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Drug delivery systems: exploring rheological properties and therapeutic effect of 5-FU chitosan gel for topical wound healing

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DRUG DELIVERY SYSTEMS: EXPLORING RHEOLOGICAL PROPERTIES AND THERAPEUTIC EFFECT OF 5-FU CHITOSAN GEL FOR TOPICAL WOUND HEALING

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Approved by
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APPROVAL OF THESIS

We, the faculty supervising the work of Samuel Tetteh-Quarshie, affirm that the thesis, *Drug delivery systems: exploring rheological properties and therapeutic effect of 5-FU chitosan gel for topical wound healing,* meets the high academic standards for original scholarship and creative work established by the Master of Science in Pharmaceutical Science and the School of Pharmacy. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Cynthia Jones, Department of Pharmaceutical Science Committee Chairperson Date 3/26/202

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DEDICATION

This work is dedicated to the Lord Almighty for His countless blessings and unfailing love. He has blessed me with an earthly family whose toil and sacrifices have brought me thus far. I pray and hope that they take joy in my work and the journey ahead.

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am fortunate to have each of you in my life and I hope I can be a blessing to all of you through Christ our Lord!

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ABSTRACT

Diabetic skin wound is a common complication of diabetes that occurs in about 15% of diabetic patients and often requires prolonged hospitalization for its management and treatment. Natural polymers are used for wound dressing due to their biological adhesiveness, non-toxicity, and biodegradable nature. 5-Fluorouracil (FU) has been shown to alter adipokine expression which is implicated in cutaneous wound repair. Thus, our overall objective was to investigate the utility of chitosan (CS) gel for topical delivery of 5-FU to treat diabetic wounds. We prepared chitosan gel (2% w/w) in serial dilutions of 5-FU (25μg/mL, 2.5μg/mL, 0.25μg/mL, and 0.025μg/mL) and evaluated their stability, antimicrobial activity, and rheological properties. Human dermal fibroblast cells were exposed to each gel formulation under high glucose conditions. MTT, real-time polymerase chain reaction, and cell migration assays were used to determine cell viability, gene expression, and migration, respectively. We showed that 2% CS gel is stable at both refrigeration (4-8^{o}C) and room temperatures (20-25^{o}C), and exhibits non-Newtonian flow property, shear thinning behavior and weak viscoelasticity. At low concentrations (0.025μg/mL), there is antimicrobial activity of 5-FU against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (RN1), and at high concentration (8.0μg/mL) against *Escherichia coli*. We also found that exposure to CS and 5-FU-CS gel at different concentrations decreases adipokine gene expression for resistin and PPARγ and improved cell migration. Our studies suggest CS can be formulated into a hydrogel for topical delivery, 5-FU-CS gel is potent against pathogenic bacteria found in diabetic wounds and has therapeutic effect on adipokine gene expression. The findings in this study offer a novel therapeutic delivery system for 5-FU for diabetic wound healing.

CHAPTER 1

INTRODUCTION

Diabetes

Diabetes mellitus is a serious disease which is characterized by insufficient production of insulin by the pancreas or ineffective use of insulin by the body (Fui et al., 2019). Therefore, the driving factor of diabetes is insulin deficiency. Diabetes is one of the most costly and prevalent chronic diseases in the United States (Narayan et al., 2003). Pathologically, diabetes can be classified as either type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) (Cebeci et al., 2019), and in some cases as gestational diabetes mellitus (GDM). Etiological studies show that GDM affects about 5 to 6% of pregnant women and is believed to be an early indicator of T2DM. T2DM accounts for most cases of diabetes mellitus (~90%), while TIDM accounts for the remaining cases (~10%) (Guthrie & Guthrie, 2004; Centers for Disease Control and Prevention, 2020).

Type 1 Diabetes Mellitus

T1DM is a chronic autoimmune disorder in genetically susceptible individuals in which one's immune system attacks and destroys pancreatic beta cells (β-cells) resulting in eventual elimination of insulin production (Fui et al., 2019). Genetically, T1DM can be defined as a heritable polygenic disease with identical twin concordance of about 30 – 70% (Redondo et al., 2008), sibling risk of 6 – 7%, and a risk of 1 – 9% for children of a parent with the disease (Pociot & Lernmark, 2016). Epidemiological data show that T1DM is slightly more common in males than in females (Diaz-Valencia et al., 2015). Furthermore, studies indicate that incidence and prevalence of T1DM is gradually on the rise, with an overall annual increase estimated to be approximately 2 – 3% per year (Mayer-Davis et al., 2017). Even though the cause of diabetes in

general is multifactorial, environmental and/or behavioral factors such as infant and adult diet, vitamin D deficiency, early exposure to islet inflammation associated viruses, and decreased gut-microbiome diversity have been implicated in the development of T1DM (Rewers & Ludvigsson, 2016).

Physiologically, these factors are believed to trigger the immune system to attack pancreatic β-cells, and cause insulin deficiency, which is the underlying factor in diabetes (Guthrie & Guthrie, 2004; Hull et al., 2017). For example, Greenbaum et al. found that people who are diagnosed with T1DM have reduced β-cell function compared to a healthy control group (Greenbaum et al., 2009). Since insulin is a driving factor for anabolic processes in the body, its deficiency, as seen in all three forms of T1DM, prevents glucose from entering the cells, which results in its accumulation in the blood. This results in hyperglycemia which causes excess urination (polyuria), excess thirst (polydipsia), and then excess hunger (polyphagia) because cells lack fuel (Guthrie & Guthrie, 2004). Despite the abnormalities of β-cell and T1DM, the methods of managing T1DM continue to improve with administration of insulin still serving as the main therapeutic approach (DiMeglio et al., 2018).

Type 2 Diabetes Mellitus

T2DM, in contrast, is a complex metabolic disease characterized by defects in insulin secretion by β-cell or insulin resistance that is mostly related to patients' lifestyle and dietary habits (Fui et al., 2019). There are several genes implicated in β-cell action, and in insulin secretion and activity at the cellular level (Guthrie & Guthrie, 2004). This means a defect in any of these genes may prevent enzyme production, which eventually blocks the action of insulin. This disrupts glucose uptake by the cell and increases the breakdown of triglycerides to produce the metabolic defects associated with T2DM (Cersosimo, 2018; Guthrie & Guthrie, 2004).

The pathophysiology of T2DM involves an interaction between genetic predisposition and environmental triggers (Javeed & Matveyenko, 2018; Smushkin & Vella, 2010). Also, the loss of first-phase insulin release and increased glucagon secretion has been implicated to accelerate the development of T2DM (Ismail-Beigi, 2012). According to Defronzo, the pathophysiology of T2DM is mainly driven by the induction of skeletal muscle, hepatic, and adipose tissue insulin resistance (Defronzo, 2009). Insulin resistance, one of the early defects of T2DM, is believed to begin many years before the onset of symptoms sufficient for diagnosis. Many genetic and environmental factors such as age, lifestyle, and obesity (Franks & McCarthy, 2016) have been implicated in the development of insulin resistance in both the peripheral cells (primarily muscle and fat cells) and the liver (Guthrie & Guthrie, 2004; Lebovitz, 2001). The development of insulin resistance forces β-cells to increase insulin production to maintain the blood glucose levels needed for normal physiological processes. However, when this phenomenon continues over time, then the β-cells begin to fail, either through genetic defects, glucose toxicity, or depletion, leading to the development of diabetes (Guthrie & Guthrie, 2004).

WOUND HEALING

Diabetic skin wound is a common complication that occurs in about 15% of diabetic patients and often requires prolonged hospitalization for its management and treatment (Albert, 2002). For instance, the management of diabetic foot ulceration alone poses a challenging financial burden on patients and the health system (Al Odhayani et al., 2017). The creation of a wound involves a disturbance in the cellular, anatomical, and functional epithelial integrity of the skin due to physical, chemical, thermal, microbial, or immunological insults, which disrupts the structure and function of the underlying normal tissue (Mekonnen et al., 2013; Oguntibeju, 2019). Thus, wound healing can be defined as an interaction between a complex cascade of cellular and biochemical activities geared towards the restoration of structural, functional integrity, and increased strength in an injured tissue (Martin, 1997; Oguntibeju, 2019; Pastar et al., 2014).

Wounds, in general, can be categorized as either acute or chronic depending on the severity of the wound. An acute wound heals in a well-organized process with predictable progress or tissue repair (Demidova-Rice et al., 2012). Conversely, a chronic wound emerges when the controlled sequence of natural wound healing is delayed and the wound shows no sign of effective healing, usually within 3 months of tissue injury (Gupta et al., 2017). These wounds are characterized by excessive levels of pro-inflammatory cytokines, proteases, reactive oxygen species, formation of drug-resistant microbial biofilms, and deficiency of functional epidermal cells (Frykberg & Banks, 2015).

Normal Wound Healing

Normal wound healing involves the repair of connective tissue and is typified by four complex and overlapping phases of hemostasis, inflammation, proliferation, and tissue

remodeling (Figure 1) (Falanga, 2005; Fui et al., 2019; Gonzalez et al., 2016). The orderly progression of the healing process is impaired in diabetic patients. For example, in diabetic patients, slight abrasion or scratch renders one susceptible to the development of chronic wounds, with an overall impaired healing (Avishai et al., 2017). These complex processes involve dynamic interactions between different cell types, the extracellular matrix (ECM), cytokines, and growth factors in distinct phases (Ezhilarasu et al., 2020). These phases of wound healing usually occur in a time-dependent manner which means any disruption in the process could potentially lead to a chronic wound or in some cases, pathological scarring (Martin, 1997; Pastar et al., 2014).

There are four phases that occur during the wound healing process in normal wounds (Figure 1). The first phase of wound healing that is initiated at the onset of an injury is the hemostatic phase. During this phase, blood platelets adhere to exposed type 1 collagen, become activated, and secrete glycoproteins to form platelet aggregation following the injury. Thus, within minutes of an injury, blood vessels constrict to reduce the extent of hemorrhage allowing hemostasis to be achieved (Oguntibeju, 2019; Pastar et al., 2014). The inflammation phase has an overlapping role with the hemostasis phase and is believed to activate the hemostasis mechanisms that rapidly halt blood loss from the wound. This phase is marked by initial vasoconstriction and platelet aggregation to initiate blood clotting, followed by vasodilation and phagocytosis to produce inflammation at the wounded site, which usually takes about two weeks (Oguntibeju, 2019). Key steps that are involved in the proliferative phase are granulation, angiogenesis, epithelialization, and contraction of wound edges. Granulation involves the formation of a collagen bed with the production of new capillaries by fibroblasts. This is followed by the contraction of wound edges to reduce the defect and an overall formation of new

epithelial and scar tissues over the wounded site (Fui et al., 2019; Oguntibeju, 2019; Pastar et al., 2014). The remodeling phase is marked by formation of new collagen with increased tensile strength due to intermolecular collagen crosslinking. This phase usually lasts anywhere between 21 days to 2 years (Fui et al., 2019; Oguntibeju, 2019).

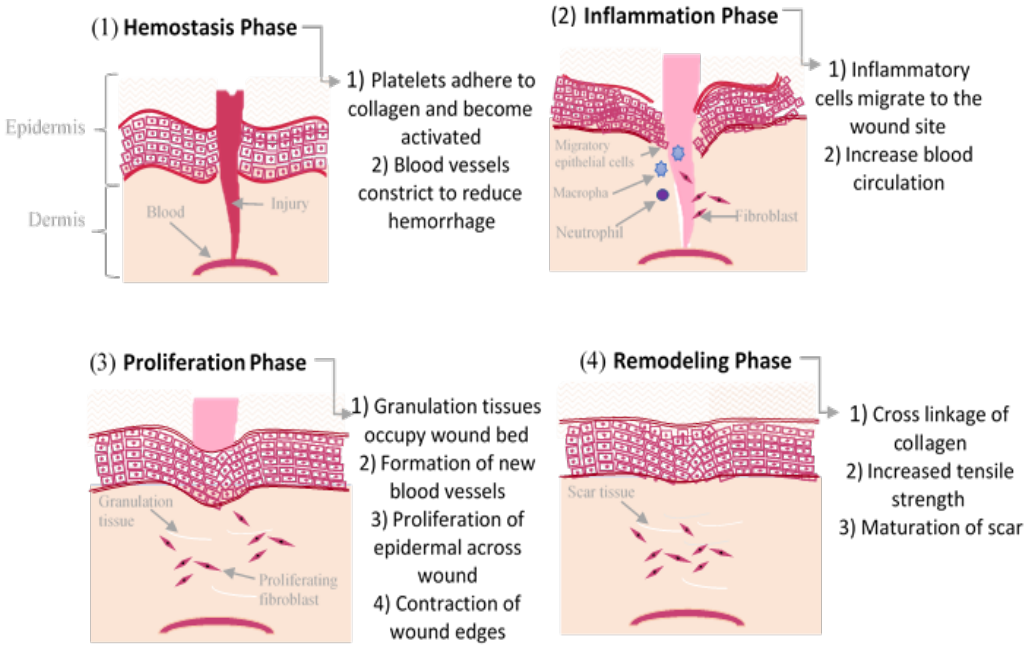

Figure 1. Four Phases of Wound Healing.

An illustration of four phases of the normal wound healing process. The first phase is represented by the hemostasis process, inflammation takes place during phase two, proliferation occurs during phase three and tissue remodeling is the final phase in the wound healing process. Illustration © 2021 Samuel Tetteh-Quarshie.

Diabetic Wounds

Diabetic wounds are one of the most severe complications of diabetes mellitus associated with high morbidity; they are difficult to manage since they are slow to heal and could last for weeks (Oguntibeju, 2019). The mechanisms responsible for the delay in wound healing in diabetic patients are not fully understood. Factors such as age, disease state, diet, reactive oxygen species (Bluher & Mantzoros), and immune state have been implicated in poor wound healing in diabetic patients (Brem & Tomic-Canic, 2007; Oguntibeju, 2019). Physiologically, a diabetic wound can be described as a microenvironment characterized by high glucose levels, advanced glycation end products (AGEs), reactive oxygen species (ROS), and inflammatory cytokines (Figure 2) (Wang & Graves, 2020). Incidences of high glucose levels have been implicated in reduced keratinocyte migration and proliferation in both in vivo and in vitro scratch wound assays (Zhu et al., 2011).

Further studies on diabetic wound healing show that prolonged exposure to high glucose can result in the loss of endothelial cell integrity which makes the cells susceptible to detachment and apoptosis (Okonkwo & DiPietro, 2017). Chronic inflammation is also implicated as one of the most common reasons for delayed wound healing observed in diabetic patients. In this instance, prolonged elevation of glucose and free fatty acids induce inflammatory processes in various tissues marked by infiltration of immune cells to tissue sites and the release of pro-inflammatory markers resulting in tissue damages (Pahwa et al., 2020).

Cutaneous wound healing requires a well-orchestrated interaction of cell migration, proliferation, and re-epithelialization from different tissues and cells. Thus, any disruption in the normal functioning of these tissues or cells is catastrophic. Among these cell types, fibroblasts play critical roles in all phases of wound healing. Following skin abrasion or wounding,

fibroblasts attracted from the edges of the wound or from the bone marrow produce chemokines and growth factors that aid in wound healing (Opalenik & Davidson, 2005). For instance, the expression of basic fibroblast growth factor (bFGF), which is generally high during the late inflammatory stage, activates macrophages to scavenger pathogens and clear cellular debris (Fui et al., 2019).

In a clinical study conducted by Matsumoto et al. (2013) patients with diabetic ulcers had sufficient granulation tissue for skin grafting after applying bFGF-impregnated gelatin sheet for three weeks. Another cell type that is critical to the wound healing process is macrophages. Macrophages are an important cell type of the innate immune system and are required for wound repair. Alterations of the normal functioning of macrophages are implicated in diabetic wounds (Mirza & Koh, 2011). Phenotypic changes in normal wounds, especially a switch from a proinflammatory to a pro-reparative phenotype supports tissue regrowth. However, in diabetic wounds, macrophage deficits result in phenotypes that fail to stimulate tissue repair (Mirza & Koh, 2011).

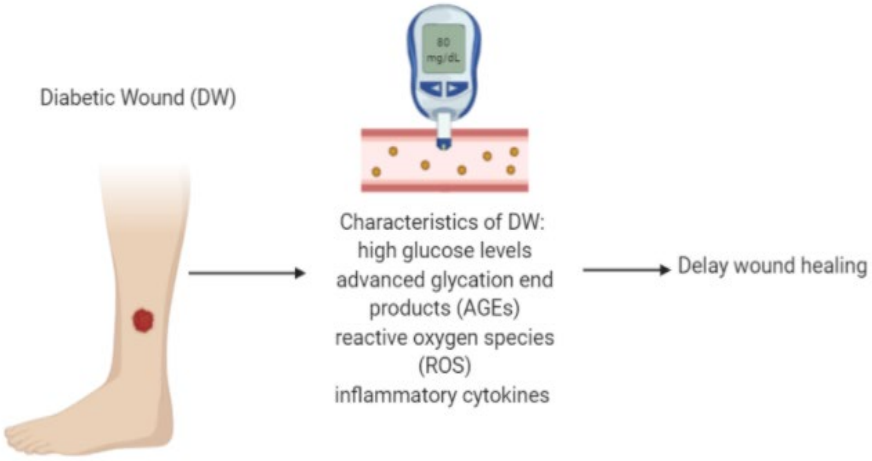

Figure 2. Delayed Wound Healing Factors in Diabetic Patients.

Several factors that are implicated in the delayed wound healing process in diabetic patients include AGEs, ROS and inflammatory cytokines (BioRender, 2021).

ADIPOKINES AND WOUND HEALING

Wound healing takes place in all three layers of the skin: the epidermis, the dermis, and the subcutaneous adipose tissue. In the physiological state, adipose tissue plays an important role, including thermoregulation, protection of internal organs, and steroid hormone production (Bergmann & Sypniewska, 2013; Bluher, 2013). More so, several studies suggest that adipose tissue is an active participant in wound repair (Ebrahimian et al., 2009; Kim et al., 2007; Nie et al., 2011). However, the importance of adipose tissue in wound healing has not been studied extensively. Adipose tissue is a major endocrine organ that releases multiple bioactive substances, known as adipose-derived secreted factors or adipokines (Ouchi et al., 2011). Studies on the relationship between adipose tissue, insulin resistance, and diabetes have created a crosstalk centered around abnormalities in adipocyte metabolism and their proinflammatory potential.

Adipokines are defined as a group of bioactive markers secreted by adipose tissue and act as paracrine and endocrine hormones (Bluher, 2014). Although the full set of human adipokines is still not entirely characterized, it has become clear that adipose tissue is a source of more than 600 potentially secretory proteins (Lehr et al., 2012). In the last few decades, the role of adipokines in immune system modulation, inflammation, and metabolic syndrome has generated an interest in adipokine studies. Examples of some of the diverse functions of adipokines include regulation of energy expenditure, insulin sensitivity, glucose and lipid metabolism, regulation of satiety and appetite, regulation of blood pressure, and hemostasis (Table 1) (Francisco et al., 2019; Gandhi et al., 2010). The ability of these bioactive markers to assume pro-inflammatory and anti-inflammatory activities make them unique (Ouchi et al., 2011).

Adipokines	Site of Expression	Functions	Therapeutic Target
Apelin	Adipose tissue, stomach, heart, lung, skeletal muscle	Regulate cardiovascular and fluid homeostasis, food intake etc.	Metabolic disorder (obesity and cardiovascular diseases)
Resistin	Adipose tissue, macrophages, spleen, and bone marrow cells	Regulate inflammation, endothelial dysfunction, smooth muscle cell dysfunction etc.	Metabolic syndrome (insulin resistance and diabetes)
Adiponectin	Adipose tissue, skeletal and cardiac myocytes, endothelial cells	Regulate lipid and glucose metabolism and fatty acid oxidation	Metabolic disorder (obesity and insulin resistance)
NAMPT	visceral adipose tissue, kidney	Regulate lipid and glucose metabolism	Metabolic syndrome (diabetic nephropathy)
IL6	Adipose tissue, smooth muscle cells and macrophages	Regulate immunity, neural development, and lipid metabolic homeostasis	Cardiac diseases caused by metabolic disorder
PPARγ	Adipose tissue, liver, kidney, heart, and skeletal muscle	Regulate glucose homeostasis, lipid metabolism and fat mass	Metabolic disorder (insulin sensitivity)
Leptin	Adipose tissue, gastric mucosa tissue	Regulate food intake and maintain metabolic homeostasis	Metabolic disorder (obesity)
TNFα	Adipose tissue, and endothelial cells	Regulate lipid metabolism, cell proliferation, apoptosis etc.	Metabolic disorder (insulin sensitivity and resistance)

Table 1. List of Various Adipokines.

