 4 provides the spectrum of elements present on the viscoelastic collagen. Results demonstrate the energy peaks or characteristic x-rays gold for the small particles observed on the viscoelastic collagen. This confirms the attachment of the gold nanoparticles using a genipin crosslinker for both 20 and 100nm size.







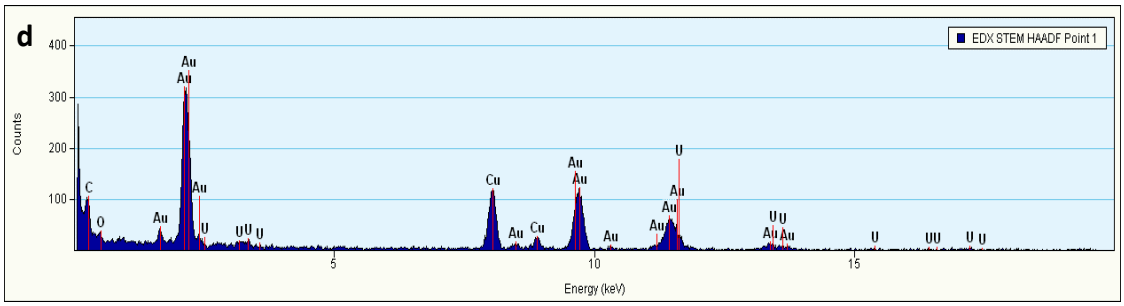


Figure 4: STEM images and EDS graphs of (a,b) 4x concentration of 20 nm AuNPs (c,d) 4x concentration of 100 nm AuNPs

3.3 Fourier-Transform Infrared Spectroscopy

The fibril structure of polymerized type I collagen and four compositions of the viscoelastic collagen was examined utilizing FTIR. The acquired FTIR spectra are shown in Figure 5 and ranged from 1000-1750 cm^{-1}. Results demonstrated similar collagen characteristic peaks between the viscoelastic collagen and polymerized collagen, as well as similarity in structure of the viscoelastic collagen to those reported in the literature. [48] Examining Figure 5, the amide III peak can be observed at around 1250 cm^{-1} and corresponds to C-N stretching, N-H bending, and C-C stretching. [48, 49] CH_2 bending and CH_2 side-chain vibrations peaks were observed at around 1338 cm^{-1}-1450 cm^{-1} [49, 50]. Amide II peaks were observed between 1500-1595 cm^{-1} [49]. These peaks can be attributed to C-N stretching and N-H bending [49]. Amide I peaks were observed at around 1595-1740cm^{-1} and correspond to C=O stretching and N-H bending [49].

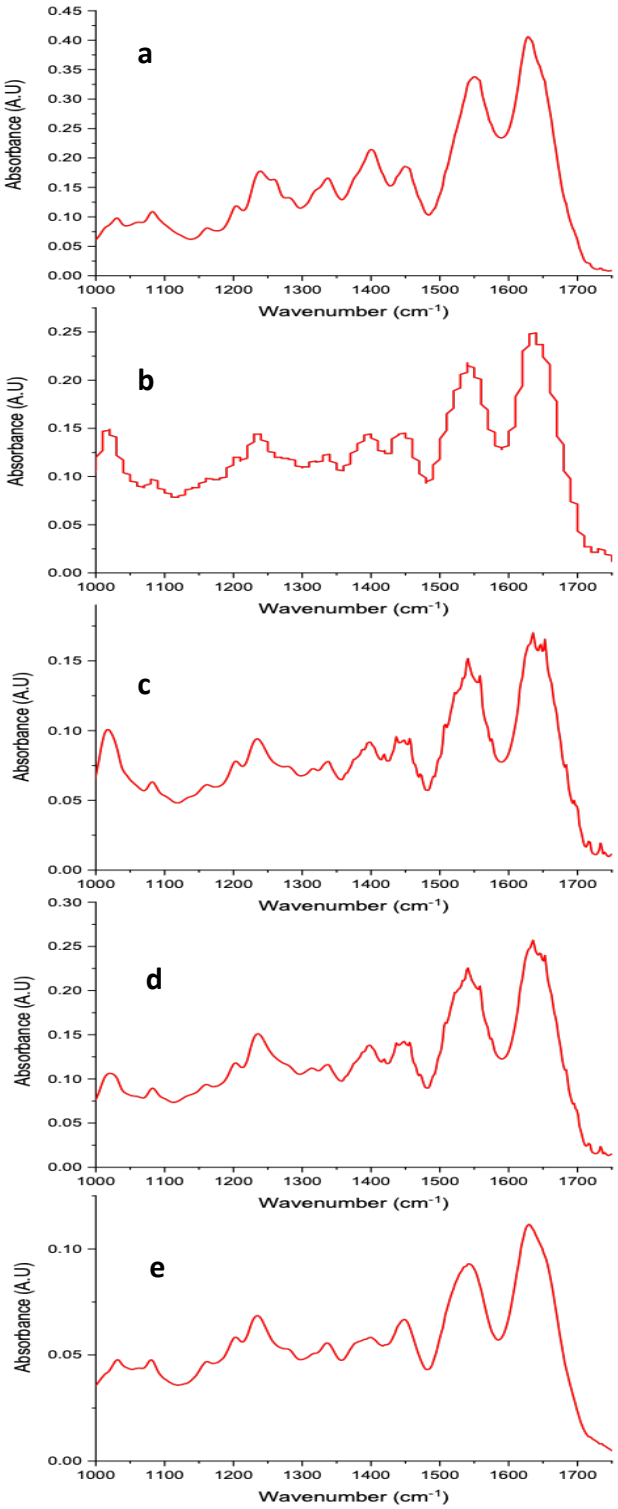


Figure 5: FTIR of (a) polymerized collagen (b) 1x, 20nm (c) 1x, 100nm (d) 4x, 20nm (e) 4x, 100nm

3.4 Neutron Activation Analysis

Neutron Activation Analysis was utilized in determining how much gold is present on the viscoelastic collagen at different concentrations. In Figure 6, an increase in the number of gold nanoparticles is seen between the 1x and 4x concentrations for both 20 and 100nm size. It is also observed that there is a higher amount of Au on the 1x, 20nm VE collagen than the 1x, 100nm collagen. A higher number of Au is seen on the 4x, 100nm VE collagen than the 4x, 20 nm collagen.

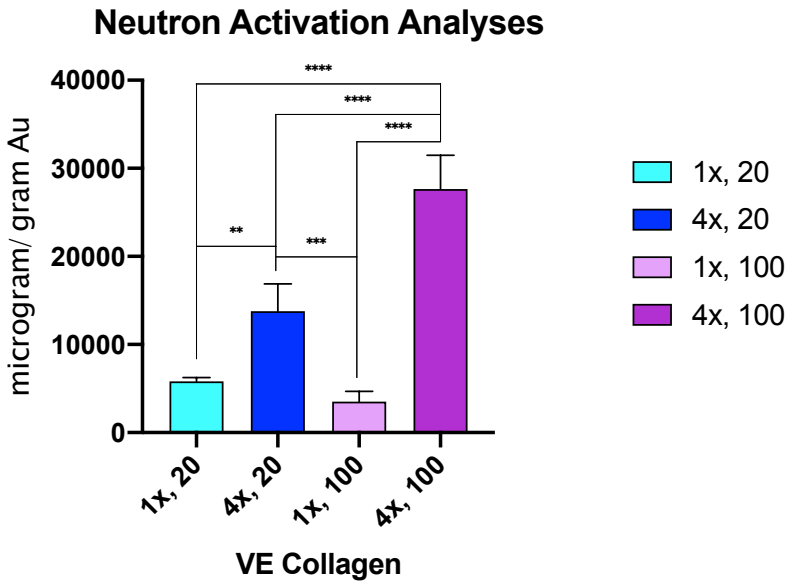


Figure 6: Results from Neutron Activation Analysis. * = p value < 0.05. ** = p value < 0.01. *** = p value < 0.001. **** = p value < 0.0001

3.5 Cell Viability

A cell viability study was performed to determine the biocompatibility of the viscoelastic collagen with gold nanoparticles and genipin crosslinker. The results, shown in Figure 7, provide a comparison of cell viability between the viscoelastic collagen

samples incubated with murine fibroblast cells and the positive control of fibroblast cells for 5- and 7-day time points. On day 5, a significant difference was only observed for the genipin crosslinked viscoelastic collagen which showed the lowest cellular viability at 86%. All VE collagen samples displayed cellular viability greater than 80%. The collagen sample with a 1x concentration of 100 nm AuNPs had the highest cell viability at 99%, followed by the 1x concentration of 20 nm AuNPs at 97%. The VE collagen crosslinked with genipin with no gold nanoparticles had the lowest cell viability at 86%. Observing the results from day 7, all VE collagen samples had cell viability greater than the positive control except for the natural viscoelastic collagen at 94% and the 4x concentration of 20 nm AuNPs at 95%. Upon further evaluation, an increase in cell viability was also observed for all the VE collagen compositions except for the natural VE collagen.

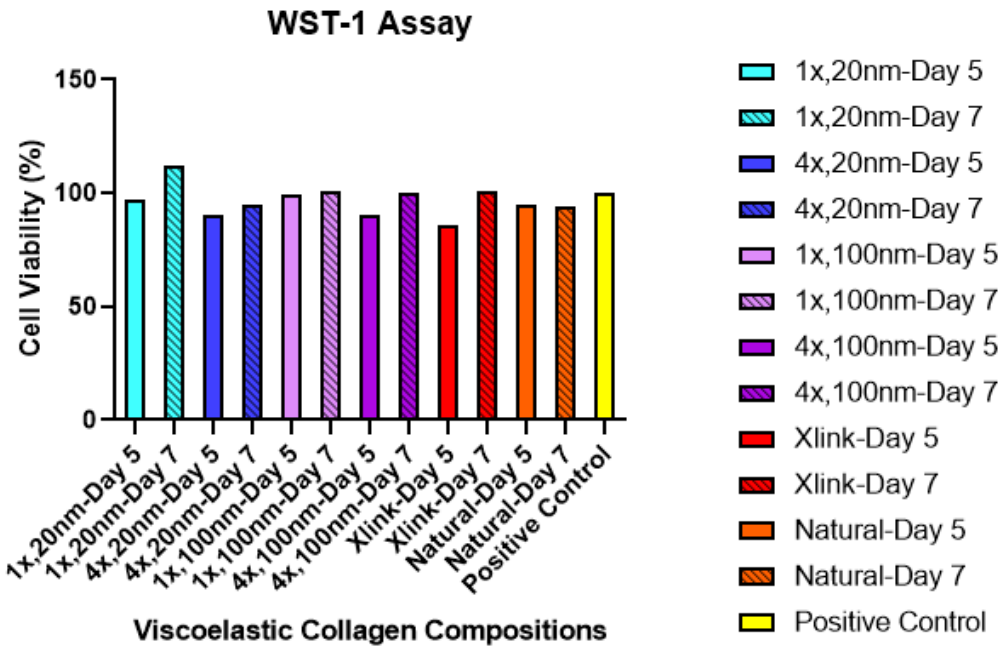


Figure 7: Cell Viability Study for 5 and 7 day

3.6 Quasi-Static Loading

Quasi-Static Loading was performed on the viscoelastic collagen samples to determine the compressive stiffness of the material. Figure 8 provides a comparison of compressive stiffness between the different concentrations and sizes of the gold nanoparticles. The data was plotted on a graph of time vs. force and the slope of the line was calculated to determine stiffness. Results show stiffness of the VE collagen samples is comparable with the 4x concentration of 100 nm AuNPs having the highest stiffness. An increase in stiffness when nanoparticles were attached vs. VE collagen with just genipin crosslinker was also noted. A significant difference was observed between the genipin only crosslinked VE collagen and the 1x and 4x concentrations of the 100nm AuNPs.

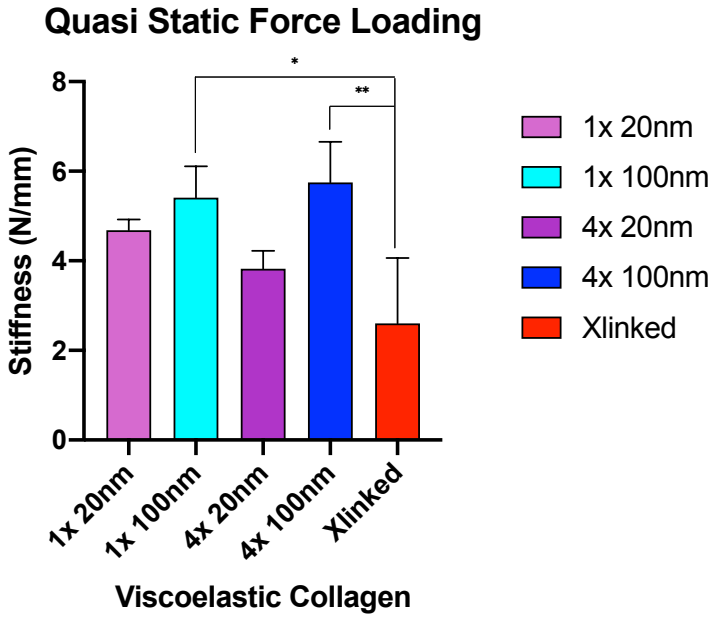


Figure 8: Results from Quasi Static Loading. * = p value < 0.05. ** = p value < 0.01

3.7 Reactive Oxygen Species Assay

ROS assay was conducted to determine the anti-inflammatory properties of the VE collagen with AuNPs. Results show a significant difference in the number of reactive oxygen species between the negative control of only cells and the collagen samples with 1x and 4x concentrations of 20nm AuNPs and 1x concentration 100nm AuNPs.

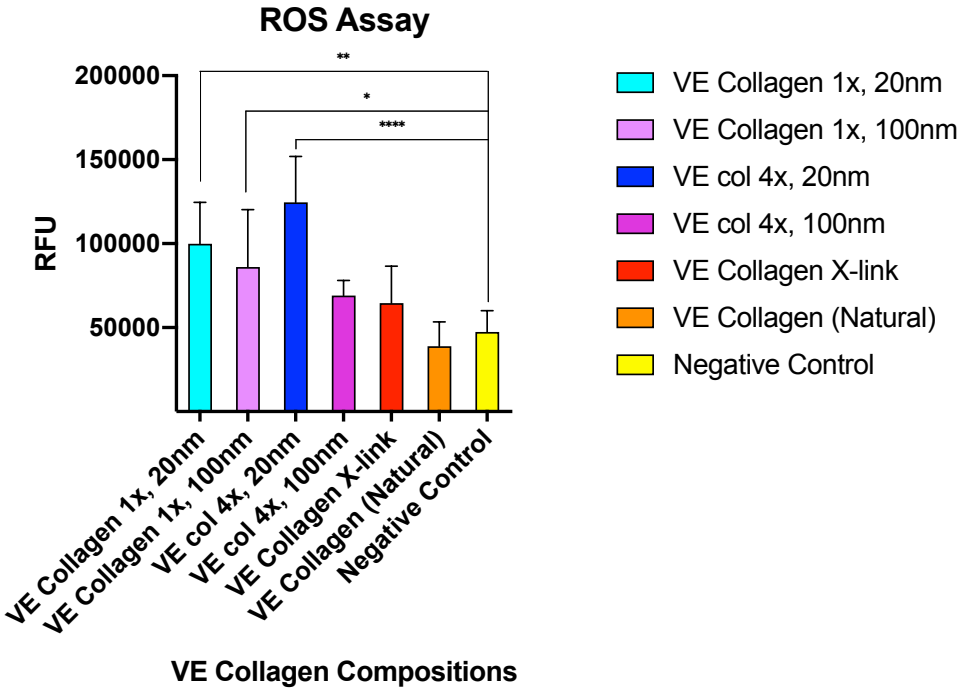


Figure 9: Results from the Reactive Oxygen Species Assay. * = p value < 0.05. ** = p value < 0.01. **** = p value < 0.0001

3.8 Differential Scanning Calorimetry

Differential Scanning Calorimetry was also performed to evaluate the thermal properties of the viscoelastic collagen and to determine the denaturation temperature.

Results from this study showed no denaturation temperature for any of the viscoelastic collagen composition types except for the pre viscoelastic collagen.

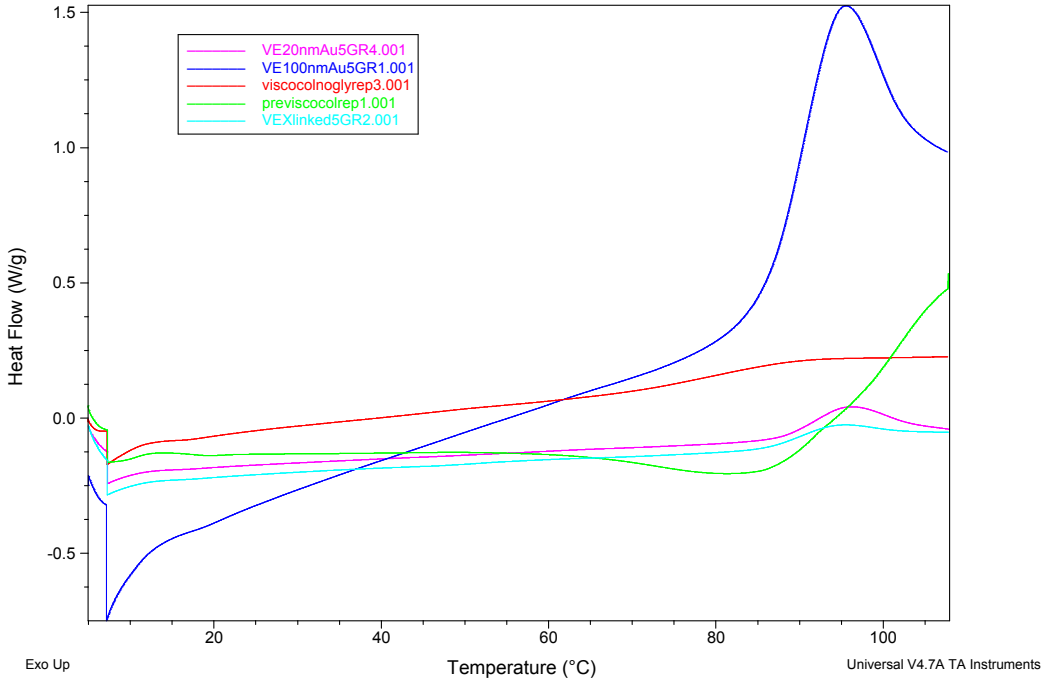


Figure 10: Differential Scanning Calorimetry for the viscoelastic collagen compositions

4. Discussion

There is a need for a less invasive treatment for intervertebral disc degeneration, with current research investigating cell and protein-based therapies. Collagen type II is prevalent in a healthy intervertebral disc; however, studies have observed the decrease of this collagen type and subsequently an increase in type I collagen synthesis during degeneration [51, 52]. This alteration in synthesis from gel collagen to the strong collagen fibers formed from type 1 collagen can impair the viscoelastic properties of the NP [51]. Prior studies have also demonstrated that during disc degeneration, a decrease in

proteoglycans is observed which may lead to a decrease in hydration and an increase in stiffness [53]. We are developing a gel-like collagen-based material to mimic the nucleus pulposus without displaying the increased stiffness and fiber formation that is observed in the nucleus pulposus of a degenerated disc. Inflammation and degradation of the extracellular matrix of the disc are also features observed in degeneration. Both gold nanoparticles and genipin have demonstrated anti-inflammatory properties and were utilized in the development of this material [54, 55].

Confirmation of the non-polymerized structure of the viscoelastic collagen and attachment of the gold nanoparticles was determined using several characterization techniques. In Figure 1, TEM images confirmed that the typical banding structure of type 1 polymerized collagen was not observed. While collagen fibrils were seen, the normal stacking or D patterns of the collagen fibrils to form collagen fibers was prevented by the addition of acyl groups during the development of the viscoelastic collagen. [56, 57, 58, 59] Buddy Ratner et al. (2004) discussed and provided images for the structure of non-banded collagen when exposing type 1 bovine hide collagen to a pH below 4.25 [60]. The TEM and STEM images from Figure 2-3 also confirmed a banding pattern of the collagen fibrils was still not present even after conjugation of the gold nanoparticles using the genipin crosslinker [47].

Observing Figure 5, results from the FTIR spectra demonstrated that each of the viscoelastic collagen composition types displayed the same characteristic peaks of collagen as the polymerized collagen. Differential Scanning Calorimetry was also performed to evaluate the thermal properties of the viscoelastic collagen and to determine the denaturation temperature. Results from this study showed no denaturation

temperature for any of the viscoelastic collagen composition types. Inferences can be made that the viscoelastic collagen's three-dimensional structure and hydrogen bonds were not disrupted; the loss of protein functionality did not occur when undergoing higher temperatures which are typically seen with polymerized collagen [50]. The results from the FTIR and DSC confirmed that the viscoelastic collagen consists of collagen fibrils, not fibers, while still maintaining a similar structure to that of polymerized collagen.

In the NAA study (Figure 6), an increase in Au was observed when the AuNP concentration was increased in the collagen. This is demonstrated for both the 20 and 100 nm size gold nanoparticles. A higher amount of 20 nm size gold nanoparticles at the 1x concentration was observed than the 100nm AuNPs. However, for the 4x concentration, a significantly higher amount of Au for the 100 nm gold nanoparticles was observed. This could be due to the larger surface area allowing for more attachment sites of the gold nanoparticles to the viscoelastic collagen.

Biocompatibility assessment is important for any material that will be implanted or injected into the body. For our study (Figure 7), we evaluated the trend in cellular viability and overall biocompatibility of the viscoelastic collagen with genipin and different sizes and concentrations of gold nanoparticles. On day 5, the collagen with just the genipin crosslinker had the lowest cellular viability at 86%, while the cellular viability was 90% or greater for the VE collagen of both sizes and concentrations, demonstrating cellular viability equivalent to the positive control. The highest cellular viabilities were observed for the 1x concentration of both the 20 and 100 nm size gold nanoparticles. From day 5 to day 7, an increase in cellular viability was observed at the

1x and 4x concentrations for both nanoparticle sizes as well as for the genipin crosslinked collagen. All VE collagen compositions had cellular viability of 94% or higher, demonstrating equivalence to the positive control. The 1x concentration of the 20 nm AuNPs showed significantly higher cell viability than the positive control, demonstrating a trend towards enhanced cellular viability. Prior studies have indicated that gold and other metallic nanoparticles promote cellular proliferation [61, 62]. This increase in cell proliferation could be a result of cellular attraction to the high surface energy of AuNPs [63]. Overall, all the viscoelastic collagen samples displayed biocompatibility with the 1x concentration of gold nanoparticles for both nanoparticles demonstrating the higher viabilities for both days 5 and 7.

The quasi-static force test was performed as a preliminary study and was conducted to evaluate the viscoelastic collagen materials under unconfined compression to determine stiffness. Upon observation, results from Figure 8 demonstrated a dose dependence for 100nm AuNPs (more NP, increased stiffness) and a size dependence (larger NP size, increased stiffness). Results also demonstrated a higher stiffness when nanoparticles were attached to the VE collagen. This higher stiffness with 100nm gold nanoparticles could be due to more attachment sites on the 100nm AuNPs versus the 20nm, leading to an increase in stiffness. This higher stiffness could also be due to the AuNPs causing a disruption to the slippage of the collagen fibrils over each other and thus increasing the stiffness. This increase in stiffness demonstrates that with different size and concentrations of gold nanoparticles, the material property can be varied and is tunable. Stiffness was determined by calculating the slope in the linear region of the force-displacement response. Prior studies have also utilized this method of using the

force-displacement curve to calculate stiffness [64]. However, stiffness was computed for the entire disc, while we want to evaluate just the nucleus pulposus, as the load placed on a multi-layer structure such as the disc is different than that placed on the nucleus pulposus [65]. Future studies would include procuring and isolating the NP from a healthy and degenerated disc. We would test the NP under confined and unconfined compression to calculate the stiffness, so a comparison between our material and the NP can be done.

A ROS assay was performed to evaluate the potential anti-inflammatory properties of the viscoelastic collagen with genipin and gold nanoparticles. The results demonstrated a significant difference between the negative control of just cells and the collagen samples with the 1x and 4x concentrations of 20nm AuNPs and the 1x concentration 100nm AuNPs. These results do not support prior studies on the anti-inflammatory properties of gold nanoparticles and genipin [66]. Studies have shown that genipin does emit a fluorescence from 380-700nm, which could contribute and influence the results of the ROS, as the fluorescence measured in the ROS experiment had a 530 nm emission and 480 nm excitation [67]. Future studies would include using a control of each of the viscoelastic collagen compositions with no cells to create a baseline to be subtracted from each sample measurement. A time point study (24, 36, 48 hours) could also be conducted to ascertain if the amount of ROS decreases over time for VE collagen samples. We could also perform the study with unsupplemented cell media instead of DPBS.