Table 1 lists examples of a variety of well-known adipokines, where they are expressed, the major systemic and tissue specific functions for each, and established or potential targets for therapeutic treatments.

ADIPOKINES AND OBESITY-RELATED DISEASES

The established relationship between adipokines and obesity-associated diseases such as T2DM, show that adipokines have an enormous potential for clinical relevance both as bioactive markers and as therapeutic agents (Bluher, 2014; Timar et al., 2014; Xu et al., 2015). Therefore, it has been proposed that adipokines could be used to determine disease progression, to monitor clinical responses to therapeutic and lifestyle interventions, and to monitor treatment adherence (Bluher et al., 2012). Most importantly, even though the mechanism of regulating molecular and physiological function has not been clearly elucidated, alterations in adipokine secretion have been implicated in obesity-related diseases (Fasshauer & Bluher, 2015). For instance, studies show the molecular mechanisms of insulin activity may be impaired by the induction of chronic low-grade inflammation in adipose tissue through the synthesis of chemotactic substances that enhance the influx of lymphocytes and monocytes (Bergmann & Sypniewska, 2013; Lumeng & Saltiel, 2011). This phenomenon highlights the importance of understanding the effect of impaired adipokine secretion in the pathogenesis of diabetes-related complications such as delayed diabetic wound healing.

Resistin

Resistin, an adipocyte-derived polypeptide, is a member of a secretory protein family commonly known as resistin-like molecules (RELMs). A distinct feature of this family of proteins is the presence of a highly conserved, cysteine-rich C terminus (Booth et al., 2015; Cebeci et al., 2019; Pang & Le, 2006). The discovery of resistin is credited to three independent groups, Kim, Holcomb, and Steppan, who were investigating different aims at the time of this discovery (Pang & Le, 2006). Resistin was first discovered by Steppan et al. and appeared in a 2001 publication (Steppan et al., 2001). Following its discovery, resistin was first considered to

mainly be a link between obesity and insulin resistance and shown to be more heavily secreted by adipocytes in a rodent model (Steppan et al., 2001). Steppan et al. showed in their earlier studies that this adipokine circulates in the serum of rodents and is increased in both genetic and diet induced obesity (Steppan et al., 2001). On the other hand, Kim and colleagues identified resistin via microarray analysis as an adipose tissue-specific secreted factor (Kim et al., 2001). Holcomb et al. initially identified resistin as an expressed sequence tag database against a related protein induced during lung inflammation (Guest et al., 2017; Holcomb et al., 2000). They later referred to this as "found in inflammatory zone 3" (FIZZ3). The expression of resistin has been identified in several cell types such as adipocytes (Savage et al., 2001; Steppan et al., 2001), intestinal epithelium, and skeletal muscle cells (Nogueiras et al., 2003), and astrocytes (Morash et al., 2002). The degree of resistin synthesis varied greatly in these sites. For instance, recent studies in humans have indicated that unlike rodents, resistin is heavily expressed in peripheral mononuclear cells, but minimally expressed in adipocytes and preadipocytes (Savage et al., 2001).

Mouse resistin is an 11 kDa cysteine-rich polypeptide, with its genes located on chromosome 8, and exclusively is expressed in high levels in white adipose tissue (Pang & Le, 2006). It contains five intramolecular disulfide bonds with multiple β-turns and is synthesized as a 114 amino acid (aa) precursor (Jamaluddin et al., 2012). Unlike in humans, the mouse RELMs family is made up of four members: resistin, RELMα, RELMβ and RELMγ (Pang & Le, 2006). More so, there is a debate about the functional roles of resistin in mouse and humans given some structural and chromosomal changes. Human resistin is a 12.5 kDa cysteine-rich polypeptide with a more mature sequence consisting of 108 amino acids (Jamaluddin et al., 2012). Also, the human resistin gene is located on chromosome 19 with only two identified family members,

resistin and RELMβ (Pang & Le, 2006). Even though mouse and human resistin gene share 64.4% sequence homology at mRNA level and 59% identity at amino acid level (Steppan & Lazar, 2004), the genes have divergent promoter regions with different mechanisms of regulation, tissue distribution, and functions (Ghosh et al., 2003). In humans, the expression of resistin is not just limited to adipose tissues but it is expressed by other cell populations such as peripheral blood mononuclear cells, macrophages, and bone marrow cells (Fain et al., 2003; Patel et al., 2003). It is worth noting while some studies have reported only human preadipocytes express resistin (Janke et al., 2002), other studies show that mature human adipocytes also produce insulin (Degawa-Yamauchi et al., 2003).

Following its discovery, several studies have explored the possible relationship between resistin and metabolic disorders in healthy subjects and diabetic patients. For instance, a study conducted by Reilly et al in healthy and T2DM patients showed an increased level of resistin in woman versus men, as well as in T2DM groups versus healthy subjects (Reilly et al., 2005). This suggests that resistin could be a key predictor of inflammatory and other metabolic disorders such as arteriosclerosis, obesity and visceral adiposity, insulin resistance, hyperglycemia, and hypertension. Conversely, one of the early findings by Steppan and colleagues show that administration of resistin to wildtype mice worsened glucose homeostasis and insulin sensitivity. However, neutralizing the expression of resistin by injection of antibodies in diet-induced obese mice decreased blood glucose and improved insulin sensitivity (Steppan et al., 2001).

This initial evidence has led to the speculation that resistin could possibly be one of the major factors contributing to insulin resistance and its related diseases. The expression of resistin in immune cells and its ability to infiltrate the adipose tissue (Lehrke et al., 2004) demonstrates that resistin is likely related to the inflammatory status of an individual. Further studies have also

explored the expression of resistin in the presence of hormones and different molecules. Among these, thiazolidinediones (TZDs), insulin, glucose, glucocorticoid, and growth hormone have been implicated to regulate resistin gene expression (Pang & Le, 2006). Experimental evidence gathered by Shojima et al. (2002) shows that resistin expression is significantly upregulated by high glucose concentrations in 3T3-L1 adipocytes. The same study also found that administering insulin to these adipocytes suppressed expression of resistin mRNA and protein (Shojima et al., 2002). Apart from its role in insulin resistance and diabetes, increasing evidence suggests resistin plays a critical role in angiogenesis and endothelial function – key steps in wound healing.

Endothelial cells form the main physical barrier between blood and the arterial wall and controls the movement of solutes and fluid from the vascular space to the surrounding tissues. This enables them to release vasoactive and trophic substances such as prostacyclin, endothelium -derived growth relaxing factor/nitric oxide (NO), and angiotensin II (Caldwell et al., 1976; Jamaluddin et al., 2012; Moncada et al., 1976). These substances aid in controlling vascular growth, platelet function, coagulation, and inflammatory responses (Libby, 2001). However, prolonged exposure to high glucose can result in a loss of endothelial cell integrity, making them susceptible to detaching and apoptosis (Okonkwo & DiPietro, 2017). When this happens, there is an alteration in endothelial metabolism and function which eventually affects the angiogenesis process (Altabas, 2015).

Studies investigating endothelial repair in diabetic patients have identified adipocytes as one of the factors involved in damaged vascular repair. These cells produce several bioactive markers that contribute to a chronic inflammatory state by secreting TNF-α, a proinflammatory adipokine that reduces proliferation of endothelial progenitor cells (Tilg & Moschen, 2008). For example, recent studies have shown that resistin strongly upregulates IL-6 and TNF-α in human

peripheral blood mononuclear cells though NF-κβ pathway (Bokarewa et al., 2005). These preliminary findings highlight the inflammatory effect of resistin on other adipokines and its overall action of endothelial tissue repair in diabetic patients.

Angiogenesis, formation of new blood vessels, is a complex process which is characterized by increased vascular permeability, matrix degradation, migration, and proliferation of endothelial cells (Jamaluddin et al., 2012). As previously established, angiogenesis is an important physiological pathway, in both normal and diabetic wound healing process. Experimental evidence by Mu et al. (2006) on human endothelial coronary artery endothelial cells show that human recombinant resistin stimulates proliferation, stimulates migration, and enhances capillary-like tube formation. Furthermore, this study reports that resistin also up-regulates gene expression of critical angiogenesis-promoting factors such as vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2) and matrix metalloproteinase (MMP-1 and MMP-2) (Mu et al., 2006). Growth factors are soluble signaling proteins whose inhibitory and stimulatory properties during the different stages of normal wound healing enhance the overall healing process (Fui et al., 2019). Major growth factors that have been extensively studied in wound healing are platelet-derived growth factors (PDGF), bFGF, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (Fui et al., 2019). Thus, the ability of resistin to stimulate the expression of these growth factors suggest that resistin could potentially play a key role in the management and treatment of diabetic wounds.

PPAR-Gamma

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate genes involved in cellular differentiation and metabolic processes such as glucose and lipid homeostasis (Janani & Ranjitha Kumari, 2015). PPARs belong to the nuclear

hormone receptor superfamily, and consist of three isotypes, namely PPAR-α, PPAR-β/δ, and PPAR-γ (Berger & Moller, 2002; Janani & Ranjitha Kumari, 2015; Michalik et al., 2006). Even though these isoforms share a high degree of structural homology, such as DNA-binding domain and ligand cofactor-binding domain with members of the superfamily (Michalik et al., 2006), they also differ from each other in relation to tissue distribution and physiological roles (Janani & Ranjitha Kumari, 2015). PPARs have been implicated in the regulation of genes that regulate homeostasis (Lefebvre et al., 2006), glucose and lipoprotein metabolism (Staels et al., 1998), fatty acid uptake, oxidation, cell proliferation, and inflammation in vascular tissue formation (Moraes et al., 2006). The discovery of genes that are regulated by PPARs and their implication in the pathogenesis of metabolic diseases such as metabolic syndrome, type 2 diabetes mellitus, nonalcoholic fatty liver disease, and cardiovascular disease (Han et al., 2017) make them important molecular targets in developing new drugs to treat these complications.

The discovery of PPAR-γ and its role in metabolism has garnered significant scientific and clinical interest in the last two decades. Unlike human PPAR-α and PPAR-β/δ, which are localized at chromosome region 6p21.2-21.1 with nine exons (Skogsberg et al., 2000), human PPAR-γ (also composed of nine exons) is localized on chromosome 3p25 (Fajas et al., 1997). Adipose tissue is the major site of PPAR-γ expression, and some of the biological functions at this site include adipose tissue differentiation, insulin sensitivity, lipid storage, glucose metabolism, and regulation of genes involved in the metabolic process (Han et al., 2017). Contrary to the high levels of PPAR-γ protein expressed in adipose tissues, the level of expression seen in liver, skeletal muscle, and heart is usually low-to-moderate. Evidence shows that under certain physiological conditions, PPAR-γ protein can be significantly upregulated in these sites. For instance, Park et al. found that PPAR-γ gene expression was upregulated in

muscle cultures from type 2 diabetic subjects when compared with lean nondiabetic subjects (Park et al., 1997). Another major site where PPAR-γ is expressed in relatively high levels apart from adipocytes is vascular cells, such as endothelial cells, smooth muscle cells, and macrophages (Han et al., 2017). The role of PPAR-γ in vasculature and inflammation has been extensively studied in the last 20 years. In endothelial cells, PPAR-γ regulates biomarkers and other molecular targets that are critical to inflammation and atherosclerosis (Marx et al., 2003). Following the discovery of PPARs, several studies have explored the physiological characteristics of each isotype in different animal models, especially PPAR-γ isoforms. For example in mouse, PPAR-γ1 and PPAR-γ2 have been identified, while in humans and non-human primates, PPAR-γ4 has also been identified in addition to PPAR-γ1 and PPAR-γ2 (Han et al., 2017).

Even though the involvement of PPAR-γ in diabetes and obesity has been extensively studied due to its role in regulating glucose metabolism, its role in wound healing is only beginning to be explored. It is well established that in skin wound healing, PPAR-α controls the early inflammation phase of healing (Michalik et al., 2001), PPAR-β regulates keratinocyte proliferation, adhesion, and migration (Michalik et al., 2001; Tan et al., 2003; Tan et al., 2004), and PPAR-δ participates in promoting fibroblast proliferation (Li et al., 2012). However, little has been elucidated about the direct role of PPAR-γ in wound healing. As already eluded to, macrophages are one of the key immune cells that are recruited to a wounded site after skin injury, and evidence shows that PPAR-γ is a key contributing factor that aids macrophages to carry out their inflammatory properties (Mahdavian et al., 2011).

In wound healing, macrophage PPAR-γ signaling is necessary for the effective clearance of apoptotic cells and the switching from proinflammatory macrophage to anti-inflammatory

macrophage. Overall, this process is important for resolving inflammation and maintaining tissue homeostasis (Chen et al., 2015). In addition to its role in enhancing macrophage polarization during wound healing, PPAR-γ has also been recognized to play an important role in immune response via its ability to inhibit the expression of inflammatory cytokines and to direct the differentiation of immune cells towards anti-inflammatory phenotypes (Tyagi et al., 2011). This transition is important for the regulation of angiogenesis, granulation tissue formation, and wound closure (Mirza et al., 2009) – key steps in normal wound healing. To further evaluate the importance of PPAR-γ activity and macrophage wound healing, Chen et al. (2015) found that macrophage PPAR-γ deficiency (PPAR-γ knock out) mice exhibited impaired skin wound healing which reduce collagen deposition, angiogenesis, and granulation formation. Therefore, understanding the physiological role of PPAR-γ activity in diabetic wound healing could enhance efforts for developing synthetic PPAR-γ agonists to combat diabetic wounds.

CHAPTER 2

TOPICAL DRUG DELIVERY FORMULATIONS AS POTENTIAL DIABETIC WOUND HEALING TREATMENTS

As discussed in Chapter 1, wound healing is a multifaceted process that involves four overlapping phases, namely hemostasis, inflammation, proliferation, and tissue remodeling (see Figure 1). These mechanisms result in the restoration of anatomical integrity with analogous function (Patel et al., 2019). Unlike acute wounds which generally heal without complications, chronic wounds are a result of poor blood circulation, diseases like diabetes, and stressed environmental conditions (Patel et al., 2019). Furthermore, they are expensive and difficult to manage. As a result, there is an alarming increase in the incidence of delayed wound healing in diabetic patients globally. For example, in the United Kingdom alone, a National Health Service report shows that up to 5 billion pounds were spent on diabetic wound treatment annually (Guest et al., 2017). Several treatments have been proposed for the management of diabetic wounds, and these are usually aimed at targeting various phases of the wound healing process (Fui et al., 2019). Examples of these treatments include customized wound dressing, hyperbaric oxygen, surgical debridement (Fui et al., 2019), and negative pressure wound therapy (Liu et al., 2017).

Customized wound dressings are used for different types of chronic wounds within 12 weeks of onset, while hyperbaric oxygen and negative pressure are usually used for diabetic wounds that persist for more than 12 weeks (Fui et al., 2019). Of these therapies, hyperbaric oxygen therapy (HBOT) has been heavily relied upon in the clinical setting. This therapy involves placing a patient in an enclosed chamber where the pressure is greater than normal pressure and supplying them with 100% oxygen. The treatment has been shown to improve tissue hypoxia, improve vessel perfusion, reduce inflammation, and increase angiogenesis

(Okonkwo & DiPietro, 2017; Tiaka et al., 2012). Unfortunately, HBOT is expensive and regardless of its track record in the clinical setting, it still does not provide a complete answer for treating chronic wounds such as diabetic foot ulceration (Okonkwo & DiPietro, 2017). Therefore, the high cost of managing diabetic wounds in a clinical setting and the challenges faced by these specialized treatments have led scientists to explore natural biodegradable products for therapy. In attempts to improve results in diabetic wound treatment, advanced therapy such as topical application of growth factors and bioengineered cellular therapies (Frykberg & Banks, 2015) employing different drug delivery systems are being investigated. Among these drug delivery systems, chitosan gel has become a focus of attention in ensuring effective delivery of drugs to the site of action without negative side effects due to its biodegradable nature.

Chitin

Chitin is a linear polysaccharide, made up of β-(1,4)-linked N-acetyl-d-glucosamine units and is considered to be one of the macromolecules with highest production rate and biodegradability existing in nature (Figure 3). Structurally, there are three different polymorphs of chitin namely, α-chitin, β-chitin, and γ-chitin (Muxika et al., 2017). At the industrial level, chitin is chemically extracted from marine shell waste streams through methods such as deproteinization, demineralization, and discoloration (Philibert et al., 2017). After the discoloration step, chitin is converted to chitosan either through enzymatic or chemical processes. In most cases, the chemical process is preferred since it is cost effective and suitable for bulk production (Meyers et al., 1975; Younes & Rinaudo, 2015). The primary chemical method used in this process is deacetylation. Chemical deacetylation involves the treatment of chitin with hydroxides at high temperatures, usually above 80°C (Muxika et al., 2017).

Chitosan

Chitosan, a linear amino polysaccharide, is composed of glucosamine and N-acetyl glucosamine units and linked by β (1–4) glycosidic bonds (Figure 3) (Aytekin et al., 2011; Dai et al., 2011; Muxika et al., 2017; Ouyang et al., 2018). As a natural copolymer, chitosan is commercially derived from chemical deacetylation of crustacean chitin under strong alkali treatment (Fai et al., 2011; Muxika et al., 2017). The presence of nitrogen in the molecular structure of chitosan and its cationic nature makes the chitosan polymer distinct from other polysaccharides (Bhattarai et al., 2010). Chemically, either acids or alkalis can be used to deacetylate chitin. However, studies show that the glycosidic bonds are very susceptible to acid, hence alkali deacetylation is most frequently employed for chitosan production (Hajji et al., 2014; Meyers et al., 1975). Dissolving chitosan in an acidic medium results in protonation of the amino acid groups in the chain. When this happens, the polymer becomes cationic and then interacts with different types of molecules (Lizardi-Mendoza et al., 2016). The production of chitosan through N-deacetylation can either occur homogeneously or heterogeneously (Lizardi-Mendoza et al., 2016). Physicochemical characteristics of chitosan, such as purity, crystallinity, inorganic matter content, degree acetylation, molecular weight, and water content, among others, have determinant influences on most of its functional properties (Dornish et al., 2001; Lizardi-Mendoza et al., 2016).

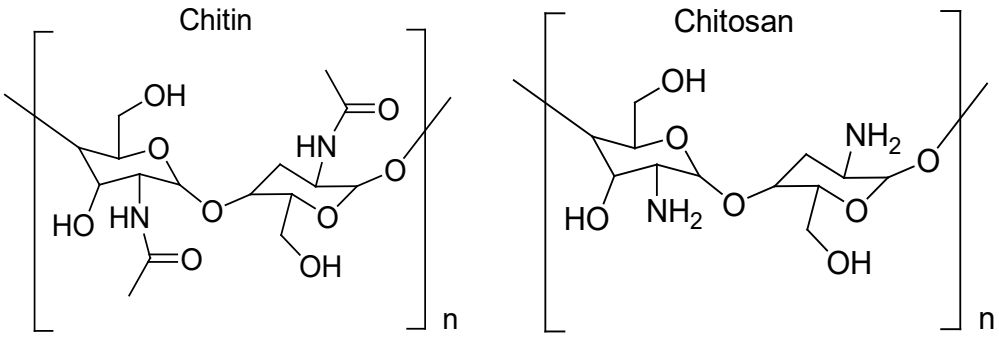

Figure 3. Chemical Structures of Chitin and Chitosan.