5. Conclusion

This research study evaluated the development of a treatment for intervertebral disc degeneration. Collagen is a component of the discs' ECM and has been utilized in tissue engineering applications. Gold nanoparticles were conjugated to the viscoelastic collagen using the genipin crosslinker. Oxidative stress and inflammation are both observed during disc degeneration. Gold nanoparticles and genipin have both demonstrated anti-inflammatory properties, while gold nanoparticles have also displayed anti-oxidative properties and served as a chemoattractant.

The goal of this study was to develop, fabricate and characterize different formulations of non-polymerized viscoelastic collagen conjugated with AuNPs and a genipin crosslinker to assess the feasibility as a tissue template for disc regeneration. Results demonstrated the attachment of gold nanoparticles, biocompatibility, and an increase in stiffness of the material with different sizes and concentrations of AuNPs. Results from the TEM and STEM demonstrated that the viscoelastic collagen that was developed did not display the characteristic D banding pattern of polymerized collagen. Qualitative (STEM) and quantitative (EDS) studies were also conducted to confirm the conjugation of the gold nanoparticles to the viscoelastic collagen. FTIR results demonstrated the same characteristic peaks of collagen as the polymerized collagen, while the NAA studies confirmed the increase in gold nanoparticle attachment to the collagen with the increase in concentration. The WST-1 assay studies confirmed the biocompatibility of the viscoelastic collagen compositions on both days 5 and 7. Finally, the quasi-static loading test was conducted to evaluate the mechanical properties of the

collagen. Results demonstrated a dose dependence for 100nm AuNPs and size dependence. The findings from this study will lead to the development of more efficient and cost-effective treatments for patients with chronic back pain caused by IVD degeneration.

Section II: Viscoelastic Collagen Studies with Glycerol

1. Introduction

This section involves the utilization of glycerol to change the physical properties or viscosity of the viscoelastic collagen. The goal is to change the viscosity of the collagen material by adding different amounts of glycerol (0, 5, 10, and 15%) to mimic the mechanical properties of the nucleus pulposus. Glycerol has been utilized in previous studies with collagen as a plasticizer [68].

2. Materials and Methods

2.1 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was utilized to determine the denaturation temperature of the viscoelastic collagen templates. The different viscoelastic collagen and glycerol compositions samples of 2– 5 mg were placed in Tzero plates/hermetic lids and sealed using the Tzero press (TA Instruments, New Castle, DE). Two microliters of double distilled water were used as the reference pan. Each of the samples underwent modulated differential scanning calorimetry using the Q2000 DSC (TA Instruments) to ramp the temperature from 5°C to 120°C at a rate of 5°C per minute.

The mean denaturation temperature (°C) is reported below in Figure 1. Five trials/repetitions of the samples were performed.

2.2 Cell Viability

L929 murine fibroblast cells (ATCC) were cultured in Eagle's Minimum Essential Media supplemented with 10% (v/v) horse serum and 200 U/mL of Penicillin Streptomycin at 36.8 ºCelsius and 5% CO_2.

A Water-Soluble Tetrazolium Salt Assay was conducted to determine the cell viability for each of the various compositions of VE collagen. Samples were sterilized with peracetic acid. The Natural VE collagen material was autoclaved for 45 minutes in order to sterilize due to its liquid form. The collagen samples were then incubated for 24 hours with supplemented cell media (horse serum and Penn strep). Cell media was then removed and L929 murine fibroblast cells were seeded at a concentration of 8.0×10^4 cells/mL. Assays were run with cells in contact with the VE collagen materials for 3 days. On day 3, the WST reagent was added and allowed to incubate for 4 hours. After 4 hours, media from each well was removed to a new well plate, and absorbance was read at 450 nm with a reference reading at 655nm using a spectrofluorometer plate reader (Cytation 5, BioTek). The baseline for 100% cell viability consisted of the positive control of fibroblast cells with no VE collagen materials.

2.3 Rheology (Viscometer)

A Haake RS100 Rheometer machine was utilized to determine the rheology properties of the glycerol and viscoelastic collagen samples. An Instron 8821s was utilized to place a 1000 N force load at a 15 cycle/min on the material.

3. Results

3.1 Differential Scanning Calorimetry

Differential Scanning Calorimetry was conducted to evaluate the thermal properties of the viscoelastic collagen and glycerol to determine the denaturation temperature. Results from this study, shown in Figure 11, demonstrated no denaturation temperature for any of the viscoelastic collagen and glycerol composition types except for the pre-viscoelastic collagen.

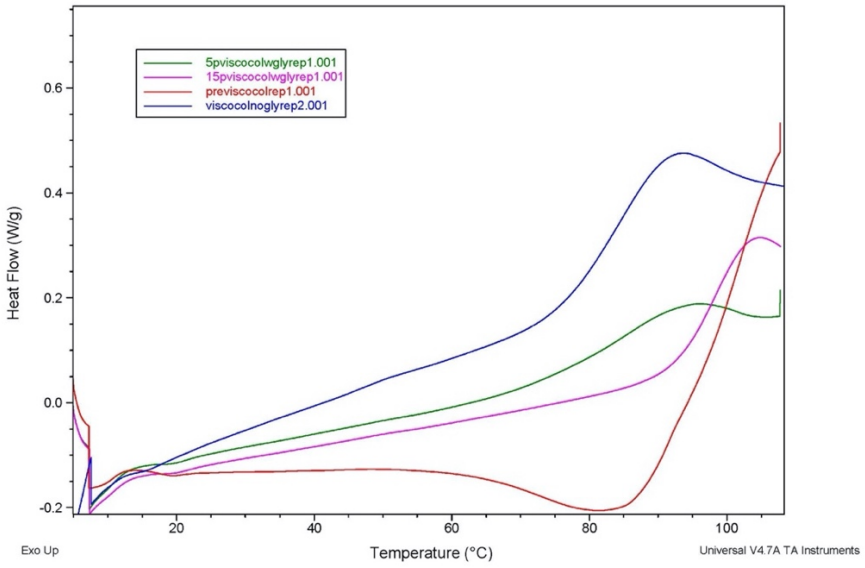


Figure 11: DSC for viscoelastic collagen and glycerol compositions

3.2 Cell Viability

A WST-1 assay was performed to evaluate the biocompatibility of the viscoelastic collagen samples with glycerol at 0, 5, and 15%. Results in Figure 12 show that as the amount of glycerol added increased, the number of viable cells decreased when compared among samples and to the positive control. Viscoelastic collagen with 0% glycerol had the highest cell viability at 129%, which surpassed the positive control. This demonstrates a trend towards enhanced cellular viability. The 5% glycerol had the second-highest number of viable cells across the samples at 94%, while the viscoelastic collagen with 15% glycerol had a cell viability of 40%, which is significantly less than the 80% threshold utilized to confirm biocompatibility. Results from this study conclude that moving further, only the 0 and 5% glycerol collagen samples should be utilized, as cytotoxicity is observed when the concentration of glycerol added to the viscoelastic collagen is at 15%.

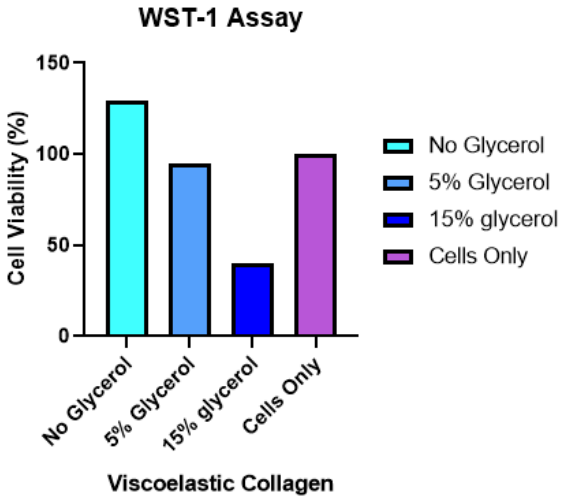


Figure 12: 3-day Cell Viability Study for Viscoelastic Collagen with glycerol

3.3 Rheology

Rheology studies were conducted to determine the physical or flow behavior of the viscoelastic collagen with different amounts of glycerol added. The shear rate was increased in increments to determine the shear stress at that respective shear rate. Results from the rheology study demonstrate that the viscoelastic collagen with the glycerol has a shear-thickening flow behavior. As the shear rate increases, an increase in shear stress is observed for both the 5% and 15% glycerol viscoelastic collagen samples, demonstrating that the glycerol-collagen sample's viscosity increases at higher shear rates. Comparing the curves for the 5% and 15% glycerol-collagen samples, the 15% glycerol collagen sample has a slightly higher viscosity than the 5%. This rheology study also demonstrates that both the 5% and 15% glycerol have similar viscosities despite the difference in glycerol added. This is important since the cell viability study showed cytotoxicity of fibroblast cells for the 15% glycerol viscoelastic collagen samples. Future studies should include a rheology study comparing the viscoelastic collagen to a healthy and a degenerated nucleus pulposus.

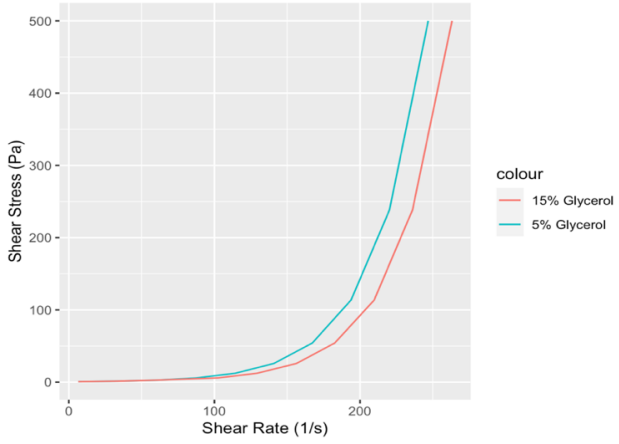


Figure 13: Rheometer data for 5% and 15% glycerol viscoelastic collagen samples

Section III: Factorial Design Study

1. Introduction

The goal of this study was to determine the main and interaction effects of nanoparticle size and concentration on cell viability for the viscoelastic collagen materials. The response variable of this study was cell viability/# of live cells=absorbance. This can be measured by performing a live/dead assay (WST-1 Assay) to determine cell viability and cytotoxicity. Fibroblast cells were seeded for 24 hours at a concentration of $8x10^4$ cells/mL and then incubated with the viscoelastic collagen materials for 3 days. The absorbance of viable cells was then measured. In this experiment, a sample size of n=4 was utilized.

2. Choice of Factors

Originally, a 2^3 factorial design was to be utilized to observe further what factors (crosslinker, nanoparticle size, nanoparticle concentration) of the different treatments was affecting cell viability. One concern with using this design is that since the templates/treatments were developed before the study, the same concentration of genipin crosslinker (12mM) was utilized for developing all the treatments with the exception of the natural VE collagen. Another study would have to be conducted where the concentration of genipin was not constant (ex. 3mM and 5mM) in order to use this factorial design. It was then decided to look at a 2^2 factorial design to investigate the effects of nanoparticle concentration, nanoparticle size, and the interaction between the two on cell viability. Equation 1 displays the regression model utilized in this study, with

β serving as the regression coefficients. The terms x^1 and x^2 represent factor A (nanoparticle concentration) and B (nanoparticle size), while the term x^1x^2 represents the interaction between nanoparticle size and concentration.

$$Y= \beta_O + \beta_1 x^1 + \beta_2 x^2 + \beta_{12} x^1 x^2 \quad (1)$$

Table 2: Material Description

Viscoelastic Collagen	**Description**
1x, 20	• 1x concentration of 20 nm size AuNPs
1x, 100	• 1x concentration of 100 nm size AuNPs
4x, 20	• 4x concentration of 20 nm size AuNPs
4x, 100	• 4x concentration of 100 nm size AuNPs
Crosslinked	• Genipin with no AuNPs
Natural	• No genipin or AuNPs

Table 3: Factorial Design

Factor A: Nanoparticle Concentration	**Factor B: Nanoparticle Size**
Low (-1): 1x concentration	Low (-1): 20 nm size AuNPs
High (+1): 4x Concentration	High (+1): 100 nm size AuNPs

Table 4: Design Matrix

Identity (one)	**Factor A**	**Factor B**
+	-	-
+	+	-
+	-	+
+	+	+
+	-	-
+	+	-
+	-	+
+	+	+

2.1 Part A

A concern with the use of collagen as a biomaterial is its limited mechanical strength and rapid biodegradation. To overcome these limitations, chemical crosslinking agents are utilized to improve mechanical strength and stability, attach nanoparticles, as well as to allow more control of the degradation rate of the collagen. The first part of this evaluated the use of a genipin crosslinker attached to the VE collagen. The viscoelastic collagen samples were sterilized with peracetic acid and aseptic techniques were utilized. A 3-day WST-1 assay was conducted for the treatments listed in Table 1. A comparison of the cell viability between VE collagen with and without the crosslinker was assessed to ascertain which treatment has higher cell viability and thus serve as a suitable treatment for disc degeneration. This preliminary analysis helped determine if there is a significant difference between the absorbance means of the VE collagen compositions/treatments (no crosslinker/no AuNPs, crosslinker/no AuNPs) vs. the positive control. The positive control consisted of just fibroblast cells with no interaction with treatments to serve as a baseline for 100% cell viability (cells that have no interruption to their environment). Biocompatibility is achieved when cell viability is greater than 80%. A Dunnett's multiple comparison One-way ANOVA test was used to analyze the results.

2.2 Analysis of Part A

Observations from Figure 14 demonstrated a significant difference (p-value < 0.0001) in absorbance for the Natural VE collagen (no crosslinker) and the positive control. There appears to be no difference between the VE Collagen with the genipin crosslinker and the positive control. Normalizing the data, there was higher cell viability

of the VE collagen with the genipin crosslinker at 98%, while the Natural VE collagen had a cell viability of 76% (which is < 80%). After analysis of this data demonstrated that the VE collagen with the genipin crosslinker is biocompatible, we chose to continue with this material.

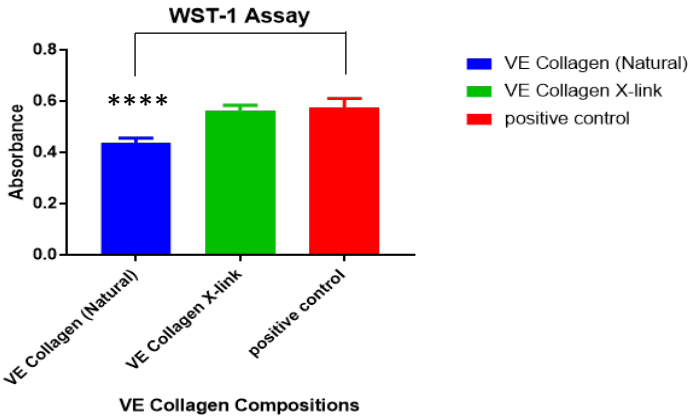


Figure 14: Biocompatibility study for natural and crosslinked viscoelastic collagen**.**

****= p-value < 0.0001

2.3 Part B

Twenty and 100 nm size AuNPs were selected due to previous studies in our lab demonstrating good biocompatibility. 1x and 4x concentrations of AuNPs were selected to observe the effects of different amounts of AuNPs attached to the VE Collagen. The absorbance and cell viability of these different VE collagen compositions was assessed to ascertain biocompatibility. This preliminary analysis helped determine if there is a significant difference between the absorbance means of the VE collagen compositions/treatments (1x and 4x concentration, 20 and 100nm AuNPs) vs. positive

control. A Dunnett's multiple comparison one-way ANOVA test was used to analyze the results.

2.4 Analysis of Part B

Observations from Figure 15 confirm that there are no significant differences in cell viability between the positive control and the VE collagen templates. Normalizing the data, results showed that the cell viability of each of the treatments is greater than 80%. The results from the ANOVA confirmed that there are no significant differences in cell viability between the positive control and the VE collagen templates. Analysis of this data demonstrated that the four different treatments were all biocompatible.

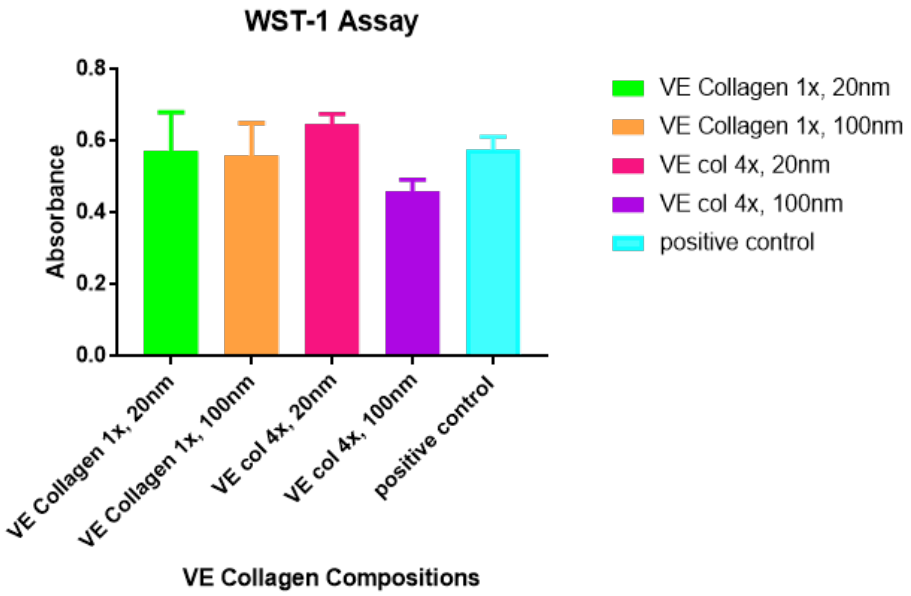


Figure 15: Biocompatibility Study for viscoelastic collagen with 1x concentration (20 and 100nm AuNPs) and 4x concentration (20 and 100nm AuNPs)

2.5 Part C

After conducting the analysis for Parts A and B, next, we evaluated whether there was a significant difference in cell viability between the different VE collagen templates from Figure 15. A Tukey's multiple comparison test was run, and the results demonstrated a significant difference in cell viability (p-value < 0.05) between the VE collagen (4x, 20nm) and VE collagen (4x, 100nm) templates.

2.6 Part D

Finally, after observing and confirming a significant difference in cell viability for the VE collagen templates (4x, 20, and 100nm), we wanted to study which factors were influencing this difference in cell viability. Though the two treatments are different because of nanoparticle size (20 and 100nm), we also wanted to look at if nanoparticle concentration played a role in influencing the difference seen in cell viability. A 2^2 factorial design was conducted.

2.7 Analysis of Part D

In Figures 16, p-values < 0.05 for nanoparticle size and the interaction between nanoparticle size and concentration. These results suggest a statistically significant main effect of the nanoparticle size on cell viability and a statistically significant interaction between the two factors (size and concentration). Figure 17 also supports this conclusion, as a statistically significant difference for the coefficient estimates (t value < 0.05) for both nanoparticle size and the interaction between size and concentration was observed. In Figure 18, the results from the interaction plot suggest that the interaction between the

4x concentrations of the 20 and 100 nm size AuNPs could attribute to the significant interaction effect observed on cell viability. Reviewing the p-values, t-values, and the interaction plot, it was determined that nanoparticle size and its interaction with nanoparticle concentration demonstrated statistically significant effects on the response variable (cell viability).

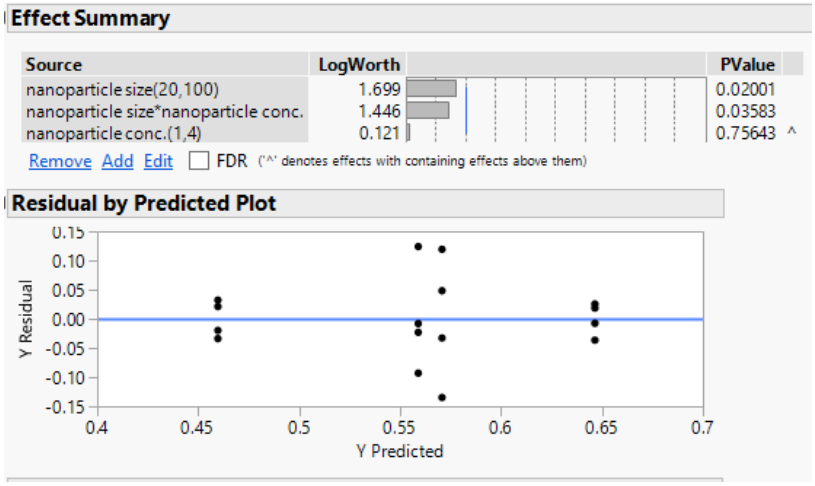


Figure 16: Effect Summary and Residual Plot

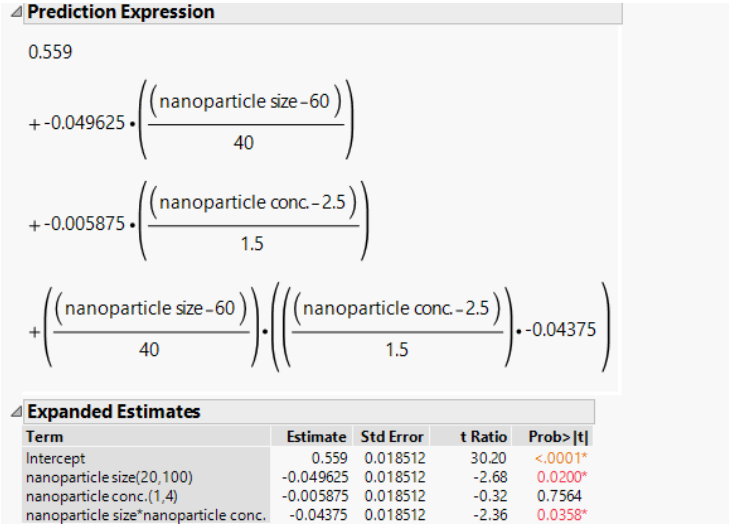


Figure 17: Coefficient Estimate Analysis

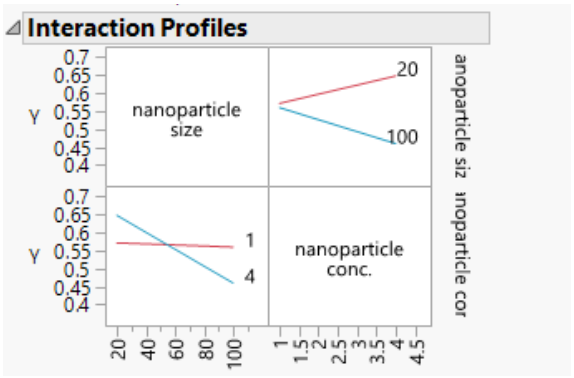


Figure 18: Interaction Plots

3. Conclusion

The results from this study suggest that nanoparticle size has a significant main effect on cell viability for the viscoelastic collagen materials. Results also suggest a significant interaction effect between nanoparticle size and concentration at the 4x concentration for both 20 and 100nm gold nanoparticles.

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Chapter Five

Increasing Adoption Rates at Animal Shelters: A Two-phase Approach to Predict Length of Stay and Optimal Shelter Allocation

Abstract

Background: Among the 6-8 million animals that enter the rescue shelters every year, nearly 3-4 million (i.e., 50% of the incoming animals) are euthanized, and 10% - 25% of them are put to death specifically because of shelter overcrowding each year. The overall goal of this study is to increase the adoption rates at animal shelters. This involves predicting the length of stay of each animal at shelters considering key features such as animal type (dog, cat, etc.), age, gender, breed, animal size, and shelter location.

Results: Logistic regression, artificial neural network, gradient boosting, and random forest was used to develop models to predict the length of stay. The performance of these models was determined using three performance metrics: precision, recall, and F1 score. The results demonstrated that the gradient boosting algorithm performed the best overall, with the highest precision, recall, and F1 score. Upon further observation of the results, it was found that age for dogs (puppy, super senior), multicolor, and large and small size were important predictor variables.

Conclusion: The findings from this study can be utilized to predict and minimize the animal length of stay in a shelter and euthanization. Future studies involve determining

which shelter location will most likely lead to the adoption of that animal. The proposed two-phased tool can be used by rescue shelters to achieve the best compromise solution by making a tradeoff between the adoption speed and relocation cost.