DEGREE OF ACETYLATION AND MOLECULAR WEIGHT

One of the major distinctions between chitin and chitosan is the degree of acetylation (DA). Chitin deacetylation reaction progresses by exposing amino groups along the molecule. The proportion of acetylated and deacetylated groups causes several changes in the main properties of the molecule. Since the amino groups are ionizable, chitosan becomes polycationic when these amino groups are in acidic pH environments. This unusual quality allows chitosan to actively interact with diverse molecules in solution. Thus, the DA regulates the solubility, extent of swelling in water, bioactivity, and biocompatibility nature of chitosan (Lizardi-Mendoza et al., 2016). The molecular weight of chitosan is a characteristic that has determinant influence in most of its functional properties. These characteristics have a marked effect on the functional properties of chitosan either in solution or solid state. The molecular weight has particular influence on the viscoelastic properties of solutions and hydrated colloidal forms. As with many other natural polymers, the molecular characteristics of chitosan are polydisperse. In chitosan, the molecular weight is also a result of depolymerization through the deacetylation process. Together with the degree of acetylation, molecular weight is the most important chemical characteristic of chitin and chitosan (Lizardi-Mendoza et al., 2016)

BIOMEDICAL APPLICATIONS

Biomedical applications of chitosan has been extensively studied and used in diverse fields such as biomedicine, cosmetology, agriculture, and pharmaceutics, among others (Muxika et al., 2017), owing to its non-toxic, biodegradable, and biocompatible properties (Philibert et al., 2017). Clinical application of chitosan includes wound dressing, drug delivery, tissue engineering, blood anticoagulant, bone regeneration biomaterial, and antimicrobial agent (Anitha et al., 2014; Azad et al., 2004; Muzzarelli et al., 1999). Chitosan has also been found to enhance the functions of inflammatory cells such as polymorphonuclear leukocytes, macrophages, and fibroblasts in promoting granulation and organization (Okamoto et al., 2003). Most importantly, studies in animal models show that it enhances blood coagulation (Okamoto et al., 2003) and accelerates wound healing (Kweon et al., 2003). In particular, Okamoto et al. (2003) reported that chitin and chitosan granules enhanced re-epithelization and regenerated normal skins in open wounds. As natural polymer and a drug delivery vehicle, chitosan and its derivatives have been widely used to deliver active ingredients such as drugs, growth factors, stem cells and peptides (Figure 4) (Bhattarai et al., 2010).

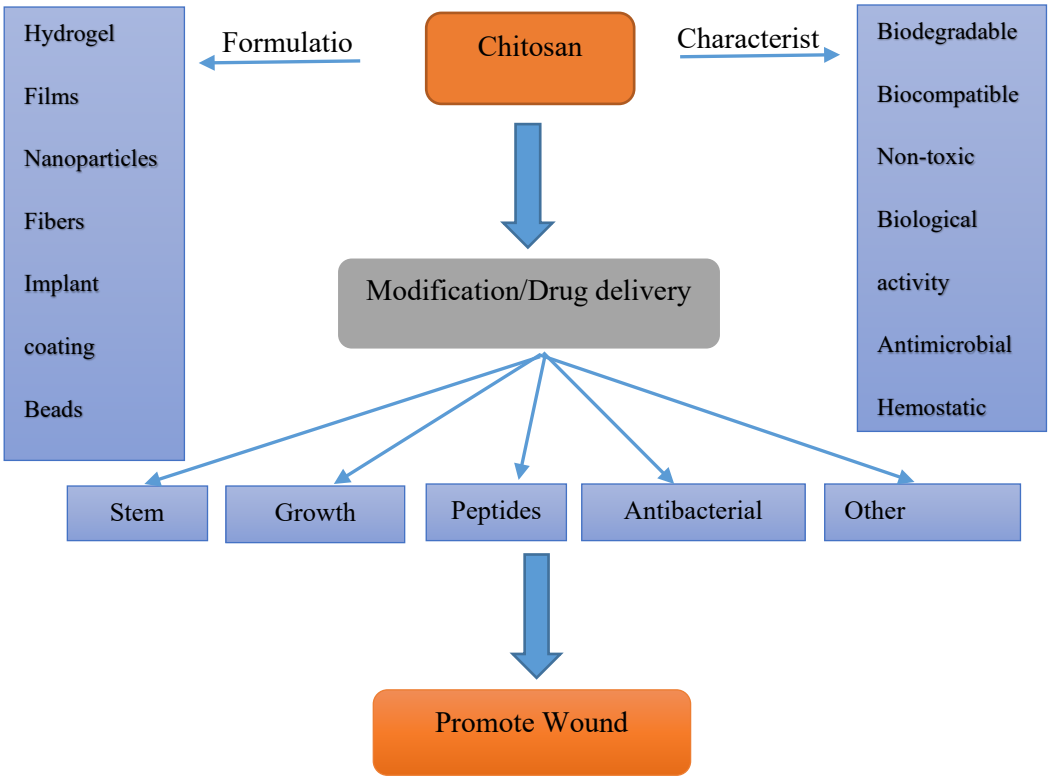

Figure 4. Utility of Chitosan for Wound Healing.

Diagram of the characteristics of chitosan-based drug formulation and the physiological applications of chitosan as a delivery vehicle. Chitosan can be used to deliver active substances, which could promote wound healing.

CHAPTER 3

DRUG DELIVERY SYSTEMS: EXPLORING RHEOLOGICAL PROPERTIES AND ANTIMICROBIAL ACTIVITY OF 5-FU CHITOSAN GEL

ABSTRACT

Background: Topical drug delivery is an attractive method for local and systemic treatments with many advantages over conventional dosage forms. Formulating drugs as topical gels can increase residence time of the drug, and improve release of the active ingredients, which enhances the overall bioavailability of the drug at the site of action. Effectively formulating topical drugs in gels or creams involves a thorough understanding of their rheological properties. Thus, the steady, dynamic rheological properties, and antimicrobial potential of chitosan gel formulated with 5-fluorouracil (5-FU), were explored. **Methods**: Chitosan gel (2% w/w) was prepared in serial dilutions of 5-FU. Stability, antimicrobial activities, and rheological properties such as flow behavior, oscillatory sweep tests (amplitude and frequency), and creep recovery of gels were analyzed. **Results**: Each concentration of 5-FU-CS gel demonstrated shear thinning behavior with a flow index < 1. CS gel formulations displayed weak gel-like properties. Storage modulus values (G') were less than loss modulus values (G"). Stability (pH and conductivity) was maintained after three weeks of storage at 4-8ºC and 20-25ºC. 5-FU and 5-FU-CS gel significantly inhibited growth of MRSA and RN1; however, there was no growth inhibition on *E. coli*. Drug-free CS gel had no growth inhibition against MRSA, RN1 or *E. coli*. The MIC for 5-FU against MRSA, RN1, and *E. coli* were 0.5µg/mL, 0.5µg/mL, and 8.0µg/mL, respectively. **Conclusions**: CS was formulated into non-Newtonian hydrogel with shear thinning properties. CS gel was viscous and less elastic. 5-FU-CS gel is stable at both room and cold temperatures after three weeks of storage. Our data show 5-FU and 5-FU-CS gel have antimicrobial activities against MRSA and RN1 at low concentrations, and against *E. coli* at higher concentrations.

INTRODUCTION

5-fluorouracil (5-FU) is a uracil analogue that can be converted into three active metabolites, namely fluorouridine triphosphate, fluorodeoxyuridine triphosphate, and fluorodeoxyuridine monophosphate via intracellular conversion (Figure 5). 5-FU is used as chemotherapeutic agent due to its ability to cause alterations in RNA processing/function and severe DNA damage which results in apoptosis (Longley et al., 2003). Even though 5-FU remains as one of the first line for chemotherapy treatments, evidence is also emerging that it has substantial antibacterial effects at high concentrations against pathogenic strains. Administering 5-FU to patients orally often results in inflammation and ulceration of the oral mucosa (Vanlancker et al., 2016). Thus, exploring the antibacterial activity of 5-FU using other routes of drug delivery is key to maximizing its therapeutic potentials.

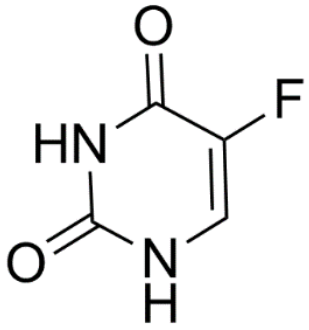

Figure 5. Chemical Structure of 5-Fluorouracil.

Topical drug delivery is an attractive method for local and systemic treatments and is commonly used in the treatment of inflammatory conditions like dermatological diseases and musculoskeletal injuries (Dantas et al., 2016). Topical application has many advantages over the conventional dosage forms, especially the avoidance of serious systemic adverse effects (Whitehouse, 2011). Topical drug delivery allows for deeper skin penetration and hence better

absorption (Glavas-Dodov et al., 2003). Topical administration prevents the metabolism of drug in the liver, avoids both gastrointestinal disorders and the risks of intravenous therapy, and avoids the risks associated with the varied conditions of absorption like pH changes, presence of enzymes, and gastric emptying time (Dantas et al., 2016). This results in an overall increase in the bioavailability of drugs at the targeted site of action. The main advantage of a topical delivery system is the avoidance of first pass metabolism. A wide variety of pharmaceutical dosage forms can be used as a delivery system for topical drugs. The most commonly used dosage forms include gels, creams, and ointments, followed by sprays and liquid preparations (Gisby & Bryant, 2000). Formulating drugs as topical gels can increase residence time of the drug and improve release of the active ingredients which enhance the overall bioavailability of the drug at the site of action. Challenges associated with the bioavailability of drugs have forced formulation scientists to explore drug delivery systems that are safe, non-toxic, and will effectively enhance the release of drugs at the intended site of action.

Chitosan is a naturally occurring compound that is present in the coating of some mollusks and insects (Escarcega-Galaz et al., 2018). Structurally, chitosan is a linear amino polysaccharide and is composed of glucosamine and N-acetyl glucosamine units and linked by β (1–4) glycosidic bonds (Aytekin et al., 2011; Dai et al., 2011; Muxika et al., 2017; Ouyang et al., 2018) (Figure 3). The presence of nitrogen in the molecular structure of chitosan and its cationic nature makes the chitosan polymer distinct from other polysaccharides (Bhattarai et al., 2010). As a natural copolymer, chitosan is commercially derived from chemical deacetylation of crustacean chitin under strong alkali treatment (Fai et al., 2011; Muxika et al., 2017). Chitosan and its derivatives have been widely used in the fields of medicine, cosmetics, wound dressing, and tissue engineering (Muzzarelli et al., 2012) as a suitable polymer for the delivery of active

ingredients such as drugs, growth factors, stem cells, and peptides (Bhattarai et al., 2010). This is because chitosan polymers are generally considered to be a biodegradable, biocompatible, non-antigenic, non-toxic, biologically adhesive, antimicrobial and biologically active compound with hemostatic effect (Dai et al., 2011; Huang & Fu, 2010; Liu et al., 2018). As part of their chemical characteristics, chitosan materials exhibit a positive charge (at typical wound pH values), film-forming capacities, mild gelation characteristics, and strong wound tissue adhesive properties (Jayakumar et al., 2011). Clinically, chitosan formulations are used in wound dressing due to its antibacterial and hemostatic activities (Zhao et al., 2017).

One of the ways to advance knowledge on the industrial use of chitosan formulations is by investigating its steady and dynamic rheological properties such as flow and deformation. Despite the numerous studies investigating biomedical application of chitosan, rheological studies on the polymer are scarce. Therefore, understanding the rheological properties of commercial chitosan is essential to designing, predicting, and characterizing most of its biomedical application (Calero et al., 2010). Rheological measurements are helpful to understand the effect of handling and preparing viscoelastic materials. They also help to assess the damage caused by heat, oxygen, etc. on the structure of different materials, including polymers. Lizardi-Mendoza et al. (2016) have reported that as the degree of deacetylation of chitosan decreases, viscosity and other flow properties tend to increase. Therefore, understanding the rheological changes that happen during phase transition of chitosan in gel formulation is important in its development and usage as a drug delivery system. Dynamic rheological measurement is useful for understanding the viscoelastic behavior of gels and pastes while allowing the continuous measurement of steady properties such as the effect of shear stress, temperature, frequency, and amplitude on these materials (Bhardwaj et al., 2019). Rheological characteristics help

formulation scientists to effectively formulate chitosan as either hydrogel, biofilm, nanosphere, implant coating, etc. Even though the FDA has cleared several chitosan based medical devices and combination products, including topical wound dressings and wound dressings containing antimicrobial drugs (Nilsen-Nygaard et al., 2015), rheological studies on the polymer are limited.

Thus, the main objective of this study was to model and study the steady shear measurements (flow behavior, apparent viscosity), dynamic measurements (oscillation sweep test of amplitude and frequency) and creep recovery properties of 2% CS gel in order to assess its potential as a topical drug delivery vehicle. A further goal was to examine the stability and antimicrobial activity of 2% CS gel formulated with different concentrations of 5-FU in vitro. To our knowledge, the antimicrobial activity of 5-FU-CS gel has not been previously reported.

MATERIALS AND METHODS

Low molecular weight chitosan powder and pure 5-Flurouracil were obtained from Sigma Aldrich (Missouri, USA, P 9012-76-4). Methicillin-resistant *Staphylococcus aureus*, (MRSA), Strain JE2, methicillin-susceptible *S. aureus* (MSSA), RN1, NR-46543 (BEI Resources, NIAID, NIH), and *Escherichia coli* K-12, Strain DC10B, NR-49804 were obtained from BEI Resources (NIAID, NIH).

5-FU-Chitosan Gel

Chitosan gel (2% w/w) was prepared by dissolving 2.0 grams of low molecular weight chitosan powder in 100 ml of 1% acetic acid. This mixture was stirred for 12 hours at room temperature. 5-Fluorouracil was dissolved in DMSO at a stock concentration of 5mg/mL and serial dilutions of the following 5-FU-CS gel formulations were prepared: 25µg/mL, 2.5µg/mL, 0.25µg/mL, and 0.025µg/mL.

RHEOLOGICAL MEASUREMENTS

The rheological properties of chitosan gel (2% w/w) were conducted on a HAAKE MARS III Rheometer (Thermo Fisher Scientific, Rheology, USA) equipped with a stainless-steel parallel plate (P35 TiL L1507) probe and 0.5 mm gap, operated through RheoWin Data Management software. The flow behavior, viscosity, stability, and rheological properties of five chitosan gel formulations (CS gel control; 25µg 5-FU-CS; 2.5µg 5-FU-CS, 0.25µg 5-FU-CS; 0.025µg 5-FU-CS) were analyzed. An adequate amount of sample was loaded onto the rheometer plate and excess material was trimmed off. All the measurements were performed at 25°C, and the samples were permitted to equilibrate at this temperature prior to the analysis. All experiments were conducted in triplicate.

STEADY SHEAR MEASUREMENTS

Flow Behavior and Viscosity

The shear stress was measured over a strain rate range of 0.1 to 200 s^{-1}. To reveal the time dependent flow behavior, samples were sheared first in ascending and then in descending (200 to 0.1 s^{-1}) shear order. To analyze flow behavior, the data generated was then fitted to Ostwald de Waele (Equation 1) and Herschel-Bulkley (Equation 2) models:

Equation 1

$$\eta = K\dot{\gamma}^{n-1}$$

Equation 2

$$\tau = \tau_0 + K\dot{\gamma}^{n}$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency coefficient (Pa.sn), n is the flow behavior index (dimensionless), and τ_0 is the yield stress (Pa).

DYNAMIC MEASUREMENTS

Amplitude Oscillation Stress Sweep

Dynamic rheological measurements are determined either at a controlled strain or controlled stress conditions, where samples are subjected to oscillatory strain or stress (Bhardwaj et al., 2019). The amplitude sweep test was performed by applying varying amplitude stress (0.5 to 150 Pa) at a constant frequency of 1 Hz (6.28 rads s^{-1}). Sufficient amounts of each formulation were loaded onto a P35 TiL L1507 plate, excess material was trimmed, and the linear viscoelastic region (LVR) was obtained. The power law analysis of storage modulus (G') and loss modulus (G") versus stress was evaluated.

Frequency Oscillation Stress Sweep

For the frequency sweep test, samples were subjected to stepwise increasing frequency (0.1 to 100 Hz) at a constant stress in the field of LVR obtained from the amplitude sweep test. Storage modulus (G') and loss modulus (G") versus stress was evaluated.

Creep Recovery

Sample was loaded onto a P35 TiL L1507 plate and kept for five minutes for structural recovery and temperature equilibration (25°C). The sample was then subjected to a constant shear stress (5 Pa) for 250 seconds. Afterward, the stress was released to allow the sample to recover for 250 seconds making the total time for creep and recovery 500 seconds. The creep compliance (J) and final percent recovery (R) (Equation 3) were determined from the creep recovery curve.

Equation 3

$$\boldsymbol{Recovery}\ (\%) = \left|\frac{\boldsymbol{J_{250} - J_{500}}}{\boldsymbol{J_{250}}}\right|$$

where J_{250} is creep compliance after 250 seconds and J_{500} is creep compliance after 500 seconds.

Antimicrobial Activity

The antimicrobial potential of drug-free CS gel, 5-FU, and 5-FU-CS gel formulations (1.25µg, 1.0µg, 0.75µg, 0.5µg, and 0.25µg) per well were tested against *Escherichia coli* (*E. coli*), methicillin-resistant *S. aureus* (MRSA), and methicillin-susceptible *S. aureus* (RN1). The bacteria cell suspensions were calibrated to 0.5 McFarland standard at 600 nm. To measure the zone of inhibition, inoculums were streaked on the surface of Muller-Hinton agar (MHA) plates using a sterile cotton swab and evaluated using the well method. Samples of each formulation were applied directly into their respective wells and the inoculated plates were incubated at 37ºC for 24 hours. The zones showing complete or partial inhibition were measured and the diameters of inhibition were recorded in millimeters. The serial dilution method in a 96-well plate format was used to determine the minimum inhibition concentration (MIC) for each sample, where vancomycin was used as the positive control. The mean zone of inhibition was recorded for all test samples (n=3). The results were analyzed using two-way ANOVA followed by Bonferroni's multiple comparison test (P<0.05) at 95% significance level using Graph Pad Prism software version 9.0 (Graph Pad Software Inc., San Diego, CA, USA).

CS GEL STABILITY

pH and Conductivity Analysis

The pH and conductivity of CS gel formulations under two temperature conditions, i.e., 4-8°C (Refrigeration; RF) and 20-25°C (Room Temperature; RT), were determined using a

digital pH meter (Accumet AB15, Fisherbrand) for a period of three weeks. Glass electrodes were calibrated, and samples were withdrawn and analyzed weekly. The conductivity measurements were recorded in millivolts. All samples were recorded in triplicate and average values ± standard error of the means were calculated. Mean pH and conductivity measurements for all test samples (n=3) were compared by two-way ANOVA followed by Bonferroni's multiple comparison test ($P<0.05$) at 95% significance level using Graph Pad Prism software version 9.0 (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Steady Shear Measurements

Shear stress (τ) versus shear rate (γ) flow curves for the control and various 5-FU-chitosan gel formulations were analyzed. The data generated were fit to Ostwald de Waele (Equation 1) and Herschel-Bulkley models (Equation 2) When comparing the two models, the Ostwald de Waele model was considered as best fit due to the high correlation coefficient number. High correlation coefficient numbers (R) were obtained for CS, 0.25µg 5-FU-CS and 0.025µg 5-FU-CS, with R values of 0.999, 0.967, and 0.911, respectively. R values for 25µg 5-FU-CS and 2.5µg 5-FU-CS were substantially less than that of CS alone.