Keywords: Animal shelter; High euthanization rates; Machine learning algorithms; Prediction models; Goal programming approach; Decision support tool.

1. Background

As the problem of overpopulation of domestic animals continues to rise, animal shelters across the nation are faced with the challenge of finding solutions to increase the adoption rates. In the United States, about 6-8 million dogs and cats enter animal shelters every year, and 3-4 million of those animals are euthanized [1]. In other words, about 50% of the total canines and felines that enter animal shelters are put to death annually. Moreover, 10 - 25% of the total euthanized population in the United States is explicitly euthanized because of shelter overcrowding each year [2]. Though animal shelters provide incentives such as reduced adoption fees and sterilizing animals before adoption, only a quarter of total animals living in the shelter are adopted.

1.1 Animal Adoption from Shelters and Rescues

There are various places to adopt an animal, and each potential owner must complete the adoption process and paperwork in order to take their new animal home [3]. Public and private animal shelters include animal control, city and county animal shelters, and police and health departments. Staff and volunteers run these facilities. Animals may also be adopted from a rescue organization, where pets are fostered in a home or a private

boarding facility. These organizations are usually run by volunteers and animals are viewed during local adoption events that are held at different locations, such as a pet store [3].

There could be several reasons for euthanization of animals in a shelter such as overcrowding, medical issues (ex. sick, disabled), or behavioral issues (ex. too aggressive). Reasons for the overpopulation of animals include failure to spay or neuter animals leading to reckless breeding habits and abandonment or surrender of offspring, animal abandonment from owners who are no longer able to take care of or don't want the animal, and owners still buying from puppy mills and pet stores [4]. This overpopulation of animals, specifically cats and dogs, leads to overcrowding in shelters with large numbers of healthy adoptable animals. This is a problem as there are a limited number of shelters that have a finite room capacity for animals that are abandoned or surrendered [5]. Though medical and behavioral issues are harder to solve, the overpopulation of healthy adoptable animals in shelters is a problem that can be addressed through machine learning and predictive analytics.

1.2 Literature Review

In this section, we describe the research conducted on animal shelters evaluating euthanasia and factors associated with animal adoption. The articles discussed provide insight into factors that influence the length of stay and what characteristics influence adoption.

Studies have been conducted investigating the positive influence of pre-adoption neutering of animals on the probability of pet adoption [2]. The author investigated the impact of the cooperation of veterinary medical schools in increasing pet adoption by offering free

sterilization. Results demonstrated that the collaboration between veterinary hospitals and local animal shelters decreased the euthanization of adoptable pets.

Hennessy et al. (2001) conducted a study to determine the relationship between the behavior and cortisol levels of dogs in animal shelters and examine its effect in predicting behavioral issues after adoption [6]. Shore et al. (2005) analyzed the reasons for returning adopted animals by owners and obtained insights for these failed adoptions to attain more successful future approvals [7]. The researchers found that prior failed adoption had led to longer-lasting future acceptances. They hypothesized that the failed adoptions might lead owners to discover their dog preferences by assessing their living situation and the type of animal that would meet that requirement. Morris et al. (2014) evaluated the trends in income and outcome data for shelters from 1989-2010 [8]. The results showed a decrease in euthanasia and intake was observed, however for cats there was an increase.

Fantuzzi et al. (2010) explored the factors that are significant for the adoption of cats in the animal shelter [9]. The study investigated the effects of toy allocation, cage location, and cat characteristics (such as age, gender, color, and activity level). Results demonstrated that more active cats that possessed toys and viewed at eye level were more likely to impress the potential adopter and be adopted. Brown et al. (2015) conducted a study evaluating the influence of age, breed, color, and coat pattern on the length of stay for cats in a no-kill shelter [10]. The authors concluded that while color did not influence the length of stay for kittens, whereas, gender, coat patterning, and breed were significant predictors for both cats and kittens.

1.3 Machine Learning

Machine learning is one possible tool that can be used to identify risk factors for animal adoption and predict the length of stay for animals in shelters. Machine learning is the ability to program computers to learn and improve all by itself using training experience [11]. The goal of machine learning is to develop a system to analyze big data, quickly deliver accurate and repeatable results, and adapt to new data independently. A system can be trained to make accurate predictions by learning from examples of desired input-output data. More specifically, machine learning algorithms are utilized to detect classification and prediction patterns from large data and to develop models to predict future outcomes [12]. These patterns show the relationship between the attribute variables (input) and target variables (output) [13].

Widely used data mining tasks include supervised learning, unsupervised learning, and reinforcement learning [14]. Unsupervised learning involves the use of unlabeled datasets to train a system for finding hidden patterns within the data [15]. Clustering is an example of unsupervised learning. Reinforcement learning is where a system is trained through direct interaction with the environment by trial and error [15]. Supervised learning encompasses classification and prediction using labeled datasets [15]. These classification and regression algorithms are used to classify the output variable with a discrete label or predict the outcome as a continuous or numerical value. Traditional algorithms such as neural networks, decision trees, and logistic regression typically use supervised learning. Figure 1 provides a pictorial of the steps for developing and testing a predictive model.

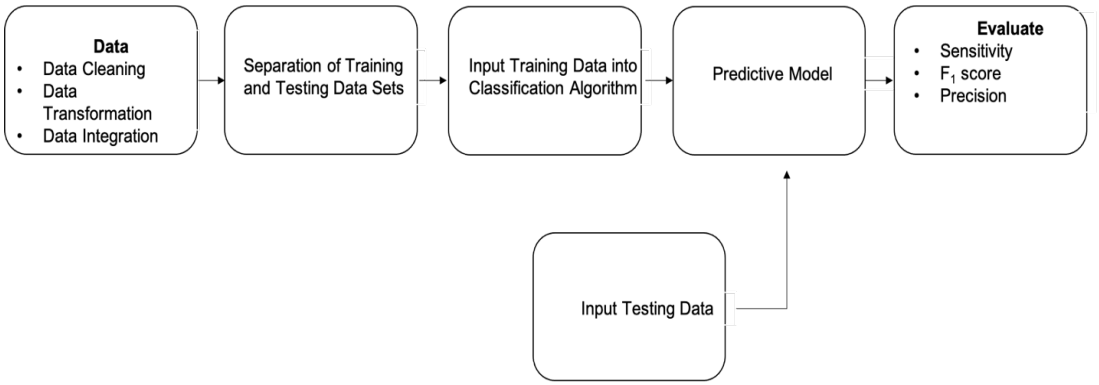

Figure 1: Pictorial Representation of Developing a Predictive Model

1.4 Contributions to the Literature

Although prior studies have investigated the impact of several factors, such as age and gender, on the length of stay, they focus on a single shelter, rather than multiple organizations, as in this study. The goal of this study is to investigate the length of stay of animals at shelters and the factors influencing the rate of animal adoption. The overall goal is to increase adoption rates of pets in animal shelters by utilizing several factors to predict the length of stay. Machine learning algorithms are used to predict the length of stay of each animal based on numerous factors (such as breed, size, and color). We address several objectives in this study that are listed below.

1. Identify risk factors associated with adoption rate and length of stay
2. Utilize the identified risk factors from collected data to develop predictive models
3. Compare statistical models to determine the best model for length of stay prediction

2. Methods

2.1 Data Description

A literature review is conducted to determine the factors that might potentially influence the length of stay for animals in shelters. These factors include gender, breed, age, and several other variables that are listed in Table 1**.** These features will be treated as input variables for the machine learning algorithms. Overall, there are eight input or predictor variables and one output variable, which is the length of stay.

Table 1: Factor Description

Variable	Description	Variable Type
Type	Cat or Dog	Categorical
Breed	Breed of the animal (e.g., labrador retriever, beagle)	Categorical
Color	Color of the animal (e.g., black, brown, white, multi-colored)	Categorical
Gender	Gender of the animal (male/female)	Categorical
Age	Age of the animal categorized as puppy/kitten, adult, senior, and super senior	Categorical
Location	Shelter location where dog or cat is housed	Categorical
Outcome Type	End outcome after the animal is brought into the shelter (euthanized, adopted, and returned)	Categorical
Length of Stay	The time that the animal spends in shelter categorized as low, medium, high, and very high (i.e., euthanized)	Categorical

Data is acquired from several online sources for animal shelters in several southern and south-western states. These online sources provide data sets for animal shelters from Kentucky (150,843 data rows), California (334,016), Texas (155,115), and Indiana (4,132). Since there is no nationwide database for animal shelters, information is also collected through individual animal shelters that conduct euthanization of animals. We contacted over 50 animal shelters across the United States and inquired for data on the factors mentioned in Table 1. We received responses from 20 of the animal shelters that were contacted. Most responses received stated there was not enough staff or resources to be able to provide this information. From the responses that were received back, only 4 shelters were able to provide any information and of that 4, only 2 of the data sets contained the factors and information needed, which are Colorado (8,488 data rows) and Arizona (4, 667 data rows).

The data that is collected from the database and animal shelters included information such as animal type, intake and outcome date, gender, color, breed, and intake and outcome status (behavior of animal entering the shelter and behavior of animal at outcome type). These data sets also included information on several types of animals such as dogs, cats, birds, rabbits, and lizards. For this study, the focus is on dogs and cats. After filtering through these data sets, we found that only California, Kentucky, Colorado, Arizona, and Indiana had all of the factors needed for the study. Upon downloading data from the database and receiving data from the animal shelters, the acquired data underwent data integration, data transformation, and data cleaning. After data pre-processing, there are over 113,000 animal records.

2.2 Data Cleaning Methods

Next, data cleaning methods are utilized to detect discrepancies in the data, such as missing values, erroneous data, and inconsistencies. Data cleaning is an essential step for obtaining unbiased results. In other words, identifying and cleaning erroneous data must be performed before inputting it into the algorithm as it can significantly impact the output results.

The following is a list of commonly used data cleaning techniques in the literature [16]:

- *Substitution with Median:* Missing or incorrect data are replaced with the median value for that predictor variable.
- *Substitution with a Unique Value:* Erroneous data are replaced with a value that does not fall within the range that the input variables can accept (e.g., a negative number)
- *Discard Variable and Substitute with a Median:* When an input variable has a significant number of missing values, these values are removed from the dataset, and the features that remain with missing or erroneous values are replaced with the median.
- *Discard Variable and Substitute with a Unique Value:* Input variables with a significant number of missing values are removed from the dataset, and the features that remain with missing or erroneous values are coded as -1.
- *Remove Incomplete Rows Entirely:* Rows that are incomplete are removed from the dataset.

2.3 Data Preprocessing

Some animal breeds are listed in multiple formats and are changed to maintain uniformity. An example of this is a Russian Blue cat, which is formatted in several ways such as “Russian”, “Russian Blue”, and “RUSSIAN BLUE”. Animals with multiple breeds such as “shih tzu/mix” or “shih tzu/yorkshire terr” are classified as the first breed listed. Other uncommon breeds are classified as "other" for the purpose of simplicity. Finally, all animal breeds are summarized into three categories (small, medium, or large) using the American Kennel Clubs’ breed size classification [17]. Part of the data cleansing process also includes categorizing multiple colors found throughout the sample size into five distinct color categories (brown, black, blue, white, and multicolor). We classified age into five categories for dogs and cats (puppy or kitten, adolescent, adult, senior, super senior). The puppy or kitten category includes data points 0-1 year, adolescent includes data points 2-3 years old, adulthood includes animals 4-7 years of age, and senior animals are 8-10 years of age. Any animal that is older than ten years are categorized as a super senior, based on the recommendations provided in Wapiti Labs [18].

As mentioned previously, the output variable is the length of stay and is classified as low, medium, high, and very high/euthanization. The length of stay is calculated by taking the difference between the intake date and the outcome date. To remove erroneous data entries and special cases, the number of days in the animal shelter is also capped at a year. The "low" category represents animals that are returned (in which case, they are assigned the days in the shelter as 0) or spent less than 8 days before getting adopted. It is important to keep these animals at the shelter so that the owner may find them or they are transferred to their new homes. Animals that stayed in a shelter for 9-42 days and are

adopted are categorized as "medium" length of stay. The "high" category is given to animals that stayed in the shelter for 43-365 days. Finally, animals that are euthanized are categorized as “very high”.

After integrating all data points from each animal shelter, the sample size includes 119,691 records. After the evaluation of these data points, 5,436 samples are found to have miscellaneous (such as a negative length of stay) or missing values. After applying data cleaning techniques, the final cleaned dataset includes 114,256 data points, with 50,466 cat- and 63,790 dog-records.