The flow curve for shear stress versus shear rate obtained from our studies shows that both CS gel alone and CS gel formulated with the different concentrations of 5-FU were non-Newtonian (Figure 7). The curves show that increasing shear rate resulted in a large increase in shear stress. The flow curves of Newtonian fluids are known to assume a linear relationship between shear stress and shear rate which means that they are independent of shear rate. Conversely, non-Newtonian fluids display a non-linear relation between shear stress and shear rate, and have a yield stress or viscosity property that is dependent on shear rate as a source of

deformation. The inverse relationship between shear stress and shear rate was observed for all CS formulations which confirms that CS gel is non-Newtonian.

The flow behavior index (n) is another parameter that helps to further classify flowing materials as either shear thinning or shear thickening in the pharmaceutical industry. The flow behavior index was evaluated for all formulations of 5-FU-CS gel. The flow behavior indexes (n) obtained for CS gel and 5-FU-CS were all less than 1 using both Herschel-Bulkley and Ostwald de Waele models (Table 2). Values of $\eta < 1$ or moving further away from 1 towards zero indicate the increase in shear thinning behavior (Bhardwaj et al., 2019). Thus, all CS formulations possessed shear thinning behavior (Figure 6), which confirms the non-Newtonian flow behavior seen in (Figure 7).

		Parameters			χ^2	R
Sample	Model	τ0 (Pa)	K (Pa.sn)	n		
CS gel	Herschel-Bulkley	-1.528	1.603	0.656	N/A	N/A
	Ostwald de Waele	N/A	1.243	0.700	26.760	0.999
25µg 5-FU-CS gel	Herschel-Bulkley	-1.309	1.233	0.682	N/A	N/A
	Ostwald de Waele	N/A	0.489	0.888	0.273	0.429
2.5µg 5-FU-CS gel	Herschel-Bulkley	-0.895	1.020	0.710	N/A	N/A
	Ostwald de Waele	N/A	0.383	0.926	0.402	0.540
0.25µg 5-FU-CS gel	Herschel-Bulkley	-0.923	1.030	0.704	N/A	N/A
	Ostwald de Waele	N/A	0.582	0.828	0.046	0.967
0.025µg 5-FU-CS gel	Herschel-Bulkley	-1.195	1.068	0.691	N/A	N/A
	Ostwald de Waele	N/A	0.503	0.848	0.091	0.911

Table 2. 5-FU-CS Gel Flow Parameters.

A list of parameters of Ostwald de Waele and Herschel-Bulkley models showing the flow behavior of formulations of chitosan gel with different concentrations of 5-fluorouracil between 0.1 to 200 1/s. Data are expressed as mean (n=3).

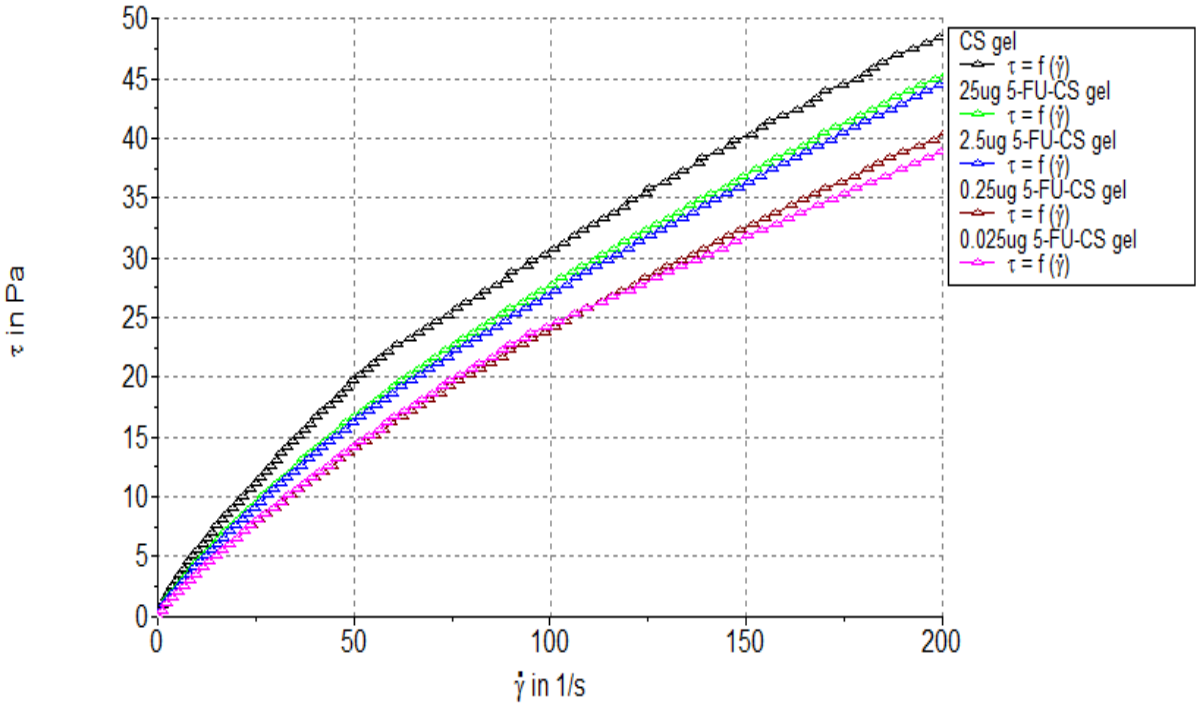

Figure 6. Flow Comparison of CS Gels.

Chitosan gel flow is compared to flow for 5-FU-Chitosan gel and shows the flow behavior of chitosan gel compared to different concentrations of 5-FU-CS gel. The graph shows a Non-Newtonian flow curve of shear stress (τ) vs shear rate (γ'), at 25 0C, for CS gel (black); 25µg/mL 5-FU-CS (green); 2.5µg/mL 5-FU-CS (blue); 0.25µg/mL 5-FU-CS (brown); 0.025µg/mL 5-FU-CS (pink). Data shown here are (n=1) of three trials for all experimental groups.

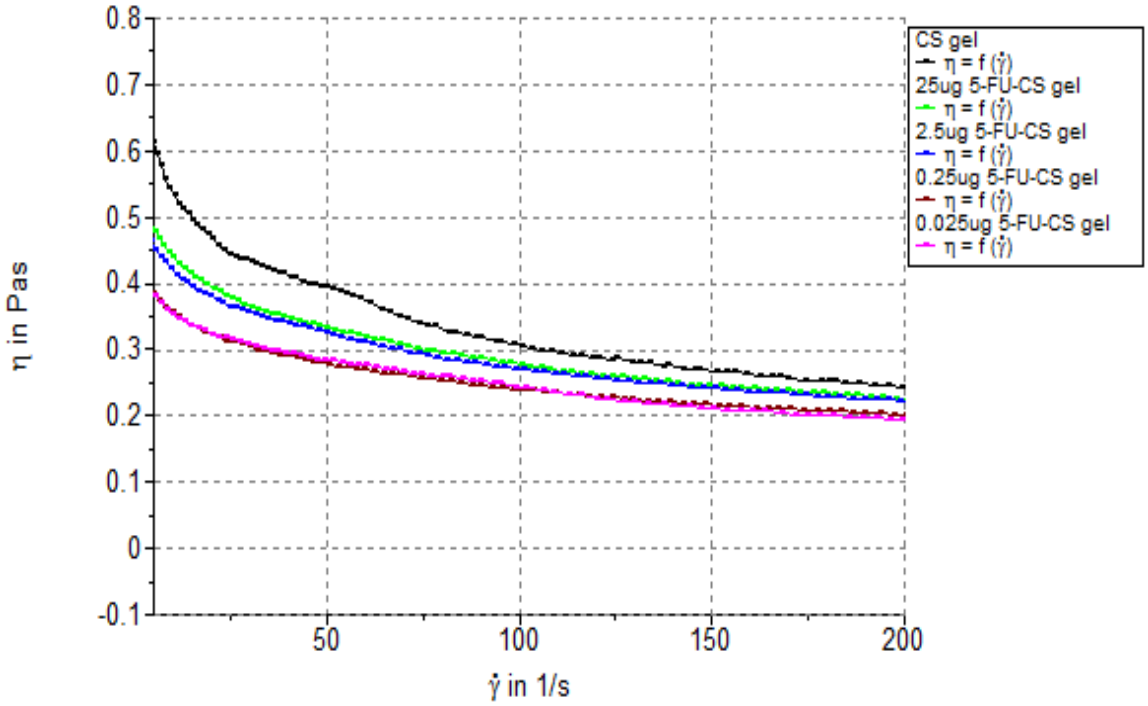

Figure 7. Chitosan Gel Apparent Viscosity.

The apparent viscosity of chitosan gel is compared to different concentrations of 5-FU-Chitosan gel. The results show the shear-thinning property of CS gel (black); 25µg/mL 5-FU-CS (green); 2.5µg/mL 5-FU-CS (blue); 0.25µg/mL 5-FU-CS (brown); 0.025µg/mL 5-FU-CS (pink). The curves show apparent viscosity (η) decreasing with increasing shear rate (γ') at a constant speed of 5 Pa, at 25ºC. Data shown here are (n=1) of three trials for all experimental groups.

Dynamic Measurements

The dynamic properties of CS gel alone and different concentrations of 5-FU-CS gel were determined by measuring the "storage modulus" (G′) and the "loss modulus" (G″). Storage modulus (G′), loss modulus (G″), and tan δ obtained during dynamic testing signify the extent of elastic character, viscous character, and physical state of the material, respectively. G′ provides information about energy stored, while G″ reveals energy dissipated by the sample (von Borries-Medrano et al., 2019). Here we show the storage modulus (G') and the loss modulus (G") of both CS gel and 5-FU-CS gels at different concentrations increased after the application of frequency as the source of deformation (0.1 Hz to 10 Hz, Figure 8). Conversely, G' and G" of CS and 5-FU-CS gels decreased after subjecting the gel to different amplitude (strain), as the source of deformation (0.1 to 100 Pa), (Figure 9).

Amplitude and frequency sweep tests are used to categorize flowing materials as either (i) dilute solutions, (ii) concentrated solutions, (iii) weak gel materials, or (iv) strong gel materials. This is achieved by identifying the linear viscoelastic region (LVE) and analyzing the loss tangent (δ values), which is the ratio of G' and G'' (Mitkari et al., 2010). Rheologically, weak or viscous gels have δ values greater than 1 ($\delta > 1$), and strong or elastic gels have δ values less than 1 ($\delta < 1$) (Bhardwaj et al., 2019; von Borries-Medrano et al., 2019). The δ values obtained for CS gel and 5-FU-CS gels were greater than 1, indicating that our CS formulation was weak and viscous (Figure 10). This affirms the results obtained in the steady shear measurements. Studies on gels and gel-like materials show that for strong or fully developed gels, G′ > G″, where these properties have the ability to be nearly independent of frequency over a large frequency range (Douglas, 2018). However, the results obtained for both amplitude and frequency sweep tests showed that the loss modulus is greater than the storage modulus (G' <

G”). This indicates that our formulation is less elastic compared to other gelation materials. For both the CS gel and the different concentrations of 5-FU-CS, the strain value of 5 Pa was within the LVE region.

For rheology, the storage modulus (G’) indicates the ability of hydrogels to store deformation energy, which represents the degree of crosslinking in the material. Therefore, the higher the degree of crosslinking in a material, the greater the storage modulus due to the presence of localized particles or molecules that store the deformation energy of the stressed material over long timescales (Douglas, 2018). An explanation for this result could stem from the swelling properties of the formulation used in this study.

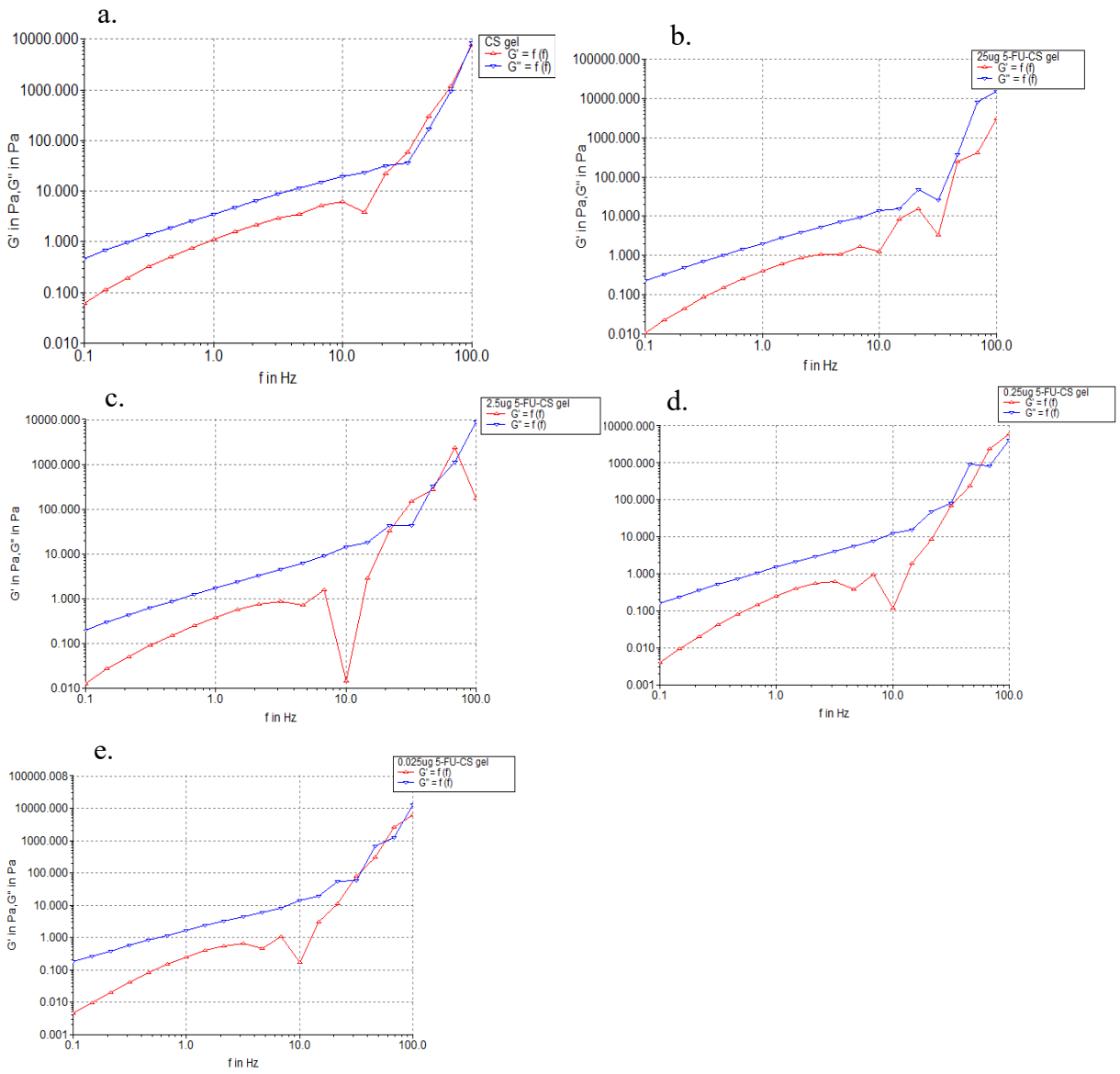

Figure 8. Chitosan Gel Storage Modulus and Loss Modulus.

The CS storage and loss modulus is compared to different concentrations of 5-FU-CS gel after oscillatory deformation of frequency. The results show loss modulus (blue) is greater than storage modulus (red) for (a) CS gel, (b) 25µg/mL 5-FU-CS, (c) 2.5µg/mL 5-FU-CS. (d) 0.25µg/mL 5-FU-CS, and (e) 0.025µg/mL 5-FU-CS. Data shown here are (n=1) of three trials for all experimental groups.

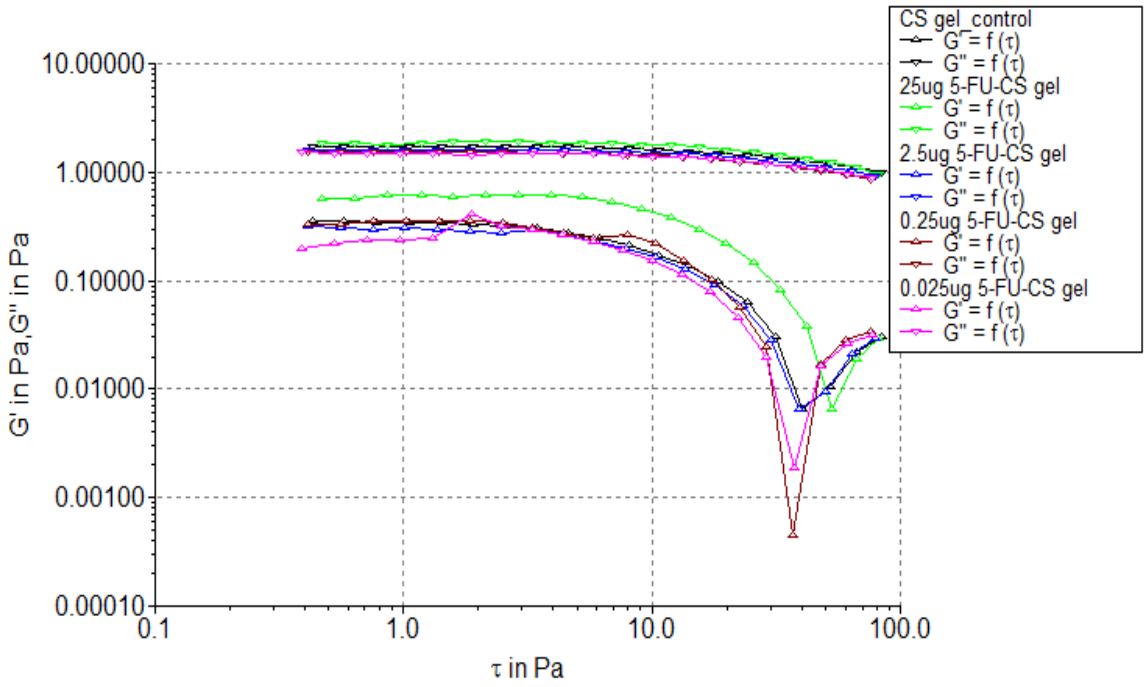

Figure 9. Chitosan Gel Deformation Amplitudes.

Storage modulus and loss modulus of CS and different concentrations of 5-FU-CS gel after oscillatory deformation of amplitude. The results show the gel-like property of CS gel (black); 25µg/mL 5-FU-CS (green); 2.5µg/mL 5-FU-CS (blue); 0.25µg/mL 5-FU-CS (brown); 0.025µg/mL 5-FU-CS (pink), at 25ºC. Data shown here are (n=1) of three trials for all experimental groups.

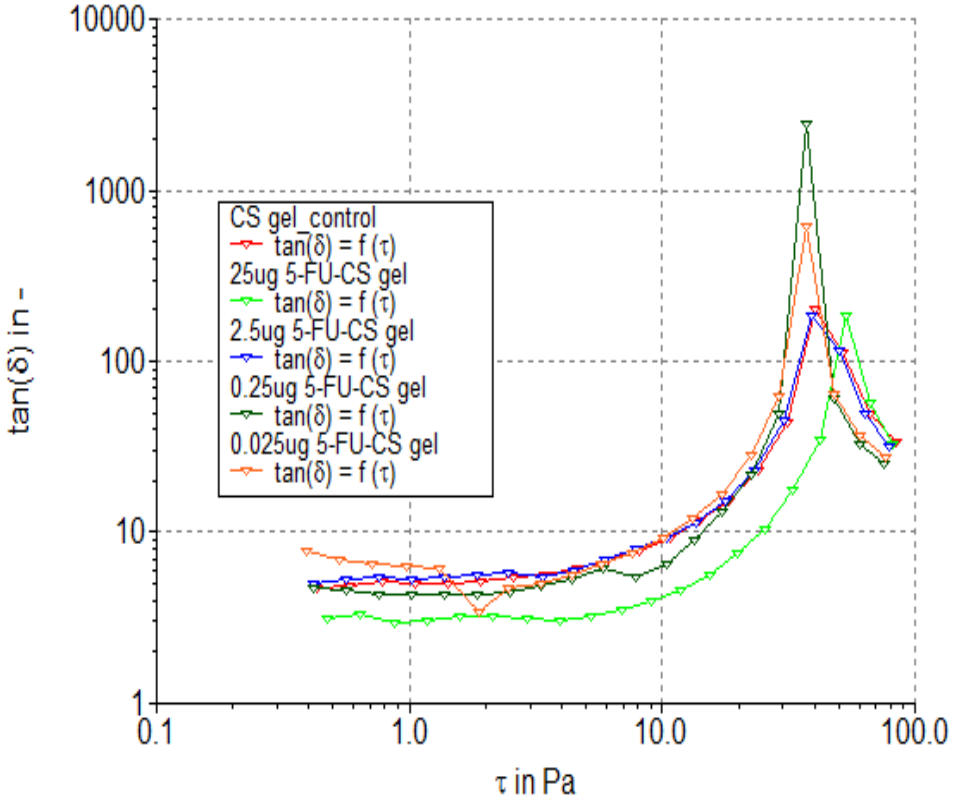

Figure 10. Plot of Tan δ vs Shear Stress (τ).

A plot of tan δ vs shear stress (τ) for CS gel and different concentrations of 5-FU-CS gel after deformation (amplitude) is shown. The results show the elastic property of CS gel (red); 25µg/mL 5-FU-CS (light green); 2.5µg/mL 5-FU-CS (blue); 0.25µg/mL 5-FU-CS (dark green); 0.025µg/mL 5-FU-CS (orange), at 25ºC. Data shown here are (n=1) of three trials for all experimental groups.

Creep Recovery

The creep compliance as a function of time for CS gel and different concentrations of 5-FU-CS gel was carried out at constant stress of 5 Pa and at a temperature of 25ºC. Figure 11 shows the creep response after 250 seconds. The highest creep compliance value was recorded for 0.25µg 5-FU-CS gel at 20.36 Pa^{-1}, and the lowest response was recorded for 0.025µg 5-FU-CS gel at 9.08 Pa^{-1} (Table 3).

Since lower creep compliance corresponds with higher elasticity (Mitkari et al., 2010), it could be concluded that of the 5-FU-CS gel formulations studied, 0.025µg 5-FU-CS demonstrated the most elastic behavior. Unlike the creep compliance measurements, the percent recovery results for all samples were negligible. The reason for these low recovery values could stem from the low elasticity of the gel. Higher percent recovery would be expected from highly elastic materials when subjected to steady deformation. The average apparent viscosity values (η in Pas) recorded after each formulation were subjected to constant strain for creep compliance analysis were recorded as follows: CS gel (η= 0.48 Pas), 25µg 5-FU-CS gel (η= 0.39), 2.5µg 5-FU-CS gel (η= 0.47), 0.25µg 5-FU-CS gel (η= 0.38), and 0.025µg 5-FU-CS (η= 0.36). For most polymeric solutions, especially pseudoplastic fluids, apparent viscosity decreases with increasing shear rate. This is because the long chain molecules in such fluids tend to align with each other at high shear rates resulting in easier flow (Stanbury et al., 2017).

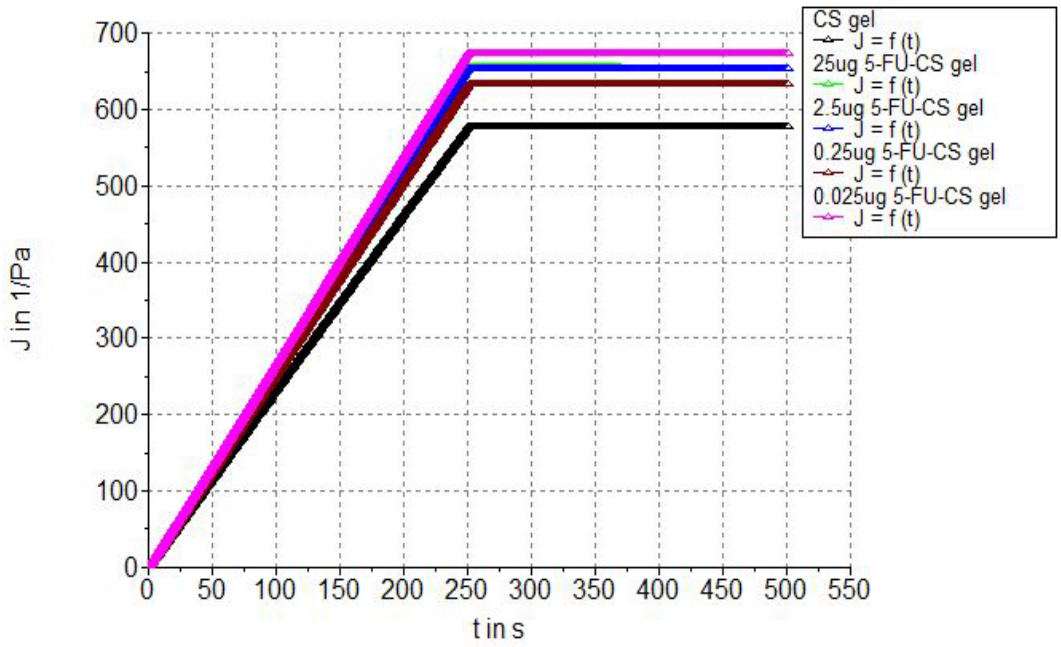

Figure 11. Chitosan Gel Creep Recovery.

Creep recovery plot of CS gel and different concentrations of 5-FU-CS gels. The results shown here are creep compliance (J) for CS gel (black); 25µg/mL 5-FU-CS (green); 2.5µg/mL 5-FU-CS (blue); 0.25µg/mL 5-FU-CS (brown); 0.025µg/mL 5-FU-CS (pink), after application of shear stress of 5 Pa, at 25°C. Data shown here are (n=1) of three trials for all experimental groups.

	Parameters					Creep
Sample	τ (Pa)	η (Pas)	γ' (s^{-1})	Je (Pa^{-1})	G (Pa)	Recovery %
CS gel	5.00	0.48	10.52	19.79	0.05	0.01
25µg 5-FU-CS gel	5.00	0.39	12.77	10.21	0.10	0.05
2.5µg 5-FU-CS gel	5.00	0.47	10.89	16.71	0.09	0.11
0.25µg 5-FU-CS gel	5.00	0.38	13.32	20.36	0.08	0.06
0.025µg 5-FU-CS gel	5.00	0.36	14.12	9.08	1.55	0.11

Table 3. Creep Recovery Parameters.

Parameters of creep analysis of 2% CS gel and 5-FU-CS gel. Data are expressed as mean (n=3).

Antimicrobial Activity

The antimicrobial potential of CS gel, and 5-FU-CS gel at different concentrations, were determined by measuring the zone of inhibition and minimum inhibitory concentration (MIC) against select pathogenic bacteria. Figure 12 shows images of the zone of inhibition for each formulation against MRSA, RN1 and *E. coli* after 24 hours of exposure. As seen in Figure 12, the degree of inhibition observed in this experiment for both formulations and vancomycin (positive control) were complete. However, unlike the positive control, we observed a double hallow effect around the zones for MRSA. This double hallow effect could be attributed to the resistant nature of the bacterial pathogens after 24 hours. Overall, the results suggest that 5-FU and 5-FU-CS gel have antimicrobial potential compared to CS gel. The average zone of inhibition measured for 5-FU against MRSA were 29.0, 28.0, 25.0, 22.0, and 15.0 mm at concentrations of 1.25µg/well, 1.0µg/well, 0.75µg/well, 0.5µg/well, and 0.25µg/well, respectively (Table 4).

Using the same treatment concentrations, the zone of inhibition recorded for 5-FU-CS gel were 29.0, 28.0, 25.0, 21.0, and 16.0 mm, respectively (Table 4). The average zones of inhibition measured for 5-FU against RN1 were 21.0, 20.0, 15.0, 11.0, 0.00 mm at concentrations of 1.25µg/well, 1.0µg/well, 0.75µg/well, 0.5µg/well, and 0.25µg/well, respectively (Table 4). The zone of inhibition recorded for 5-FU-CS gel at the same concentrations were 21.0, 19.0, 15.0, 11.0, 0.00 mm, respectively (Table 4). Even though chitosan hydrogel is known to exhibit antimicrobial activities against Gram-positive bacteria (Masood et al., 2019), no zone of inhibition was recorded with the CS gels used against methicillin-resistant *S. aureus* (MRSA), and methicillin-susceptible *S. aureus* (RN1). Additionally, the CS gels had no effect on Gram-negative *E. coli.*

Figure 13 and Figure 14 show the zone of inhibition of 5-FU and 5-FU-CS against MRSA and RN1. The results also showed that, as the concentration decreases for each treatment (i.e., from 1.25µg/well to 0.25µg/well), the zone of inhibition significantly decreased ($P < 0.05$). Comparing the zone of inhibition at similar concentrations of 5-FU to that of 5-FU-CS gel, there was no statistically significant difference between values measured against MRSA and RN1, respectively (Figure 13). However, there was a statistically significant difference when the zone of inhibition measured against MRSA is compared to that of RN1 ($p<0.001$). The ability of 5-FU-CS gel to induce antimicrobial effects could be due to the poly-cationic property of chitosan that allows it to interact with negatively charged cell membranes of microorganisms, thus inducing its antimicrobial effects. Moreover, several accounts have been reported on the effect of a lower percentage of CS formulation on both Gram-positive and Gram-negative bacteria. For example, Zheng & Zhu (2003) report that 1% chitosan solution could inhibit both bacteria types completely. In the same study, it was observed that increasing the MW of chitosan induces significant antimicrobial effect on *E. coli* and *S. aureus* (Zheng & Zhu, 2003).

To further evaluate the antimicrobial activity of 5-FU against common bacterial infections in diabetic wounds, the minimum inhibitory concentration (MIC) of 5-FU was assessed. Table 5 shows the MIC for 5-FU and vancomycin against MRSA, RN1, and *E. coli* using the serial dilution method. The MIC for 5-FU and vancomycin against MRSA was 0.5µg/mL, and 1.0µg/mL, respectively. The MIC for 5-FU and vancomycin against RN1 was 0.5µg/mL, and 1.0µg/mL, respectively. Unlike MRSA and RN1, the MIC for 5-FU against *E. coli* was 8.0µg/mL. No MIC was verified for vancomycin against *E. coli.* Since vancomycin is often used as the drug of choice for treating MRSA, extensive studies about its MIC have been reported. The MIC of vancomycin against MRSA and RN1 was 1.0µg/mL which corresponds

with the literature. For example, Kshetry et al. (2016) found that the minimal inhibitory concentrations of vancomycin to 47 strains of MRSA ranged from 0.125µg/mL to 1.0µg/mL. To our knowledge, there are no reports on the antibacterial activity of 2% CS gel on MRSA in the literature. Thus, the result obtained here is a novel therapeutic approach for the antimicrobial potential of 2% CS gel on MRSA. Taken together, our experimental data show that 5-FU and 5-FU-CS gel have antimicrobial potential on MRSA and RN1 at low concentrations and *E. coli* at high concentrations.

Treatment (μg/well)	Species		Zone of Inhibition			
	MRSA		RN1		*E. coli*	
	5-FU	5-FU-CS	5-FU	5-FU-CS	5-FU	5-FU-CS
1.25	29.33±0.33	29.33±0.33	21.33±0.67	20.67±0.33	0.00	0.00
1.0	27.67±0.33	27.67±0.67	19.67±0.33	19.33±0.33	0.00	0.00
0.75	25.00±0.58	24.67±0.33	15.00±0.58	15.33±0.33	0.00	0.00
0.5	21.67±1.20	20.67±0.33	11.33±0.33	10.67±0.33	0.00	0.00
0.25	14.67±0.88	16.00±0.58	0.00±0.00	0.00±0.00	0.00	0.00

Table 4. Zones of Inhibition.

The measurement for the zone of inhibition is recorded for 5-FU and 5-FU- CS gel formulations at different concentrations against bacterial pathogens. Data are expressed as mean ±SEM (n=3). Bacterial strains evaluated were methicillin-resistant *Staphylococcus aureus* (JE2, NR-46543), methicillin-susceptible *S. aureus* (RN1), and *Escherichia coli* (K-12, DC10B, NR-49804). Compounds tested were 5-fluorouracil (5-FU) and 5-fluorouracil chitosan (5-FU-CS) formulated with DMSO and chitosan gel, respectively as drug delivery vehicles. Vancomycin was used as a positive control.

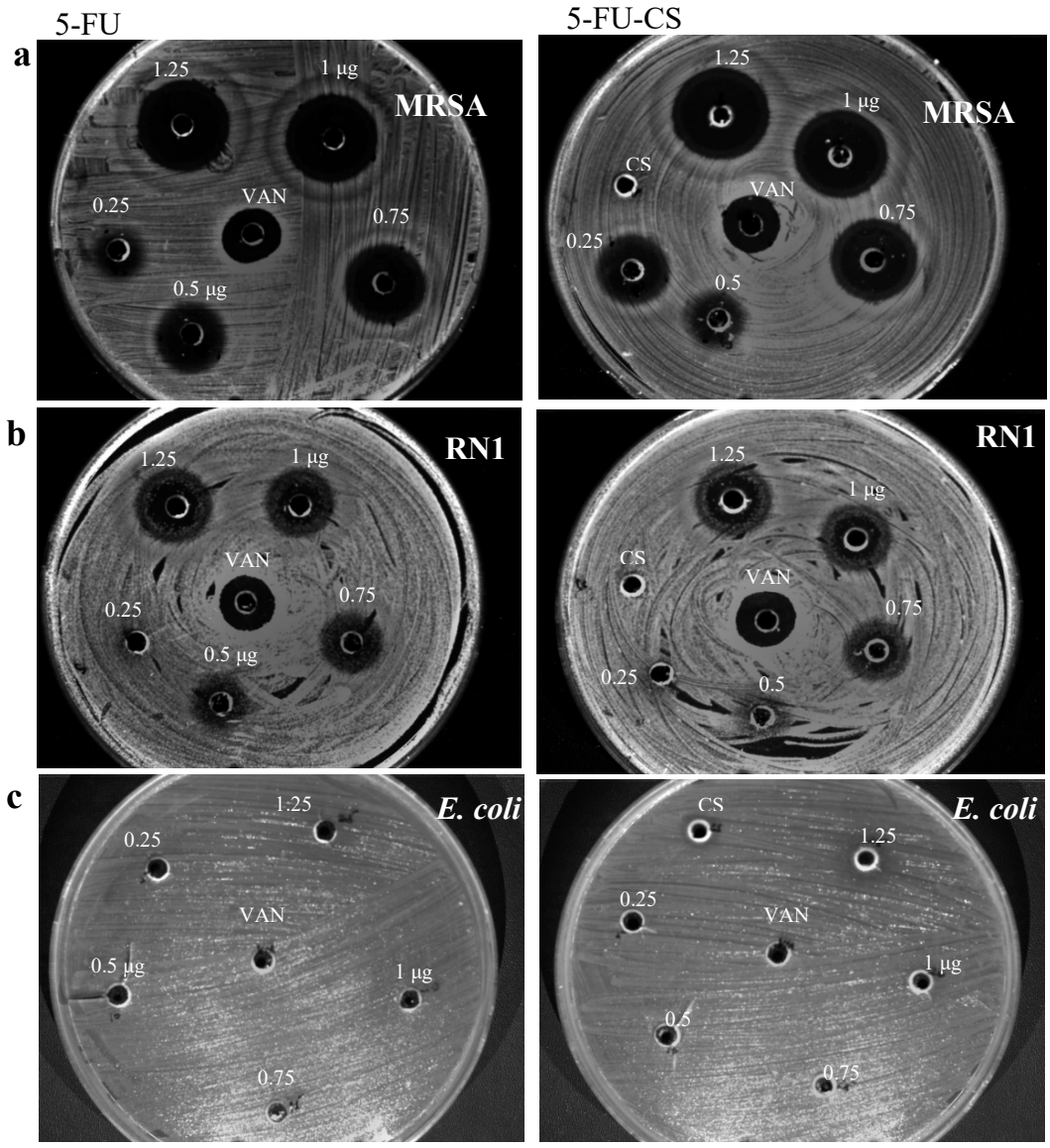

Figure 12. Antimicrobial Activity of Chitosan Gels.

Petri plates of CS gel, 5-FU, and different concentrations of 5-FU-CS gel were evaluated by the well method. Vancomycin was used as the positive control. The results shown here are (a) images of the zone of inhibition after 5-FU and 5-FU-CS gel were exposed to MRSA culture plate for 24 hours, (b) the zone of inhibition after 5-FU and 5-FU-CS gel were exposed to RN1 culture plate for 24 hours, and (c) the zone of inhibition after 5-FU and 5-FU-CS gel were exposed to *E. coli* culture plate for 24 hours. All the experiments were repeated three times. Zones of inhibition were measured in mm.

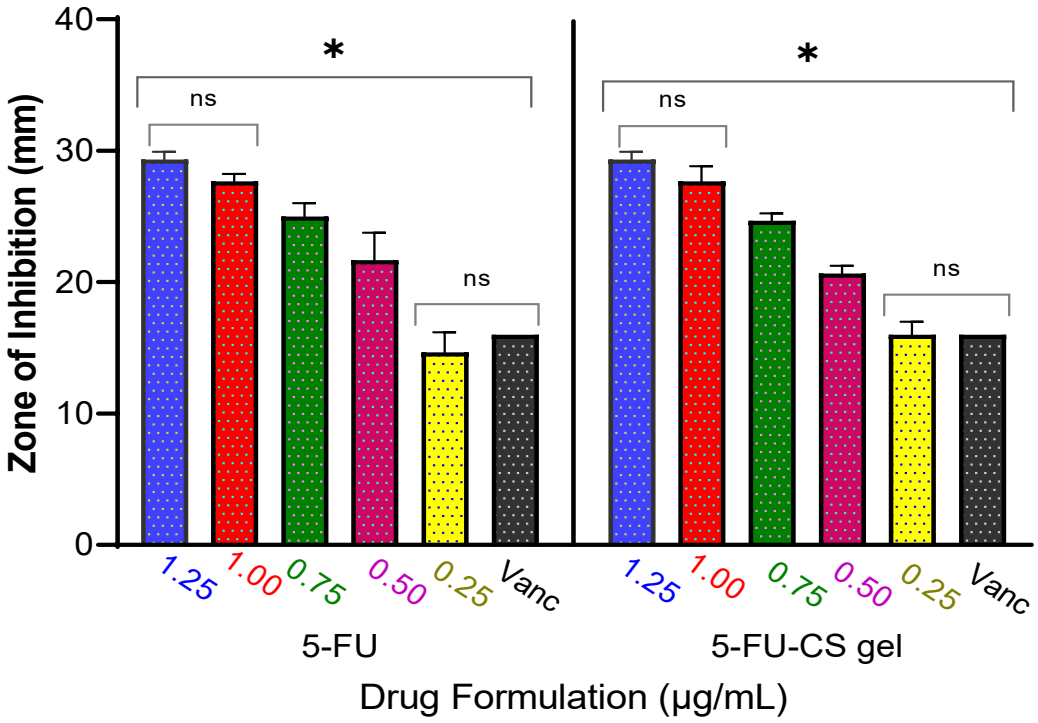

Figure 13. Chitosan Gel Antimicrobial Activity on Methicillin-Resistant *S. aureus* (MRSA). Zones of inhibition for 5-FU, and different concentrations of 5-FU-CS gel against methicillin-resistant *Staphylococcus aureus* (MRSA). Vancomycin was used as positive control. All experiments were repeated three times and data expressed are Mean ± SEM (n=3). Data were analyzed by two-way ANOVA with a post hoc Bonferroni multiple comparisons test. *Denotes a significant difference between 1.25µg 5-FU vs. different concentrations of 5-FU, and 5-FU-CS gel, respectively. $P<0.05$ was considered as statistically significant difference.