2.4 Machine Learning Algorithms to Predict the Length of Stay

The preprocessed records are then separated into training and testing datasets based on the type of classification algorithm used. Studies have demonstrated the need for testing and comparing machine learning algorithms, as the performance of the models depends on the application. While an algorithm may develop a predictive model that performs well in one application, it may not be the best performing model for another. A comparison between the statistical models is conducted to determine the best performing model. In this section, we provide a description as well as the advantages of each classification algorithm that is utilized in this study.

2.4.1 Logistic Regression

Logistic regression (LR) is a machine learning algorithm that is used to understand the probability of the occurrence of an event [19]. It is typically used when the model output variable is binary or categorical, unlike linear regression, where the dependent variable is numeric [20]. Logistic regression involves the use of a logistic function, referred to as a

"sigmoid function" that takes a real-valued number and maps it into a value between 0 and 1 [21]. The probability that the length of stay of the animal at a specific location will be low, medium, high, or very high is computed using the input features discussed in Table 1.

The linear predictor function to predict the probability that the animal in record i has a low, medium, high, and very high length of stay categories is given by Equations (1) – (4), respectively.

$$f(low, i) = \beta_{0,\text{low}} + \beta_{\text{type,low}} x_{\text{type,i}} + \beta_{\text{breed,low}} x_{\text{breed,i}} + \cdots \quad (1)$$

$$f(med, i) = \beta_{0,\text{med}} + \beta_{\text{type,med}} x_{\text{type,i}} + \beta_{\text{breed,med}} x_{\text{breed,i}} + \cdots \quad (2)$$

$$f(high, i) = \beta_{0,\text{high}} + \beta_{\text{type,high}} x_{\text{type,i}} + \beta_{\text{breed,high}} x_{\text{breed,i}} + \cdots \quad (3)$$

$$f(v.high, i) = \beta_{0,\text{v.high}} + \beta_{\text{type,v.high}} x_{\text{type,i}} + \beta_{\text{breed,v.high}} x_{\text{breed,i}} + \cdots \quad (4)$$

Where $\beta_{v,l}$ is a set of multinomial logistic regression coefficients for variable v of the length of stay category l, and $x_{v,i}$ is the input feature v corresponding to data observation i.

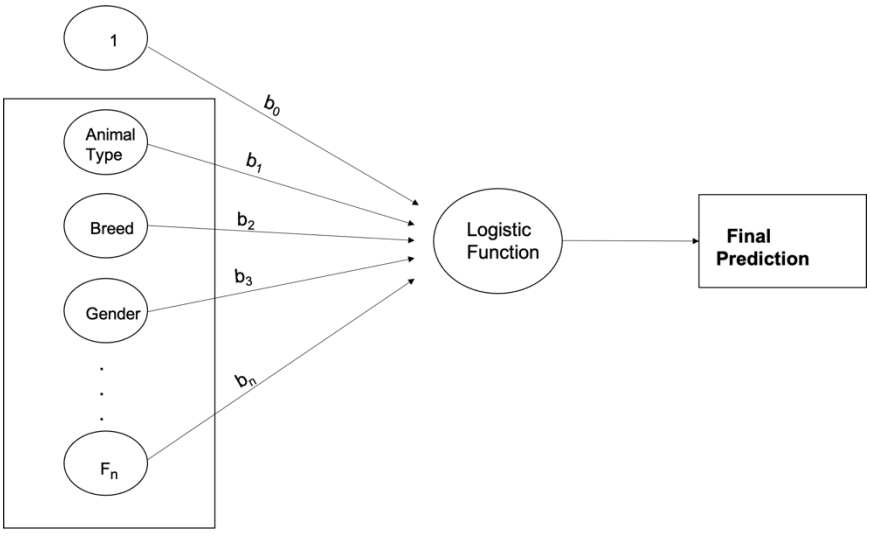

Figure 2: Pictorial Representation of the Logistic Regression Algorithm

2.4.2 Artificial Neural Network

Artificial Neural Network (ANN) algorithms were inspired by the brain's neuron, which transmits signals to other nerve cells [20]. ANN's were designed to replicate the way humans learn and was developed to imitate the operational sequence in which the body sends signals in the nervous system [22]. In an ANN, there exists a network structure with directional links connecting multiple nodes or "artificial neurons". These neurons are information-processing units, and the ties that connect them represent the relationship between each of the connected neurons. Each ANN consists of three layers- the input layer, the hidden layer, and the output layer [23]. The input layer is where each of the input variables is fed into the artificial neuron. The neuron will first calculate the sum of multiple inputs from the independent variables. Each of the connecting links (synapses) from these inputs has a characterized weight or strength that has a negative or positive value [23]. When new data is received, the synaptic weight changes, and learning will occur. The hidden layer learns the relationship between the input and output variables, and a threshold value determines whether the artificial neuron will fire or pass the learned information to the output layer. Finally, the output layer is where labels are given to the output value, and backpropagation is used to correct any errors.

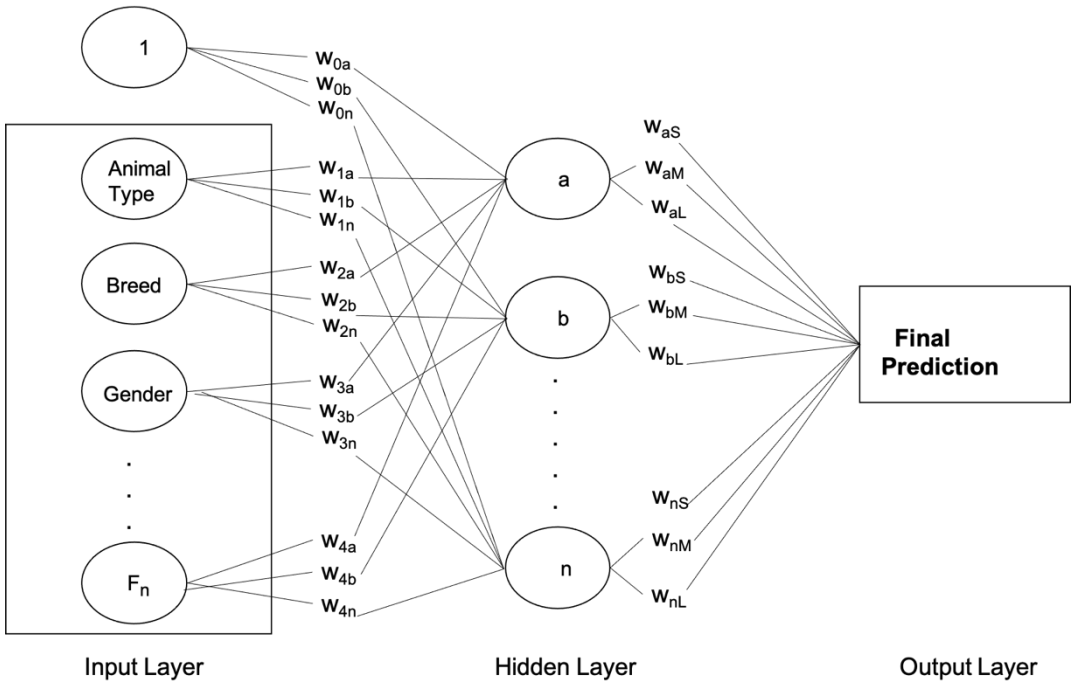

Figure 3: Pictorial Representation of the Artificial Neural Networks

2.4.3 Random Forest

The Random Forest (RF) algorithm is a type of ensemble methodology that combines the results of multiple decision trees to create a new predictive model that is less likely to misclassify new data [24]. Decision Trees have a root node at the top of the tree that consists of the attribute that best classifies the training data. The attribute with the highest information gain (given in Equation 6) is used to determine the best attribute at each level/node. The root node will be split into more subnodes, which are categorized as a decision node or leaf node. A decision node can be divided into further subnodes, while a leaf node cannot be split further and will provide the final classification or discrete label. RF algorithm uses *mtree* and *ntry* as the two main parameters in developing the multiple parallel decision trees. Mtree specifies how many trees to train in parallel, while ntry

defines the number of independent variables or attributes to choose to split each node [24]. The majority voting from all parallel trees gives the final prediction.

$$\text{Entropy} = \sum_{i} -p_i \log_2 p_i \tag{5}$$

$$\text{Information Gain} = \text{Entropy (parent)} - \text{Weighted Average [Entropy (children)]} \tag{6}$$

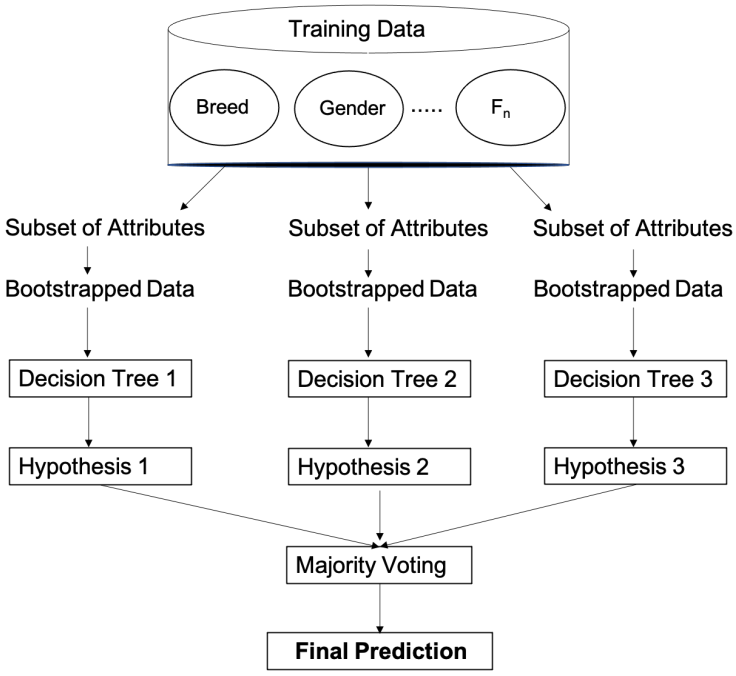

Figure 4: Pictorial Representation of the Random Forest Algorithm

2.4.4 Gradient Boosting

Boosting is another type of ensemble method that combines the results from multiple predictive algorithms to develop a new model. While the RF approach is built solely on decision trees, boosting algorithms can use various algorithms such as decision

trees, logistic regression, and neural networks. The primary goal of boosting algorithms is to convert weak learners into stronger ones by leveraging weighted averages to identify "weak classifiers" [25]. Samples are assigned an initial uniformed weight, and when incorrectly labeled by the algorithm, a penalty of an increase in weight is given [26]. On the other hand, samples that are correctly classified by the algorithm will decrease in weight. This process of re-weighing is done until a weighted vote of weak classifiers is combined into a robust classifier that determines the final labels or classification [26]. For our study, gradient boosting (GB) will be used on decision trees for the given dataset.

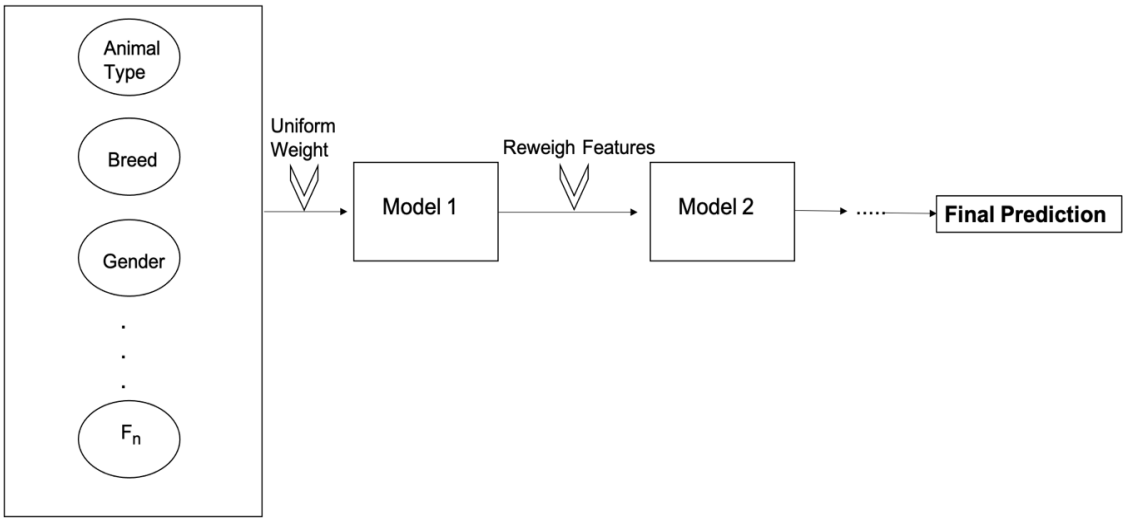

Figure 5: Pictorial Representation of Boosting Algorithm

2.5 Machine Learning Model Parameters

The clean animal shelter data is split into two datasets; training and testing data. These records are randomly placed in the two groups to train the algorithms and to test the model developed by the algorithm. 80% of the data is used to train the algorithm, while the other 20% is used to test the predictive model. To avoid overfitting, a tenfold cross-

validation procedure is used on the training data. There are no parameters associated with the machine learning of logistic regression algorithms. However, a grid search method is used to tune the parameters of the random forest, gradient boosting, and artificial neural network algorithms. This allows the best parameter in a specific set to be chosen by running an in-depth search by the user during the training period.