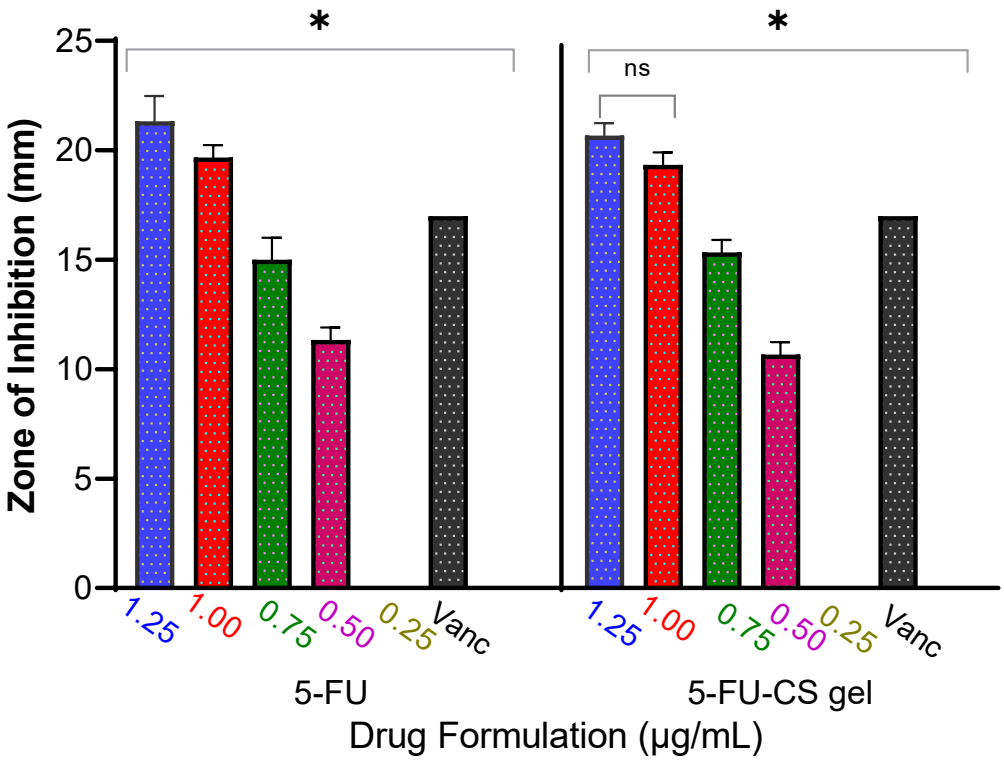

Figure 14. Chitosan Gel Antimicrobial Activity on Methicillin-Resistant *S. aureus* (RN1). Zones of inhibition for 5-FU, and different concentrations of 5-FU-CS gel against methicillin-susceptible *Staphylococcus aureus* (RN1). Vancomycin was used as positive control. All experiments were repeated three times and data expressed are Mean (n=3). Data were analyzed by two-way ANOVA with a post hoc Bonferroni multiple comparisons test. *Denotes significant difference between 1.25µg 5-FU vs. different concentrations of 5-FU, and 5-FU-CS gel, respectively. $P<0.05$ was considered as statistically significant difference.

	Species MIC (μg/mL)		
Compound	MRSA	RN1	*E. coli*
5-FU	0.5	0.5	8.0
Vancomycin	1	1.0	–

Table 5. 5-FU Minimum Inhibitory Concentration (MIC).

Minimum Inhibitory Concentration (MIC) of 5-FU against methicillin-resistant *Staphylococcus aureus* (JE2, NR-46543), methicillin-susceptible *S. aureus* (RN1), and *Escherichia coli* (K-12, DC10B, NR-49804). The test compound was 5-fluorouracil (5-FU) and vancomycin was used as a positive control.

pH and Conductivity Analysis

pH is an important physiochemical factor that influences metabolic processes and immune function, especially in the micro-compartments of the stratum corneum. This highlights the importance of analyzing absolute pH measurements in topical formulations as part of quality criteria for optimized preparations (Wohlrab & Gebert, 2018). In this study, all CS formulations were stored at different storage conditions for three weeks and stability indicators such as pH, conductivity, and change in color were assessed. The stability test for each CS formulation was carried out at room temperature (RT) (20-25ºC) and refrigeration temperature (4-8ºC). The average pH and conductivity measurements obtained at storage conditions are shown in Table 6 and Table 7, respectively.

There was no change in color in CS gel or all 5-FU-CS gel formulations at the end of the experimental period, indicating the physical stability of the vehicle. The average conductivity of CS gel and all formulations of 5-FU-CS gel were in the range of 140 to 143 mV, with significant fluctuations weekly (Figure 15). However, comparing the conductivity values for formulations

stored at RT to those stored at RF shows no substantial differences at different time points (Figure 16). The results obtained for pH measurements for CS gel and 5-FU-CS gels significantly increased each week (i.e., week 0 vs. week 1; week 0 vs week 2; week 0 vs week 3) after storage at 20-25^{o}C ($p < 0.05$, Figure 17A). Similarly, the pH values significantly increased each week after storage at 4-8^{o}C ($p < 0.05$, Figure 17B). Comparing pH measurements for each formulation at the respective temperatures (RT vs RF), showed that there was a significant difference in pH values for 25µg/mL 5-FU-CS gel (during week 3), 2.5µg/mL 5-FU-CS gel (during week 2), and 0.25µg/mL 5-FU-CS gel (during weeks 1-3), Figure 18A-E. Overall, the average pH values of CS gel and 5-FU-CS gels formulations were in the range of 4.45 to 4.57 after three weeks of storage at 20-25^{o}C and 4-8^{o}C, respectively. This was expected because CS gels were formulated with 2% acetic acid. The physiological pH of the stratum corneum is within 4.1 to 5.8 (Proksch, 2018); thus, our gel formulation is unlikely to cause skin irritation, making it a desirable topical vehicle. In addition, performing stability tests of our chitosan gels provide reliable evidence on the life cycle or shelf life of our chitosan gel when subjected to different environmental factors such as temperature changes over a period of time (Szymanska & Winnicka, 2015). Studies on CS showed that exposure to elevated temperatures (40°C) caused a significant loss of moisture (dehydration of chitosan powder), which resulted in a decrease in hardness and mechanical tablet strength (Viljoen et al., 2014). Therefore, understanding the impact of varied temperatures on CS gel is necessary for its application as a topical delivery system.

Weeks	CS gel		25µg/mL 5-FU-CS		2.5µg/mL 5-FU-CS		0.25µg/mL5-FU-CS		0.025µg/mL 5-FU-CS	
	pH	Conductivity	pH	Conductivity	pH	Conductivity	pH	Conductivity	pH	Conductivity
0	4.45±0.00	141.0	4.45±0.00	141.0	4.45±0.00	142.0	4.45±0.00	143.0	4.45±0.00	143.0
1	4.53±0.00	141.0	4.54±0.00	139.7	4.51±0.01	141.0	4.52±0.00	141.0	4.52±0.00	141.0
2	4.54±0.01	141.7	4.53±0.00	142.0	4.52±0.00	143.0	4.52±0.01	142.7	4.53±0.00	142.0
3	4.57±0.02	140.3	4.57±0.01	139.7	4.56±0.01	141.0	4.55±0.01	141.0	4.56±0.00	141.0

Table 6. Chitosan Gel pH and Conductivity at Room Temperature.

Stability test of CS gel formulation at room temperature showing conductivity and pH measurements for three weeks. Data expressed as mean ±SD (n=3).

Weeks	CS gel		25µg/mL 5-FU-CS		2.5µg/mL 5-FU-CS		0.25µg/mL5-FU-CS		0.025µg/mL 5-FU-CS	
	pH	Conductivity	pH	Conductivity	pH	Conductivity	pH	Conductivity	pH	Conductivity
0	4.45±0.00	141.0	4.45±0.00	141.0	4.45±0.00	142.0	4.45±0.00	143.0	4.45±0.00	143.0
1	4.54±0.00	140.0	4.54±0.00	140.0	4.51±0.00	141.0	4.53±0.00	140.0	4.52±0.00	141.0
2	4.55±0.00	141.0	4.55±0.00	141.0	4.54±0.01	141.0	4.56±0.01	140.0	4.53±0.00	141.0
3	4.57±0.01	140.0	4.57±0.00	140.0	4.56±0.00	140.7	4.57±0.00	140.0	4.56±0.00	140.0

Table 7. Chitosan Gel pH and Conductivity at Refrigerated Temperature.

Stability test of CS gel formulation at refrigerated temperatures showing conductivity and pH measurements for three weeks. Data expressed as mean ±SD (n=3).

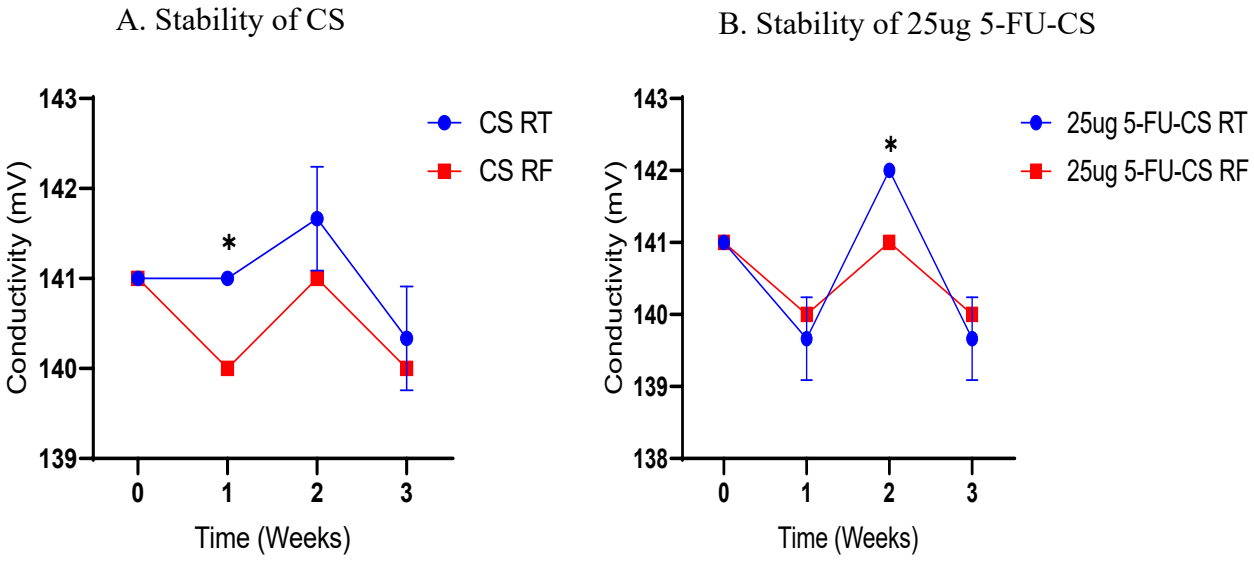

Figure 15. Chitosan Gel Conductivity after Long-Term Storage.

Conductivity measurements after long-term storage at room temperature (RT), 20-25ºC and refrigeration temperature, 4-8ºC for three weeks. The results are (A) weekly conductivity measurements for CS gel at RT vs RF, and (B) weekly conductivity measurements for 25ug 5-FU-CS gel at RT vs RF. All values are expressed as Mean ± SEM at 95% confidence intervals. Data were analyzed by two-way ANOVA with a post hoc Sidak's multiple comparisons test. *Denotes significant difference between CS RT vs CS RF; and 25µg 5-FU-CS RT vs. 25µg 5-FU-CS RF, respectively. $P<0.05$ was considered as statistically significant.

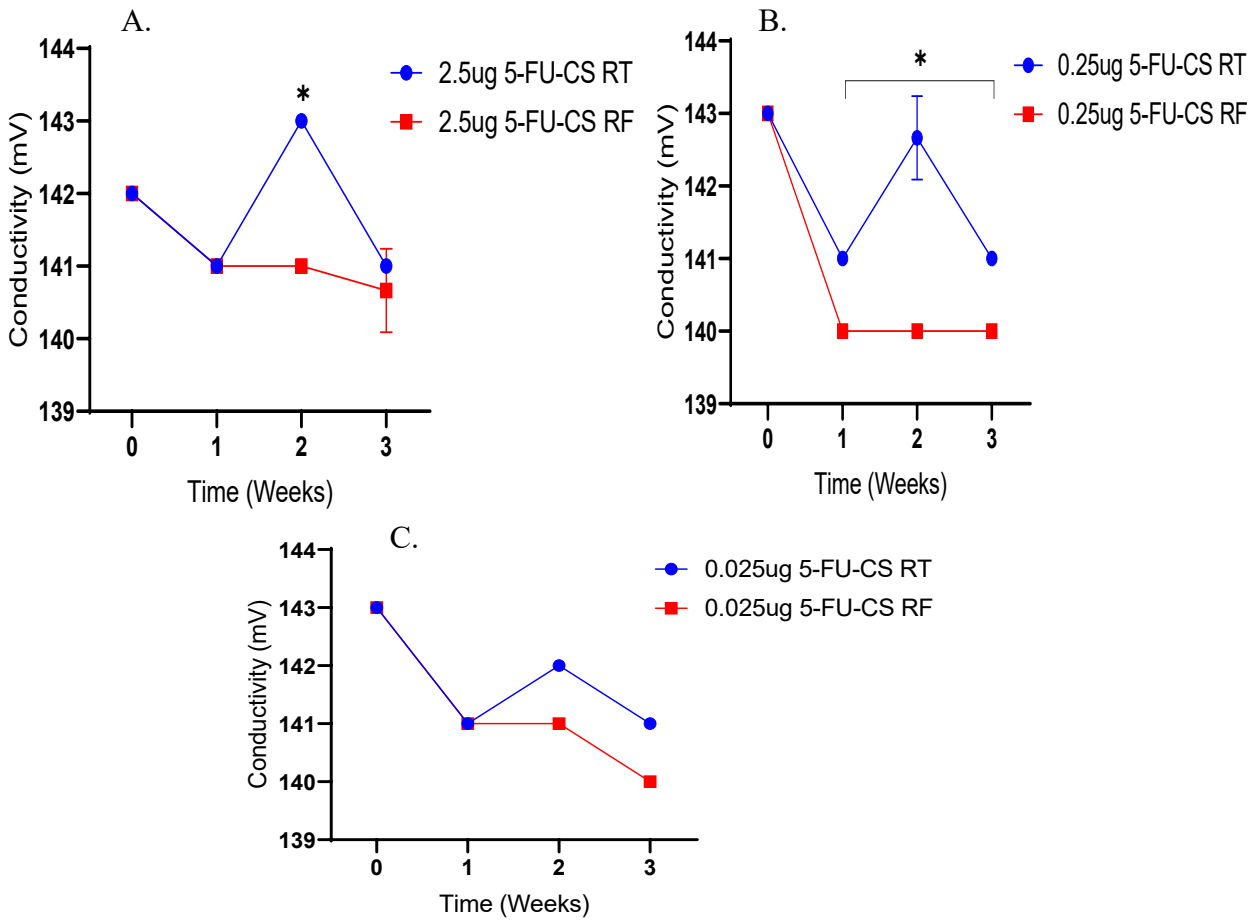

Figure 16. Chitosan Gel Conductivity at Different Temperatures.

Conductivity of CS gel formulation was measured at room temperature (RT) 20-25ºC and refrigeration temperature 4-8ºC after three weeks. The results show (A) weekly conductivity measurements for 2.5ug 5-FU-CS gel at RT vs RF, (B) weekly conductivity measurements for 0.25ug 5-FU-CS gel at RT vs RF, and (C) weekly conductivity measurement for 0.025ug 5-FU-CS gel. All values are expressed as Mean ± SEM at 95% confidence intervals. Data were analyzed by two-way ANOVA with a post hoc Sidak's multiple comparisons test. *Denotes significant difference between 2.5µg 5-FU-CS RT vs 2.5µg 5-FU-CS RF; 0.25µg 5-FU-CS RT vs 0.25µg 5-FU-CS RF; and 0.025µg 5-FU-CS RT vs 0.025µg 5-FU-CS RF, respectively. $P<0.05$ was considered as statistically significant.

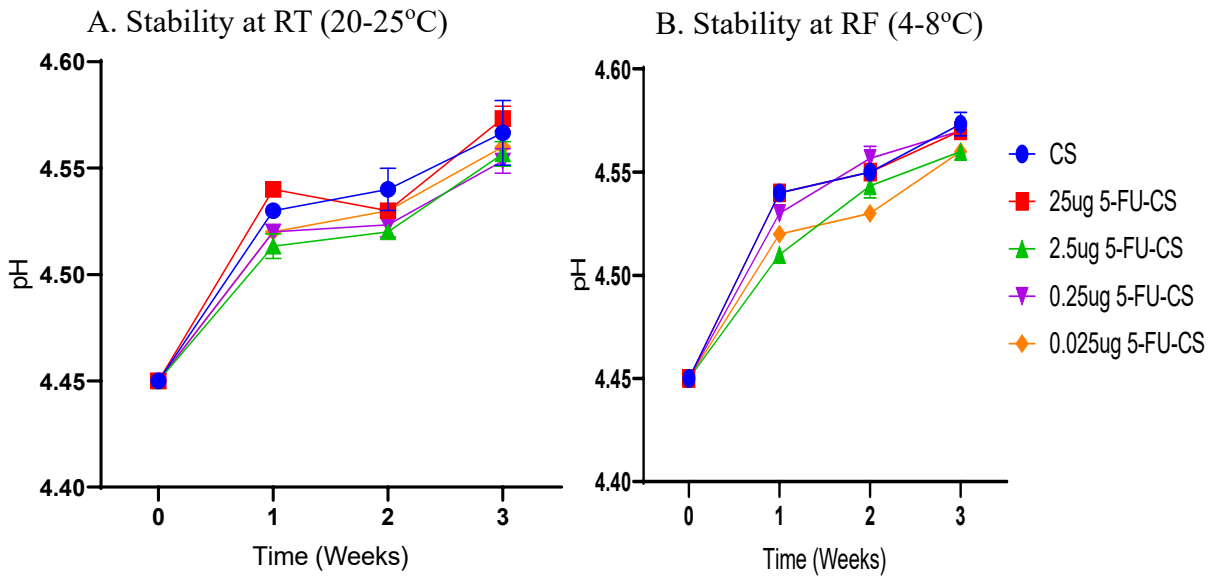

Figure 17. Stability Tests Comparing pH Measurement of CS Gel Formulation.

pH measurement of CS formulation after three weeks storage at room temperature and refrigeration. Results show (A) weekly pH measurements of samples after storage at room temperature (20-25°C), and (B) weekly pH measurements of samples after storage at refrigeration temperature (4-8°C). All the pH measurements were recorded three times per week. All values are expressed as Mean ± SEM with 95% confidence intervals. Data were analyzed by two-way ANOVA with a post hoc Sidak's multiple comparisons test. $P<0.05$ was considered as statistically significant.