The number of trees in the random forest and gradient boosting algorithms is changed from 100 to 1000 in increments of 100. A learning rate of 0.01, 0.05, and 0.10 is used based on the recommendations of previous studies [27]. The minimum observations for the trees' terminal node are set to vary from 2 to 10 in increments of one, while the splitting of trees varies from 2 to 10 in increments of two. A feed-forward method is used to develop the predictive model using the artificial neural network algorithm. The feed-forward algorithm consists of three layers (input, hidden, output) as well as backpropagation learning. The independent and dependent variables represent the input and output layers. Since the input and output layers are already known, an optimal point is reached for the number of nodes when between 1 and the number of predictors. This means that for our study, the nodes of the hidden layer vary from 1 to 8. The learning rate values used to train the ANN are 0.01, 0.05, and 0.10. To find the optimal setting for each machine learning algorithm, a thorough search of their corresponding parameter space is performed.

2.6 Performance Measures

In this study, we use three performance measures to evaluate the ability of machine learning algorithms in developing the best predictive model for the intended application. The measures considered are precision, F1 score, and sensitivity/recall to determine the

best model given the inputted data samples. Table 2 provides a confusion matrix to define the terms used for all possible outcomes.

Table 2: Confusion Matrix

		Predicted	
		Negative	**Positive**
Actual	**Negative**	True Negative	False Positive
	Positive	False Negative	True Positive

The first measure, precision, evaluates the number of correct, true positive predictions by the algorithm while still considering the incorrectly predicted positive when it should have been negative (Equation 7). By having high precision, this means that there is a low rate of false positives or type I error. Sensitivity or recall evaluates the number of true positives that are correctly predicted by the algorithm while considering the incorrectly predicted negative when it should have been positive (Equation 8). Recall is a good tool to use when the focus is on minimizing false negatives (type II error). F1 score (shown in Equation 9) evaluates both types I and type II error and assesses the ability of the model to resist false positives and false negatives. This performance metric evaluates the robustness (low number of missed classifications), as well as the number of data points that are classified correctly by the model.

$$Precision = \frac{True\ Positive}{True\ Positive + False\ Positive} \tag{7}$$

$$Sensitivity/Recall = \frac{True\ Positive}{True\ Positive + False\ Negative} \tag{8}$$

$$F1\ Score = \frac{2(True\ Positive)}{2(True\ Positive)+False\ Positive+False\ Negative} \quad (9)$$

3. Results

3.1 Exploratory Data Results

From Figure 6, it is evident that the returning of dogs is the highest outcome type at 43.3%, while Figure 7 shows that the adoption of cats is the highest outcome type at 46.1%. Both figures illustrate that the euthanization of both cats and dogs is still prevalent (~20%). The results from Table 3 demonstrate that the longest time spent in the shelter is at 355 days by a male cat that is adopted and a female dog that is euthanized. Observing the results, adoption has the lowest variance among all animal types compared to the other outcome types. Adopted male cats have the lowest variance for days spent in the shelter, followed by female dogs. Female cats that are returned have the highest variance for days spent in the shelter.

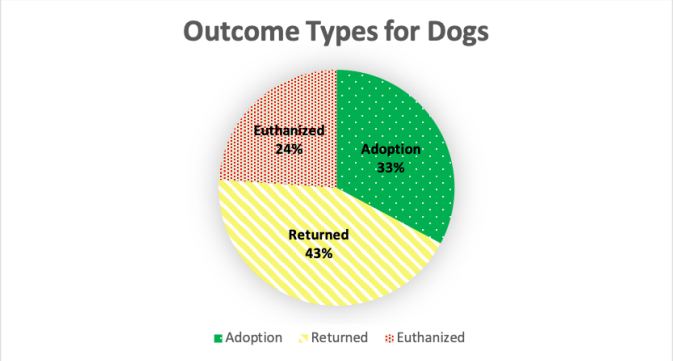

Figure 6: Distribution of Outcome Types for Dogs

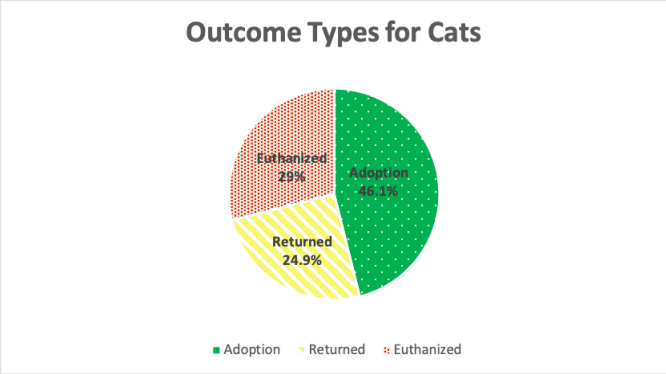

Figure 7: Distribution of Outcome Types for Cats

Table 3: Data Summary

Type	Adopt			Return			Euthanize		
	Mean	St. Dev	CV	Mean	St. Dev	CV	Mean	St. Dev	CV
Male Dogs	21.87	24.74	1.13	8.52	15.74	1.85	10.69	20.75	1.94
Female Dogs	21.17	23.65	1.12	8.56	15.31	1.79	9.64	20.00	2.07
Male Cats	37.75	40.78	1.08	6.32	13.69	2.17	6.59	11.73	1.78
Female Cats	29.68	33.63	1.13	7.53	16.83	2.24	7.15	11.02	1.54

Figure 8 shows a comparison of cats and dogs for the three different outcome types. It is observed from the data that there are more dogs returned than cats. From Figure 9, it is observed that the number of days a dog stays in the shelter decreases as the age increases. This is not expected, as it is predicted that the number of days in a shelter would be lower for younger dogs and puppies. This observation could be due to having more data points for younger dogs.

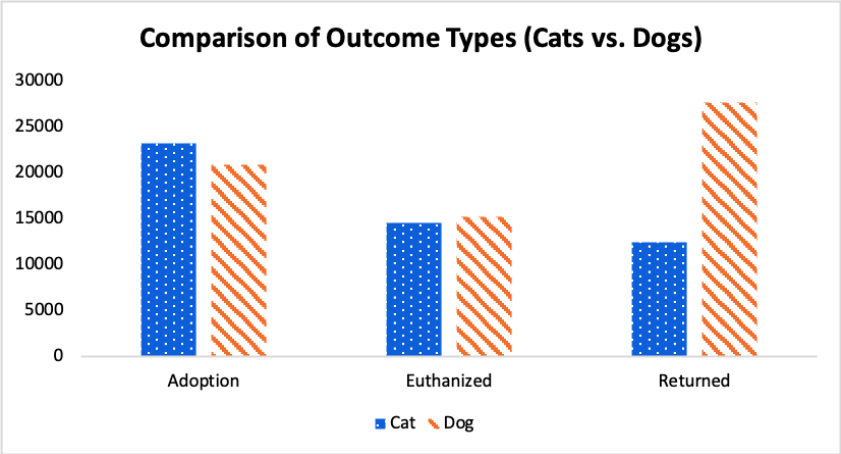

Figure 8: Comparison of Outcome Types for Cats and Dogs

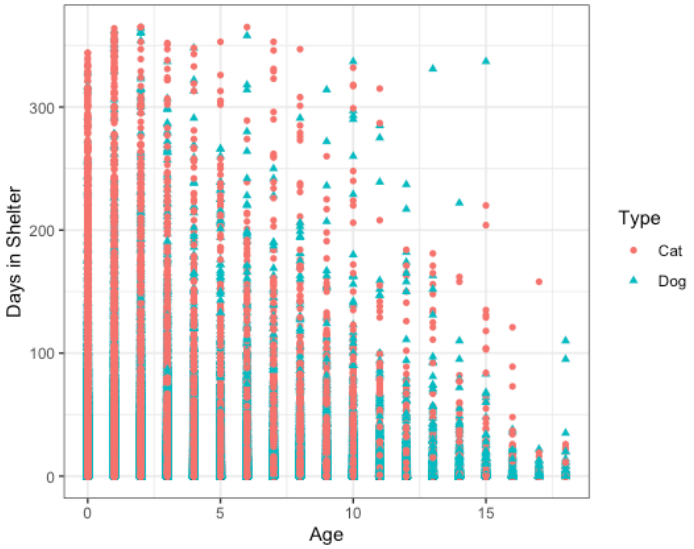

Figure 9: Age vs. Days in Shelter for Cats and Dogs

3.2 Machine Learning Results

Examining Table 4, it is clear that the most proficient predictive model is developed by the gradient boosting algorithm for this dataset, followed by the random forest algorithm. The logistic regression algorithm appears to perform the worst with low precision, recall, and F1 score performance metrics for all categories of length of stay. For the prediction of low length of stay in a shelter, the random forest algorithm is the best

performing model in comparison to the others at around 64-70% performance for precision, recall, and F1 score. The ANN algorithm is found to be the best when evaluating the precision and F1 score for medium length of stay, while the random forest algorithm is better for assessing recall. However, the performance of these models in predicting the medium length of stay for the given dataset is low for all three-performance metrics. The gradient boosting algorithm performs the best when predicting the high length of stay. Finally, the gradient boosting and random forest algorithms perform well when predicting the very high length of stay at around 70-80%.

Table 4: Consolidated Results

Length of Stay	LR			ANN			RF			GB		
	Precision	Recall	F1 Score	Precision	Recall	F1 Score	Precision	Recall	F1 Score	Precision	Recall	F1 Score
Low	0.4171	0.3852	0.4005	0.5508	0.513	0.5312	0.6441	0.703	0.6723	0.6264	0.4631	0.5325
Medium	0.386	0.3716	0.3786	0.4181	0.5146	0.4614	0.3068	0.5533	0.3948	0.2254	0.5043	0.3116
High	0.3072	0.4145	0.3529	0.4155	0.2707	0.3278	0.3832	0.6197	0.4736	0.7251	0.7188	0.7219
Very High: Euthanization	0.4536	0.4213	0.4369	0.5612	0.5209	0.5403	0.7031	0.7138	0.7084	0.7902	0.7096	0.7477
Average	0.391	0.3981	0.3922	0.4864	0.4548	0.4652	0.5093	0.6474	0.5623	0.5918	0.5989	0.5784
Computation Time (s)	9.41			2023			2139			2009		

Results from Table 4 also demonstrate that the model developed from the gradient boosting algorithm has a higher performance when predicting the high length of stay that leads to adoption, and when the outcome is euthanization. Evaluating the average of all three-performance metrics for all algorithms, the gradient boosting is the most proficient

model at almost 60%, while logistic regression appears to be the worst. Table 4 also provides the computational time for each machine learning algorithm. For the given dataset, logistic regression runs the fastest at 9.41 seconds, followed by gradient boosting, artificial neural network, and finally, random forest running the longest. The gap in the performance measure (pm) is calculated by $\frac{pm_{best}-pm_{worst}}{pm_{best}}$, and is nearly 34%, 39%, and 32% for precision, recall, and F1 score, respectively.