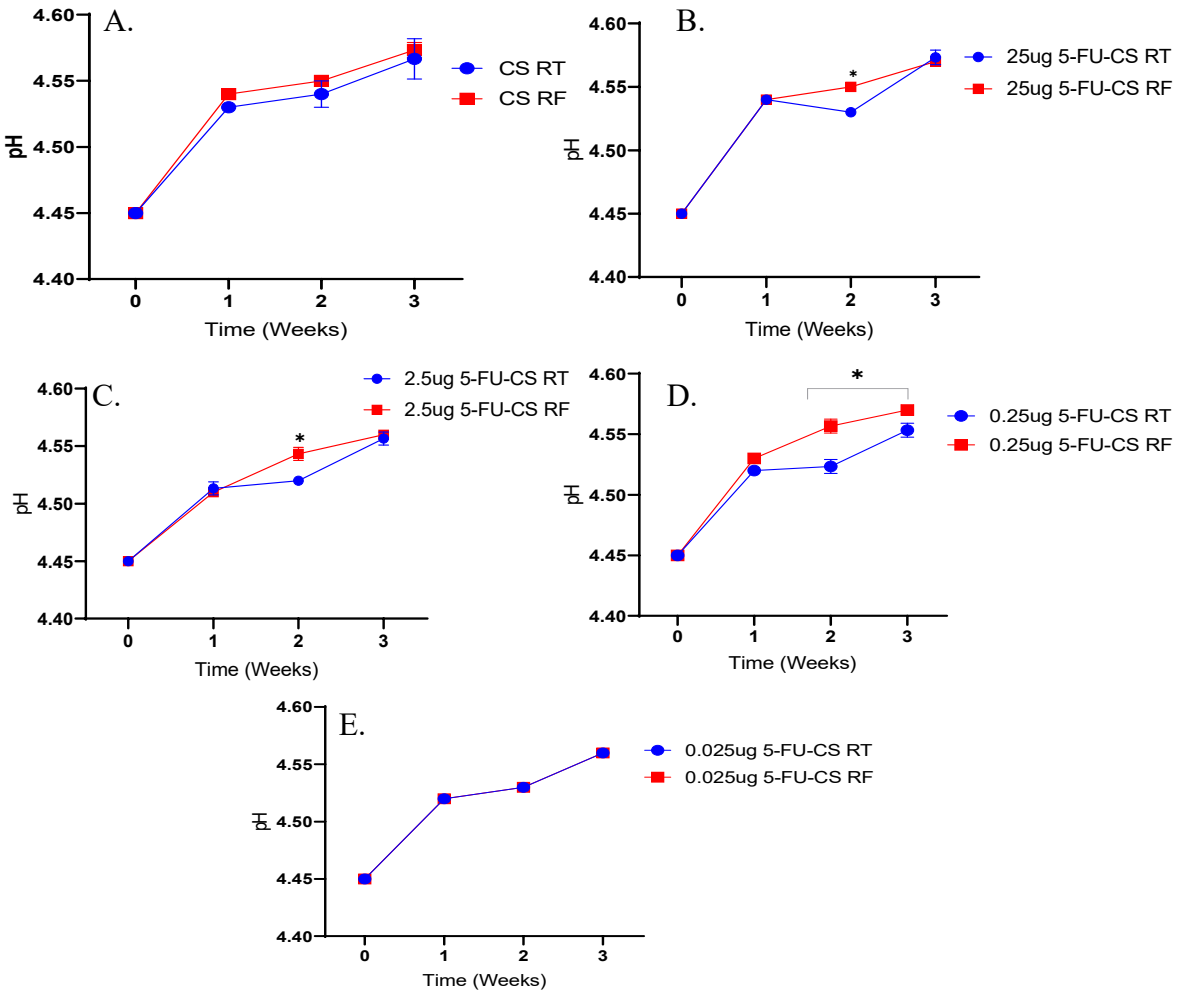

Figure 18. pH Analysis at Room Temperature (RT), 20-25°C and Refrigeration (RF), 4-8°C.

The results show (A) pH measurements for CS gel at RT vs RF, (B) pH measurements for 25ug 5-FU-CS gel at RT vs RF, (C) pH measurements for 2.5ug 5-FU-CS gel at RT vs RF, (D) weekly pH measurements for 0.25ug 5-FU-CS gel at RT vs RF, and (E) pH measurements for 0.025ug 5-FU-CS gel at RT vs RF. All values (n=3) are expressed as Mean ± SEM with 95% confidence intervals. Data were analyzed by two-way ANOVA with a post hoc Sidak's multiple comparisons test. *Denotes significant difference between 25µg 5-FU-CS RT vs 25µg 5-FU-CS RF; 2.5µg 5-FU-CS RT vs 2.5µg 5-FU-CS RF; 0.25µg 5-FU-CS RT vs 0.25µg 5-FU-CS RF; and 0.025µg 5-FU-CS RT vs 0.025µg 5-FU-CS RF, respectively. $P<0.05$ was considered as statistically significant.

DISCUSSION

Delivering drugs to the topical route is an attractive method for both local and systemic treatments (Dantas et al., 2016), with many advantages over other routes. Specifically, topical drug delivery allows for deeper skin penetration, better absorption, and avoidance of serious systemic adverse drug effects (Glavas-Dodov et al., 2003; Whitehouse, 2011). Topical administration prevents the metabolism of drugs in the liver, avoids both gastrointestinal disorders and risks of intravenous therapy, and avoids risks associated with the varied conditions of absorption (Dantas et al., 2016). This is most often characterized by the overall increase in the bioavailability of drugs at the targeted site of action. Issues pertaining to the bioavailability of drugs at the active site have led formulation scientists to explore topical formulations such creams, gels, and ointments, with the goal of increasing residence time. In light of this notion, several studies are exploring natural polymers due to their non-toxic and biodegradable nature. In this study, topical formulations of chitosan gels were formulated, and their rheological properties evaluated.

Chitosan is a natural polymer that is known to exhibit biodegradable, biocompatible, and non-toxic properties (Dai et al., 2011; Huang & Fu, 2010; Liu et al., 2018). As part of their chemical characteristics, chitosan materials exhibit a positive charge, film-forming capacities, mild gelation characteristics, and strong wound tissue adhesive properties (Jayakumar et al., 2011). Chitosan formulations are used in wound dressing due to its biological adhesiveness, antimicrobial, and hemostatic activities (Zhao et al., 2017). To investigate the inherent wound healing effect of CS and 5-FU-CS gel, rheological properties such as steady shear measurements and dynamic measurements of CS gel were analyzed. Results obtained from our studies show that CS gel demonstrates non-Newtonian flow behavior with weak viscoelastic properties. One

of the true marks of non-Newtonian flow materials is the flow behavior index, where values less than one indicate shear thinning behavior (Bhardwaj et al., 2019). As seen throughout our rheological studies, CS gel demonstrates shear thinning behavior.

In rheological studies, parameters such as storage modulus (G′), loss modulus (G″) and tangent δ provide information pertaining to the extent of elasticity, viscosity, and physical state of the material. For example, G′ gives information about energy stored by the material, G″ reveals energy dissipated by the sample, and loss tangent (δ values) is the ratio of G' and G'' (von Borries-Medrano et al., 2019). Rheologically weak or viscous gels have δ values greater than 1 ($\delta > 1$), and strong or elastic gels have δ values less than 1 ($\delta < 1$) (Bhardwaj et al., 2019; von Borries-Medrano et al., 2019). Here we find the δ values obtained for CS gel and 5-FU-CS gels were all greater than 1, indicating that our CS formulation is a weak and viscous gel. Studies on gels and gel-like materials show that for strong or fully developed gels, G′ > G″ these properties have the ability to be nearly independent of frequency over a large frequency range (Douglas, 2018). However, the results obtained for both amplitude and frequency sweep tests showed that the loss modulus is greater than the storage modulus (G' < G"). This indicates that our formulation is less elastic compared to other gelation materials.

5-FU is known to have an antibacterial effect at high concentrations, but its effect at physiologically relevant concentrations is still underexplored (Vanlancker et al., 2016). We show that at low concentrations (0.025μg/mL), there is antimicrobial activity of 5-FU against pathogenic bacterium such as methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (RN1), and at high concentration (8.0μg/mL) against *E. coli*. Even though MRSA is known to develop short-term drug resistance to commonly used antibiotics (Deurenberg et al., 2007), there was complete zone of inhibition after 24 hours exposure to our topically formulated

5-FU-CS gel, with a minimum inhibition concentration of 1.0μg/mL. Examples of proposed antimicrobial mechanisms of CS is centered around the formation of polymer membrane of CS on the surface of the cell which prevents nutrients from reaching the cells or, the ability of lower MW CS to enter the cell via pervasion to adsorb electronegative substances therein leading to disturbance of their physiological activities (Zheng & Zhu, 2003). Taken together, the zone of inhibition and minimum inhibition concentration results demonstrate that our topically formulated 5-FU-CS gel is potent and effective against these pathogenic bacteria at low concentrations.

CONCLUSION

In this study, CS gel was formulated with different concentrations of 5-FU and its rheological properties and antimicrobial activity were analyzed against pathogenic bacteria. It was shown that CS gel and 5-FU-CS gel formulated demonstrated non-Newtonian properties with shear thinning flow behavior. Dynamic rheological measurements revealed that the formulations have weak viscoelastic properties with G' < G", and a loss tangent value greater than 1. However, the gel formulations were fairly stable at both room and refrigeration temperatures. We also show that 5-FU and 5-FU-CS gels have significant antimicrobial effect against common bacterial infections such as MRSA and RN1 at low concentrations, and *E. coli* at higher concentrations. Understanding viscosity as a property of gels is important in topical delivery systems. This is because very low viscous products may easily flow from the surface of application, while extremely high viscous products like creams are challenged with poor spreading on surface.

CHAPTER 4

DRUG DELIVERY SYSTEMS: EXPLORING THE THERAPEUTIC EFFECT OF 5-FU CHITOSAN GEL FOR TOPICAL WOUND HEALING UNDER DIABETIC CONDITIONS.

ABSTRACT

Background: Diabetes is one of the most prevalent and costly chronic diseases in the United States. Diabetic wounds are one of the most severe complications of diabetes mellitus and often require prolonged hospitalization. The relationship between adipose-derived biomarkers, adipokines, and metabolic disorders have been well studied; however, little is known about the role of resistin and PPAR-γ in wound healing. Even though natural polymers such as chitosan have been extensively studied for their wound healing properties, there is no report on the effect of 5-fluorouracil (5-FU) formulated chitosan gel in wound healing under diabetic conditions. **Methods:** Chitosan gel (2% w/w) was prepared and formulated with different concentrations of 5-FU (25µg/mL, 2.5µg/mL, 0.25µg/mL, and 0.025µg/mL). Human dermal fibroblast cells (HDFa) were treated with each formulation for 24 hours in DMEM growth media. An MTT assay was run to determine the effect of each formulation on cell viability. The scratch wound healing assay was used to determine cell migration/wound closure and mRNA gene expression was analyzed by RT-qPCR. **Results:** 5-FU and 5-FU-CS gel induced statistically significant dose-dependent effects on HDFa cell viability. High-glucose media did not impair HDFa cell migration. 5-FU induces dose-dependent effect cell migration and adipokine mRNA gene expression in vitro. Drug free CS gel had no impact on cell migration. **Conclusion:** The results suggest that 5-FU has an effect on adipokine expression, and migration of HDFa cells under diabetic conditions, which could be further explored with the aim of understanding the mechanism involved in this process and any potential therapeutic effect on diabetic wound healing.

INTRODUCTION

Diabetes mellitus is a serious disease which is characterized by insufficient insulin production by the pancreas or the ineffective use of insulin by the body (Fui et al., 2019). Epidemiologically, diabetes is considered as one of the most costly and prevalent chronic diseases in the United States (Narayan et al., 2003). Diabetes has become a major public concern with considerable social, health, and economic consequences globally (Diez & Iglesias, 2003; Qi et al., 2004). According to World Health Organization, in 2016 alone, diabetes was the direct cause of death of 1.6 million patients (World Health Organization, 2020). Examples of diabetes-related complications include cardiovascular disease, stroke, chronic renal failure, peripheral neuropathy, and diabetic skin wound. Among these complications, diabetic skin wound is the most frequent cause of hospitalization (Nathan, 1993).

Foot ulceration, an example of diabetic skin wound, is a common complication that occurs in about 15% of diabetic patients, and often requires prolonged hospitalization for its management and is a major disease-associated amputations in developed countries (Albert, 2002). The management of diabetic foot ulceration poses a debilitating financial burden on patients and the health system (Al Odhayani et al., 2017). The mechanisms responsible for delays in wound healing in diabetic patients are not fully understood. However, factors such as age, disease state, diet, reactive oxygen species (Bluher & Mantzoros, 2015), and immune state have been implicated in poor wound healing in diabetic patients (Brem & Tomic-Canic, 2007; Oguntibeju, 2019). The relationship of adipose-derived biomarkers, adipokines, and metabolic disorders have been well studied, especially the role of resistin and PPAR-γ in immune system modulation, inflammation and metabolic syndrome.

The importance of avoiding wound infection during the wound healing process makes chitosan a suitable material for wound dressings due to its inherent antibacterial activity and

many other advantages, such as analgesic effect and hemostatic activity (Zhao et al., 2017). Over the last decade, several therapies such as debridement, negative pressure wound therapy, and hyperbaric oxygen have been proposed for the treatment and management of diabetic wounds (Fui et al., 2019). Even though these unconventional treatments have yielded some positive results, the high cost of managing diabetic wounds in clinical settings, and the challenges posed by these treatments, have forced scientists to explore other novel therapeutic treatments that could enhance wound healing in diabetic patients.

Chitosan is a naturally occurring compound that is present in the coating of some mollusks and insects (Escarcega-Galaz et al., 2018). Chitosan is a conglomerate copolymers of glucosamine and N-acetyl glucosamine units linked by b-1,4-glycosidic linkages and is generally considered to be a biodegradable, biocompatible, non-antigenic, non-toxic, biological adhesiveness, antimicrobial, and biologically active compound with hemostatic effect (Dai et al., 2011; Huang & Fu, 2010; Liu et al., 2018). The cationic nature of chitosan as well as the presence of nitrogen in its molecular structure makes the polymer distinct from other polysaccharides (Bhattarai et al., 2010). Chitosan and its derivatives have been widely used in the fields of medicine, cosmetics, wound dressing, and tissue engineering (Muzzarelli et al., 2012) as a suitable polymer for the delivery of active ingredients such as drugs, growth factors, stem cells, and peptides (Bhattarai et al., 2010). As part of their chemical characteristics, chitosan materials exhibit a positive charge (at typical wound pH values), film-forming capacities, mild gelation characteristics, and strong wound tissue adhesive properties (Jayakumar et al., 2011). Several studies have demonstrated that chitosan has inherent antibacterial activity and is clinically useful for developing hydrogel wound dressings due to its analgesic effect and hemostatic activity (Zhao et al., 2017). From their antimicrobial studies of chitosan in solution,

powders, and edible films, Friedman et al. (2010) showed that low-molecular-weight chitosan at a pH < 6.0 presents ideal conditions for achieving suitable antimicrobial and antioxidative, preservative effects in liquid and solid foods.

In this study, we investigated the therapeutic effect of 5-FU-Chitosan gel on wound healing under diabetic conditions in vitro. Chitosan gel (2% w/w) was prepared in acetic acid prior to mixing serial dilutions of 5-FU. The flow behaviors of gels were determined by rheological analysis. Human dermal fibroblast cells were exposed to each formulation and cell migration was recorded for 24, 48, and 72 hours. It was hypothesized that 5-FU-Chitosan gel would upregulate resistin and PPAR-γ expression in HDFa cells and enhance wound closures.

MATERIALS AND METHODS

Normal adult primary human dermal fibroblast (HDFa) (ATCC® PCS201012™), dimethyl sulfoxide (DMSO) (ATCC® 4-X™), fibroblast basal medium, fibroblast growth kit–low serum, Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), penicillin-streptomycin solution, and fetal bovine serum (FBS) were purchased from American Type Culture Collection. D-(+)-Glucose, ≥99.5% (GC), acetic acid, low molecular weight chitosan powder, and 5-Fluorouracil powder were obtained from Sigma Aldrich.

5-FU-Chitosan Gel Formulation

Chitosan gel (2% w/w) was prepared by dissolving 2.0 grams of low molecular weight chitosan powder in 100 mL of 1% acetic acid and stirring for 12 hours at room temperature. The pH of gel was 4.03. 5-Fluorouracil was dissolved in DMSO at a stock concentration of 5mg/mL and serial dilutions of the following 5-FU-CS gel formulations were prepared: 25µg/mL, 2.5µg/mL, 0.25µg/mL, and 0.025µg/mL.

Cell Culture

For all experiments, normal adult primary human dermal fibroblast (HDFa) cells were grown in fibroblast basal medium supplemented with Low-serum growth Kit. Fetal bovine serum (2%), 1% penicillin, streptomycin sulfate, 2 Mm L-glutamine, and Dulbecco's modified eagle medium (DMEM) containing high D-glucose was used to simulate diabetic conditions. The growth medium was replaced every two days. The cells were washed with DPBS prior to treatment with 5-FU and CS gel formulations. The primary fibroblasts were grown in a humidified incubator at 5% CO2 and 37ºC.

Cell Proliferation Assay

HDFa cells were seeded in 6-well plates at a density of $7.92x10^5$ cells/mL and incubated overnight. The culture medium was modified to simulate diabetic condition as described above. After 24 hours of incubation, the cells were treated with 1.0 mL of each formulation of 5-FU-CS gel for 24 hours. Cells were rinsed three times with DPBS and harvested using 0.53 mM Trypsin-EDTA. The number of cells for each experimental group were counted in a microscopic counting chamber.

MTT Cell Viability Assay

Viability of HDFa cells after 24 hours exposure to 5-FU and 5-FU-CS gel at different concentrations (25µg/mL, 2.5µg/mL, 0.25µg/mL, and 0.025µg/mL) was analyzed using a MTT assay kit. 200 µL of MTT solution was added to 100µL of cell culture and incubated for four hours in a 96-well plate. 500µL of 10% SDS solution was added to the cell culture to dissolve the formation of purple formazan crystals and incubated at 37ºC for 24 hours. Absorbance values of the MTT were read at 570 nm and background absorbance was read at 690 nm. The background absorbance was subtracted from the 570 nm absorbance.

Wound Healing Assay

Normal adult primary human dermal fibroblasts were seeded in 6-well plates under standard culture conditions for 24 hours. The culture medium was modified as described previously. The cells were wounded across the monolayer with a linear scratch using a sterile pipette tip (p20) to simulate a wound and washed with DPBS to remove any floating cells and other cellular debris. The simulated wound was treated with 1.0 mL of each formulation as described above in the absence of mitomycin C. Images of the wound closure and cell migration were taken using an inverted fluorescent microscope at 24-hour intervals (i.e., D1, D2, D3, etc.) until complete closure/migration across the wounded area.

Evaluation of RNA Quality

Cellular RNA of 5-FU, CS treated, and control cells were extracted using the RNeasy Kit as described by the supplier. RNA was eluted in RNase-free water. The RNA concentration and purity were determined using the NanoDrop1000 Spectrophotometer (NanoDrop Lite, Wilmington, USA).

cDNA Synthesis

Based on the total RNA concentration, samples were reverse transcribed into first-strand cDNA using the DC Protein Assay Kit (BioRad, Hercules, CA) as described by the supplier. For the synthesis, RNA Standard Mix (2X RT Mix, RT Enzyme) and nuclease free water were utilized in a total volume of 20 µL. The real time reaction was quantified overnight using the Real Time PCR Software (QuantStudio).

RT-qPCR for mRNA Gene Expression

RT-qPCR was performed using a Touch real-time detection cycler, and TaqMan® Gene Expression Master Mix (Fisher Scientific, USA) was used for gene expression profiling. 10 µL

of RNA Master Mix, and 9.0 µL of cDNA sample was added to 1.0 µL of the target gene primer (Life Technologies), and PCR was quantified overnight. The target gene primers that were analyzed were Resistin and PPARγ. The threshold frequency (CT) values of the PCR data were analyzed by comparing it to the house keeping gene (beta actin). Three independent cell culture experiments were conducted, and RNA was extracted as mentioned above.

Statistical Analysis

Data are reported as mean ± standard error of the mean. Data from the MTT cell viability assay of experimental groups were compared using two-way ANOVA followed by Sidak's multiple comparisons test. mRNA gene expression of experimental groups was compared using the student's t test. Statistical analysis and creation of figures were performed with Graph Pad Prism software version 9.0 (Graph Pad Software Inc., San Diego, CA, USA). For all tests (n=3), $P < 0.05$ was considered statistically significant.

RESULTS

MTT Cell Viability

Viability of HDFa cells as a consequence of exposure to CS gel, 5-FU and 5-FU-CS gel formulations were analyzed using MTT assay with a calorimetric reaction. Results from the MTT assay show that 5-FU decreased HDFa cell growth after 24 hours exposure (Figure 19). When we compared 5-FU treated samples to the vehicle (control group), there was a statistically significant difference in cell growth ($P<0.05$), as seen in Figure 19. This effect was evidenced by the gradual increase in relative percent viability values from 67% for 25µg/mL 5-FU-exposed cells and 73% for 0.025µg/mL 5-FU-exposed cells. Similar to the 5-FU values, results from our MTT assay indicate that CS gel and 5-FU-CS gel significantly hinder cell viability ($P<0.05$, Figure 19). Among the 5-FU-CS treatment groups, cells exposed to 0.025µg/mL 5-FU-CS gel

had the highest relative percent viability. However, comparing the different concentrations of 5-FU to that of 5-FU-CS gel, showed that 5-FU-CS has a significant impact on cell viability after 24 hours exposure.

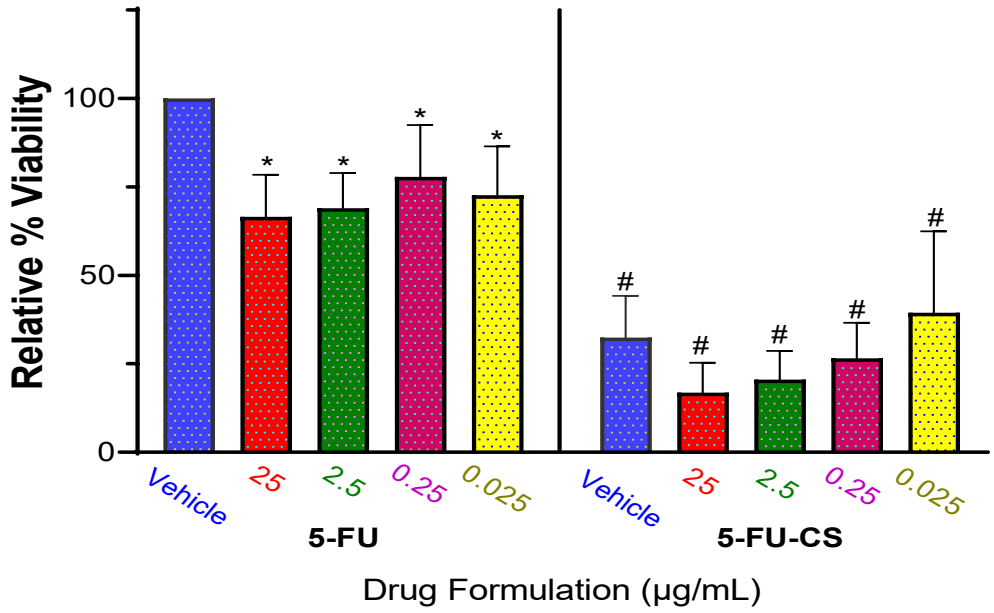

Figure 19. HDFa Cell Viability after 5-FU Exposure.

MTT Assay cell viability of HDFa cells after 24 hours exposure to different formulation of chitosan gel. The results show relative percent viability after exposure to control (vehicle) and 5-FU, and vehicle (CS gel) and 5-FU-CS gel. All values are expressed as Mean ± SEM with 95% confidence intervals. Data were analyzed by two-way ANOVA with a post hoc Sidak's multiple comparisons test. *Denotes significant difference between 5-FU (Vehicle) vs. 25µg/mL 5-FU, 2.5µg/mL 5-FU, 0.25µg/mL 5-FU, and 0.025µg/mL 5-FU, respectively. $^{\#}$Denotes significant difference between 5-FU vs 5-FU-CS: (5-FU) Vehicle vs (5-FU-CS) Vehicle; 25µg/mL 5-FU vs 25µg/mL 5-FU-CS; 2.5µg/mL 5-FU vs 2.5µg/mL 5-FU-CS; 0.25µg/mL 5-FU vs 0.25µg/mL 5-FU-CS; and 0.025µg/mL 5-FU vs 0.025µg/mL 5-FU-CS. $P<0.05$ was considered as statistically significant.

HDFa Wound Closure/Migration

Migration of fibroblast is one of the key steps involved in normal wound healing. Therefore, to investigate the therapeutic effect of topically formulated CS gel and 5-FU on human dermal fibroblast migration, an in vitro wound-healing assay was performed. The migratory ability of dermal fibroblast was not impaired in the presence of high glucose media. As seen in Figure 20, column A (control group), there was full migration of dermal fibroblast cells across wounded areas after two days. Even though high glucose is implicated as one of the factors that delay wound healing, exposing dermal fibroblast to high glucose media resulted in no negative effect on cell migration in vitro.

CS is a well-known biodegradable polymer with several biomedical applications. Here, we investigated the therapeutic effect of CS gel and different formulations of 5-FU-CS gel on migration of human dermal fibroblast under high-glucose conditions. Migration was measured by movement of cells across a wounded area using the scratch assay. The results from our scratch assay showed a gradual increase in the rate of cell migration from day one to day five when cells were exposed 25μg/mL and 0.025μg/mL of 5-FU (Figure 20). Comparing the rate of migration after treatment with 5-FU, results show a dose dependent effect on cell migration. Cells treated with 0.025μg/mL 5-FU migrated across the wounded area after day three, compared to those that were exposed to higher concentrations. Conversely, cells that were exposed to 2% CS gel showed no sign of migration from day one to day five (Figure 20). This was unexpected because chitosan gel is known to exhibit inherent wound-healing properties. However, this could be due to the pH of the gel and possible gel-induced changes in osmotic pressure. These results suggest that the migratory ability of human dermal fibroblast is enhanced by low concentrations of 5-FU, and significantly impaired by 2% CS gel.

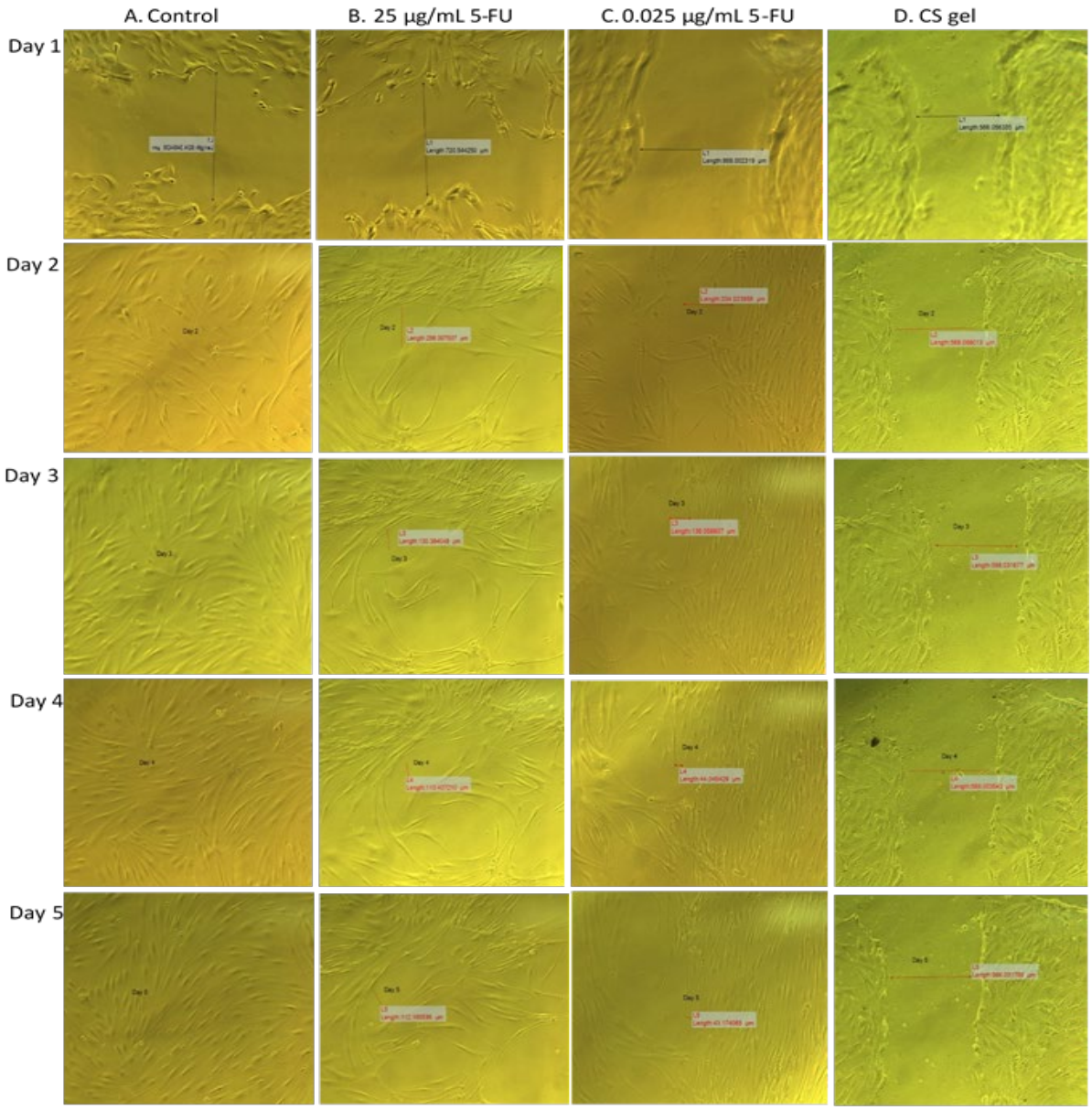

Figure 20. Effect of 5-FU and CS Gel on HDFa Cell Migration.

HDFa cell migration after treatment with 5-FU and CS gel under diabetic condition at different time points. The result shown are microscopic images (A) cell migration after exposure to high glucose media (control), (B) cell migration after exposure to 25µg/mL 5-FU-CS, (C) cell migration after exposure to 0.025µg/mL 5-FU-CS gel, and (D) cell migration after exposure to CS gel.

mRNA Gene Expression

Prolonged high glucose exposure has been implicated in the loss of endothelial cell integrity and reduced keratinocyte migration and proliferation in both in vivo and in vitro scratch wound assays (Okonkwo & DiPietro, 2017; Zhu et al., 2011). Therefore, to examine the therapeutic effect of 5-FU on wound healing, human dermal fibroblast cells were treated with multiple doses of 5-FU in 2% CS vehicle in the presence of high-glucose media, and mRNA gene expression of resistin and PPARγ were analyzed. There was a dose-dependent effect of 5-FU on resistin and PPARγ gene expression after 24 hours of treatment. Figure 21 shows that expression of PPARγ increases with decreasing concentration of 5-FU. However, the level of gene expression was significantly lower than the control group ($P<0.05$). Similar dose-dependent effects were recorded for gene expression of resistin. However, there was upregulation of resistin gene expression after 24 hours exposure to 25μg/mL and 2.5μg/mL 5-FU. This change in gene expression was not significant but is worth further explorations in different models. Conversely, there was a statistically significant decreased in resistin gene expression after treatment with 0.25μg/mL 5-FU.

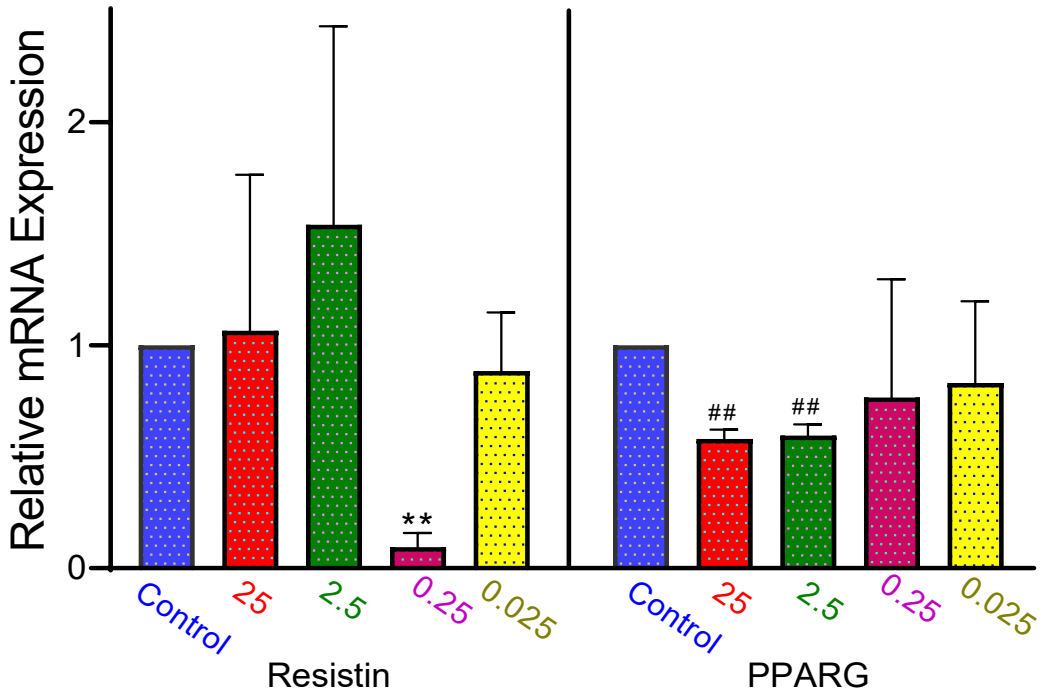

Figure 21. Effect of 5-FU on HDFa Gene Expression after 24 Hours Exposure.

The result show relative mRNA gene expression of resistin and PPARγ after exposure to control, 25µg/mL 5-FU, 2.5µg/mL 5-FU, 0.25µg/mL 5-FU, and 0.025µg/mL, respectively. All values are expressed as Mean ± SEM with 95% confidence intervals. Data were analyzed by student's t test. **Denotes significant difference in resistin expression for control vs. 0.25µg/mL 5-FU. ##Denotes significant difference in PPARγ expression for control vs 25µg/mL 5-FU, and control vs 2.5µg/mL 5-FU. $P<0.05$ was considered as statistically significant.

DISCUSSION

Normal wound healing involves the repair of connective tissue and is typified by four complex and overlapping phases that consists of hemostasis, inflammation, proliferation, and tissue remodeling (Falanga, 2005; Fui et al., 2019; Gonzalez et al., 2016). One of the critical things in normal wound healing is timing. Especially, the timing of wound re-epithelialization and fibroblast proliferation (Cam et al., 2020). Even though the mechanisms responsible for the

delay in wound healing in diabetic patients are not fully understood, several factors have been implicated in poor wound healing in diabetic patients (Brem & Tomic-Canic, 2007; Oguntibeju, 2019). Evidence suggests high glucose levels are connected to reduced keratinocyte migration and proliferation in both in vivo and in vitro scratch wound assays (Zhu et al., 2011). However, in our studies, exposing human dermal fibroblast cells to high-glucose media did not impair in vitro cell migration.

Cutaneous wounds require a sequential interaction of cell migration and proliferation from numerous different tissues and cell types. Among these, fibroblasts have been shown to play critical roles in all phases of wound healing. For example, following skin abrasion or wounding, fibroblasts are attracted from the edges of the wound or from the bone marrow to produce chemokines and growth factors that aid in the wound-healing process (Opalenik & Davidson, 2005). The proliferation and migration of dermal fibroblasts are essential for cutaneous wound repair because dermal fibroblasts migrate to damaged sites, repopulate the wound, and remodel fibrin and collagen deposits (Shi et al., 2015). Diabetes is known to have adverse effects on fibroblast proliferation which is marked by reduced cell migration abilities (Lamers et al., 2011; Lerman et al., 2003). In this present study, migratory abilities of human dermal fibroblast were analyzed using an in vitro scratch assay after exposure to CS gel and 5-FU. Fibroblast cells demonstrated migratory abilities in a timely manner depicting observations seen in normal wound healing.

The established relationship between adipokines and obesity-associated diseases such as T2DM, show that adipokines have an enormous potential to be clinically relevant both as bioactive markers and as therapeutic compounds (Bluher, 2014; Timar et al., 2014; Xu et al., 2015). Alterations in adipokine secretion have been implicated in obesity-related disease. For

example, human recombinant resistin has been shown to stimulate proliferation, stimulate migration, and enhance capillary-like tube formation by upregulating gene expression of angiogenesis-promoting factors (Mu et al., 2006). Further studies also show that PPARα controls the early inflammation phase of healing (Michalik et al., 2001), PPARβ regulates keratinocyte proliferation, adhesion, and migration (Michalik et al., 2001; Tan et al., 2003; Tan et al., 2004), and PPARδ participates in promoting fibroblast proliferation (Li et al., 2012) in normal skin wound healing. In this study, gene expression of resistin and PPARγ were measured in relation to cell migration/proliferation after exposure to 5-FU and 2% CS gels. We found increased expression in resistin after exposure to 25μg/mL and 2.5μg/mL 5-FU. However, we found decreased expression in PPARγ gene after exposure to all the doses of 5-FU. Even though this result is contradictory to previous studies, there was an overall improvement in cell migration or wound closure.

CONCLUSION

5-FU was formulated with 2% CS gel at different concentrations and its therapeutic potentials on in vitro wound healing and effect on adipokine expressions were examined. It was shown that 5-FU induces a dose-dependent effect on HDFa cell viability, as well as on adipokine mRNA gene expression of resistin and PPARγ. Exposure of human dermal fibroblast cells with 5-FU resulted in cell migration in a timely fashion compared to exposure to bare CS gel alone. Therefore, our experimental results suggest 5-FU is beneficial against adipokine expression, migration of HDFa cells under diabetic conditions, and could be further explored for its potential therapeutic effect on diabetic wound healing.

SUMMARY AND FUTURE DIRECTIONS

Diabetic wounds are one of the most severe complications of diabetes mellitus and clinically difficult to manage. Unfortunately, the mechanisms responsible for the delay in diabetic wound healing are not fully understood. However, evidence shows that factors such as high glucose levels and increased expression of pro-inflammatory adipokines reduce migration of key cells involved in normal wound healing. These initial studies have paved the way for target drug delivery through topical applications. Delivering drugs via topical routes is distinguished by many advantages over conventional routes, especially when drugs are formulated with non-toxic vehicles. Among these non-toxic vehicles, chitosan is a natural polymer that has gained more interest as a suitable drug delivery vehicle due to its biodegradable, biocompatible, and biological properties. Thus, in our study, we show rheological properties of a 2% CS gel is a suitable vehicle for topical drug delivery. This was achieved by examining the viscoelastic properties of the gel. After identifying the gel-like properties of CS gel, it was then formulated with different doses of 5-FU with the aim of improving wound healing in vitro. Results gathered from our studies show that 5-FU has a dose dependent effect on HDFa cell migration/wound closure and mRNA adipokine expression.

This preliminary finding has created the platform for further exploration of CS gel as a drug delivery vehicle for in vivo diabetic models. This would involve evaluating rheological properties of CS gel at different concentrations, investigating the release profile of 5-FU from CS gel and biofilm, and testing the therapeutic effect of 5-FU CS gel in animal models.

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APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

March 31, 2021

Samuel Tetteh-Quarshie
2557 3rd Avenue
Huntington, WV 25703

Dear Mr. Tetteh-Quarshie:

This letter is in response to the submitted thesis abstract entitled *"Drug Delivery Systems: Exploring Rheological Properties and Therapeutic Effect of 5-FU Chitosan Gel for Topical Wound Healing."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
bFGF	Basic fibroblast growth factor
CS	Chitosan
DA	Degree of acetylation
DM	Diabetes mellitus
ECM	Extracellular matrix
G’	Storage modulus
G’’	Loss modulus
GDM	Gestational diabetes mellitus
HBOT	Hyperbaric oxygen therapy
HDF	Human dermal fibroblast
J	Creep compliance
LVR	Liner viscoelastic region
MIC	Minimum inhibition concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MW	Molecular weight
n	Flow behavior index
η	Apparent viscosity
Pa	Pascal
PPAR	Peroxisome proliferator-activated receptor
RF	Refrigeration
RT	Room temperature

SEM	Standard error of mean
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
γ’	Shear rate
τ	Shear stress