Table 5: Top Three Features using Different Machine Learning Algorithms

Algorithm	**Top Three Features**		
	#1	**#2**	**#3**
Logistic Regression	Multicolor	Small Size	Senior
ANN	Large Size	Super Senior	Senior
RF	Puppy	Multicolor	Super Senior
GB	Small Size	Super Senior	Large Size

Table 5 provides information on the top features or factors from each machine learning algorithm. Observing the table, we find that age (senior, super senior, and puppy), size (large and small), and color (multicolor) has a significant impact or influence on the length of stay. Specifically, we observe that older-aged animals (senior and/or super senior) appear as a significant factor for every algorithm. For the artificial neural network, older age is the #2 and #3 predictor, and super senior is the #2 predictor for the gradient boosting algorithm. Large and small-sized animals are also observed to be important features, as both are shown as the #1 predictor in the gradient boosting and ANN algorithms. The

results also demonstrate that gender, animal type, other colors besides multicolor, middle age, and medium-sized animals did not significantly impact the length of stay.

4. Discussion

Results from our study provided information on what factors are significant in influencing length of stay. Brown et al. (2015) conducted research that found that age, breed designation, coat color, and coat pattern influenced the length of stay for cats in animal shelters [10]. Similar to these studies, observations from our study also suggest that age and color have a significant impact or influence on the length of stay.

Determining which algorithm will develop the best model for the given set of data is critical in order to predict the length of stay and minimize the chances of euthanization. The goal of predictive analytics is to develop a model that best approximates the true mapping function for the relationship between the input and output variables. In order to approximate this function, parametric or non-parametric algorithms can be used. Parametric algorithms simplify the unknown function to a known form. Non-parametric algorithms do not make assumptions about the form of the mapping function, allowing free learning of any functional form. In this study, we utilize both parametric (logistic regression and artificial neural network) and non-parametric (random forest and gradient boosting) algorithms on the given data. Observing the results from Table 4, the gradient boosting and random forest (non-parametric algorithms) perform the best on the data set. It is observed from the results that using a non-parametric approach leads to a better approximation of the true mapping function for the given data set. These results also support prior studies on parametric versus non-parametric methods. Neely et al. (2013)

discusses the theoretical superiority of non-parametric algorithms for detecting pharmacokinetic and pharmacodynamic subgroups in a study population [28]. The author suggests this superiority comes from the lack of assumptions made about the distribution of parameter values in a data set. Bissantz et al. (2003) discusses a resampling algorithm that evaluates the deviations between parametric and non-parametric methods to be noise or systematic by comparing parametric models to a non-parametric "super model" [29]. Results demonstrated the non-parametric model to be significantly better. The use of algorithms that do not approximate the true function of the relationship between input and output provided better performance results for this application as well.

Current literature also supports the use of ensemble methods to increase prediction accuracy and performance. Dietterich et al. (2000) discuss the ongoing research into developing good ensemble methods as well as the discovery that ensemble algorithms are oftentimes more accurate than individual algorithms that are used to create them [30]. Pandey et a. (2014) conducted a study to compare the accuracy of ensemble methodology on predicting student academic performance as research has demonstrated better results for composite models over a single model [31]. This study applied ensemble techniques on learning algorithms (AdaBoost, Random Forest, Rotation Forest, and Bagging). For our study with the given data set, the results support this claim. Both the gradient boosting and random forest algorithms are ensemble algorithms and performed the best on the animal shelter data.

Results from Table 4 demonstrate the best performance of the gradient boosting and random forest algorithm when the length of stay was classified very high or the animal was euthanized. This is beneficial as the models can predict long stays where the outcome

is euthanasia. This can lead to shelters identifying at-risk animals and implementing methods and solutions to ensure their adoption. These potential methods are the second phase of this research study which will involve relocating animals to shelters where they will more likely be adopted. This phase is discussed in the future directions section.

The job of utilizing euthanasia of these animals in the shelter falls on the workers and volunteers. Not only is there an overpopulation problem leading to euthanasia but euthanizing these animals can cause mental and emotional problems for the workers. Studies have been conducted evaluating euthanasia-related stress on workers [1]. Reeve et al. (2005) evaluated the strain related to euthanasia among animal workers [32]. Results demonstrated that euthanasia related strain was prevalent and an increase in substance abuse, job stress, work causing family conflict, complaints, and low job satisfaction was observed. Predicting the length of stay for animals will aid in them being more likely to be adopted and will lead to fewer animals being euthanized, adding value not only to animals finding a home but also less stress on the workers.

One of the limitations of this study is the lack of behavioral data of the animal during intake and outcome, which would be beneficial to develop a more comprehensive model. Though behavioral problems are harder to solve, having data would provide insight on how long these animals with behavioral issues are staying in shelters and what is the outcome. Studies have shown that behavioral issues play a significant role in preventing bonding between owners and their animals and one of the most common reasons cited for animal surrender [33, 34]. These behavioral problems can include poor manners, too much energy, aggression, and destruction of the household. Dogs surrendered to shelters because of behavioral issues have also been shown to be less likely to be adopted or rehomed and the

ones that are adopted are more likely to be returned [34]. Studies have also been conducted to evaluate the effect of the length of time on the behavior of dogs in rescue shelters [35, 36, 37]. Most of them concluded that environmental factors led to changes in the behavior of dogs and that a prolonged period in a shelter may lead to unattractive behavior of dogs to potential owners. Predicting how long an animal will stay in a shelter could also aid in adoption by making sure healthy animals are not developing behavioral problems in the shelters. It is not only important for the animal to be adopted, but also that the adoption is a good fit between owner and pet so that the animal is less likely to be returned. Having this information will also allow shelters to find other shelters close by where animals with behavioral issues are more likely to be adopted. To overcome this limitation of the lack of data on behavioral problems, behavioral issues will be used as a factor and will be specifically asked for when acquiring data from shelters.

Another limitation includes collecting more data from animal shelters across the United States, allowing for more representative data to be collected and inputted into these algorithms. However, this presents a challenge due to most shelters being underfunded and low on staff. Though we reached out to shelters, most replied that they lacked the resources and staff to provide the information needed. Future work would include applying for funding to provide a stipend to staff for their assistance in gathering the data from respective shelters. With more data, the algorithm has more information to learn on, which could improve the performance metrics of the predictive models developed. There may also be other factors that show to be significant as more data is collected.

5. Conclusion

Nearly 3-4 million animals are euthanized out of the 6-8 million animals that enter shelters annually. The overall objective of this study is to increase the adoption rates of animals entering shelters by using key factors found in the literature to predict the length of stay. The second phase determines the best shelter location to transport animals using the goal programming approach to make relocation decisions. To accomplish this objective, first, the data is acquired from online sources as well as from numerous shelters across the United States. Once the data is acquired and cleaned, predictive models are developed using logistic regression, artificial neural network, gradient boosting, and random forest. The performance of these models is determined using three performance metrics: precision, recall, and F1 score.

The results demonstrate that the gradient boosting algorithm performed the best overall, with the highest precision, recall, and F1 score. Followed closely in second is the random forest algorithm, then the artificial neural network, and then finally, the logistic regression algorithm is the worst performer. We also observed from the data that the gradient boosting performed better when predicting the high or very high length of stay. Further observing the results, it is found that age for dogs (e.g., puppy, super senior), multicolor, and large and small size are important predictor variables.

The findings from this study can be utilized to predict how long an animal will stay in a shelter, as well as minimize their length of stay and chance of euthanization by determining which shelter location will most likely lead to the adoption of that animal. For

future studies, we will implement phase 2, which will determine the best shelter location to transport animals using the goal programming approach to make relocation decisions.

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Chapter Six

Future Directions

1. Intervertebral Disc Degeneration

1.1 Phase 1

One direction for future studies is to collect more data on patients with disc degeneration. The SMOTE technique was applied to the data set to fix the problem of an unbalanced data set; however, it would be beneficial to collect additional data on IVDD patients to provide more information for the algorithms to learn and predict. This acquired information should also be collected from medical facilities across Missouri and the United States. By collecting more information from across the nation, collecting this data will not only provide the machine learning algorithms with more data to train on, but it would also provide insight on how IVDD patients may differ based on region, socioeconomic status, access to healthcare, and the type of occupations in a particular state or region. For example, socioeconomic status may affect the patient's ability to go visit the doctor for back pain or the availability of medical facilities in the area. It is important to gather more information to truly develop a representative model.

Another direction for this study is to collect additional data on patients where disc degeneration is hereditary or genetic. Studies have shown that there is a correlation between genetics and intervertebral disc degeneration [1]. Having more information about this factor for patients can aid in developing the predictive model for the risk of disc degeneration. Finally, it would be beneficial to continue developing the user-friendly

app for patients to utilize on the first onset of back pain so that doctors can identify patients at risk for disc degeneration.

1.2 Phase 2

Future studies for Phase 2 include developing the viscoelastic collagen utilizing type II collagen and a blend of type I and type II to determine which is a better material for disc regeneration. Though the nucleus pulposus is composed of type II, it may be found that utilizing collagen type I or a blend of the two types would provide better support. Another focus is to utilize cell-based therapies in our studies. As mentioned in Chapter 2, both cell-based and protein-based therapies are currently being investigated as potential treatments for disc degeneration. Future studies can include (1) running cell viability studies on stem cells (such as mesenchymal or chondrocytes) and (2) embedding stem cells into our viscoelastic collagen material.

Another future direction would be to go more in-depth for mechanical testing of the viscoelastic collagen material, by collaborating with those in the field of biomechanics. Not only is it important for the material to be biocompatible and function properly within the body, but it is also just as important that the material can withstand the biomechanics placed on the disc. To understand this, we would evaluate and test for the material properties of both healthy and degenerated NP, as well as the viscoelastic collagen so that we can make a comparison. These tests could include rheology and confined and unconfined compression tests on the NP and the viscoelastic material, and injectability studies.

Finally, another focus would evaluate incorporating injectable materials for both the damage done to the nucleus pulposus and the annulus fibrosus during disc degeneration and/or disc herniation. Prior studies have evaluated and developed materials for repairing tears in the annulus fibrosus, which can be a later effect of disc degeneration [2, 3]. However, these studies only investigate and heal the tear but do not address the issue of the degenerated nucleus pulposus. While the tear may be healed, the root problem is not addressed.

2. Animal Shelter

One future direction for this study is to address the lack of behavioral data of the animal during intake and outcome, which would be beneficial to develop a more comprehensive model. Though behavioral problems are harder to solve, having data would provide insight into how long these animals with behavioral issues are staying in shelters and what is the outcome. Studies have shown that behavioral issues play a significant role in preventing bonding between owners and their animals and one of the most common reasons cited for animal surrender [4, 5]. Predicting how long an animal will stay in a shelter could aid in adoption by making sure healthy animals are not developing behavioral problems in the shelters. It is not only important for the animal to be adopted, but also that the adoption is a good fit between owner and pet so that the animal is less likely to be returned. Having this information will also allow shelters to find other shelters close by where animals with behavioral issues are more likely to be adopted.

Another future focus includes collecting more data from animal shelters across the United States, allowing for more representative data to be collected and inputted into these

algorithms. However, this presents a challenge due to most shelters being underfunded and low on staff. Though we reached out to shelters, most replied that they lacked the resources and staff to provide the information needed. One solution to this problem is to find funding so that we can pay the shelter volunteers for their time in collecting the data for us.

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VITA

Janae Bradley is a native of St. Louis, Missouri, and attended Hazelwood East High School. She received her Bachelor of Science in BioEngineering with a minor in Mathematics from the University of Missouri. Janae was selected to participate in the National Institutes of Health (NIH) IMSD EXPRESS program. Participating in this program allowed her to learn more about research, graduate school, and helped her secure a position in a biomaterials and biosensors research lab. Janae also served as a Peer Mentor for the IMSD EXPRESS program and was a co-founder for the dance outreach program called First Position at the Boys and Girls Club of Columbia. During her undergraduate years, Janae received several awards such as the Undergraduate Research Top Presenter award at the American Physical Society. She also won first place in the engineering category at the Undergraduate Research and Creative Achievements Forum at Mizzou. Janae pursued a Doctor of Philosophy in BioEngineering at the University of Missouri with an emphasis in biomaterials and data analytics.

She has won several awards in her graduate career such as the NIH IMSD Graduate Fellowship, the GAANN fellowship, Delta Sigma Theta's Ursula Burn's Women in Engineering award, and the College of Engineering's 2016 Women in Engineering award. Janae has also been awarded the Gus T. Ridgel fellowship, Suggs fellowship, and was inducted into the Rollins Society. She was also a recipient of the Class of 2021 Mizzou 18 award and the 2021 Outstanding PhD Student in the Biological Engineering department. Janae has served in numerous leadership roles on campus including the Graduate Scholars of Excellence, Mizzou Fellowship Office Ambassador, the College of Engineering Student Services Inclusivity Board, MOLSAMP Graduate

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