Evaluating the Osteogenic Potential of Raspberry Ketone and Simvastatin Dual-Loaded Electrospun Chitosan Membranes on Preosteoblasts

Matthew T. Atwill

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Evaluating the Osteogenic Potential of Raspberry Ketone and Simvastatin Dual-Loaded Electrospun Chitosan Membranes on Preosteoblasts

By

Matthew T. Atwill

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Dedication

I wish to dedicate this thesis to the memory of my grandparents, Jack and Theresa Haddad, as well as my parents that have lovingly supported me through this journey.
Acknowledgements

I want to thank my advisor, Dr. Joel D. Bumgardner, for allowing me the opportunity to work with him and for his guidance and teaching throughout my undergraduate and graduate studies. This thesis and all the experiences that came with it would not have been possible without his hard work and dedication. I also want to thank my committee members, Dr. Judith Cole, Dr. J. Amber Jennings, and Dr. Tomoko Fujiwara, for their insight, advice, and patience. I would like to acknowledge Priya Murali for taking me in as an undergraduate and teaching me the fundamentals for lab work and cell culture. Lastly, I want to acknowledge my fellow lab mates, Alex Bryan and Blass Watson, for their comradery and insight during my graduate studies.
Preface

The second chapter of this thesis is a journal article entitled “Evaluating the Osteogenic Potential of Raspberry Ketone and Simvastatin Dual-Loaded Electrospun Chitosan Membranes on Preosteoblasts.” This manuscript will be submitted to the Journal of Biomedical Materials Research Part B: Applied Biomaterials.
Abstract


This study evaluated the osteogenic potential of dual-loading raspberry ketone (RK) and simvastatin (SMV) onto electrospun chitosan membranes (ESCMs) to impart bioactivity onto guided bone regeneration (GBR) membranes and give them a more active role in osteodifferentiation. In this study, W-20-17 murine preosteoblast stromal cells in media exposed to 12-100µg/ml RK in combination with 75-150ng/ml SMV resulted in increased basal alkaline phosphatase (ALP) expression, whereas RK at any dose alone had no effect on ALP expression. Additionally, there was a dose-dependent combinatory cytotoxic effect in which SMV in combination with increasing concentrations of RK resulted in decreased viability and vice versa. RK+SMV combinations were loaded onto hexanoic anhydride modified ESCMs and evaluated for cytotoxicity and osteogenic potential. The RK dose used had minimal effect on viability, whereas the higher SMV dose had significant cytotoxic effects even with daily media change. The RK+SMV ESCM groups with lower SMV loading had minimal effect on osteodifferentiation, and combinations with higher SMV loading inhibited ALP and calcium expression below basal level. It is speculated that RK has a minimal effect on preosteoblast differentiation and may have positive effects on less differentiated cell types. Additionally, it is possible the SMV elution kinetics when combined with RK resulted in exposure to higher SMV concentrations. With some modification of RK+SMV combination concentrations and better understanding of the specific mechanistic effects of RK, RK+SMV dual loaded ESCMs may have potential to be used in GBR applications.
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Chapter 1: Introduction

Problem Statement

Untreated caries, periodontitis, and trauma are leading causes for loss of teeth and surrounding alveolar bone [1]. If left untreated, alveolar bone resorption can lead to additional tooth loss. Dental implants are an increasingly common treatment option to replace the function and aesthetic of missing teeth. It is estimated that over 5 million dental implants are placed each year worldwide, reaching an estimated global market cost of nearly $5 billion [7]. However, dental implants require sufficient alveolar bone volume to successfully remain anchored into the jaw. Guided bone regeneration is one of the most common methods to restore lost alveolar bone volume. In guided bone regeneration, a bone graft material fills the alveolar defect and is covered with a guided bone regeneration membrane, which physically isolates the defect and prevents infiltrations from rapidly proliferating gum tissue [11].

Current membranes are made of titanium-reinforced polytetrafluoroethylene, collagen, polylactic acid, or polyglycolic acid and can have undesirable degradation rates or inflammatory degradation products. The guided bone regeneration membrane complication rate, including membrane exposure, soft tissue dehiscence, and acute infection, has been estimated to be approximately 17% [15].

In addition to providing a physical barrier, recent attempts have been made to impart bioactivity onto guided bone regeneration membranes to enhance bone regeneration. Recombinant bone morphogenetic protein-2 has been investigated for its ability to impart osteoinductive activity onto membranes, and while it is a very potent osteoinductive protein, it is very expensive and has undesirable release patterns from clinical membranes [19]. Therefore, there is a clinical need to improve the bioactivity of guided bone regeneration membranes to enhance bone wound healing and regeneration at a defect site.

Chitosan and electrospun chitosan membranes have recently emerged as a potential alternative to clinical guided bone regeneration membrane materials. Chitosan has demonstrated desirable
biocompatibility and biodegradability and has been studied for potential innate immunoregulatory, anticoagulant, antibacterial, and wound healing properties [24]. Electrospun chitosan are biocompatible, successfully provide barrier function, and have desirable degradation properties in vivo [28]. Additionally, their highly nanofibrous structure make electrospun chitosan membranes ideal for loading and delivery of drugs. Many drugs and growth factors have been investigated for loading and delivery, and two such potentially osteoinductive compounds are raspberry ketone and simvastatin [28],[38]. Raspberry ketone has shown potential to induce osteodifferentiation in vitro and has also been shown to promote immunomodulatory response in vitro and in vivo [35], [38]. Raspberry ketone is widely used as a nutraceutical and flavoring agent that can be inexpensively synthesized, and preliminary studies have linked raspberry ketone to a several metabolic effects such as macrophage polarization and adipogenesis [34]. Simvastatin has been widely studied for its pleiotropic effects on a wide variety of metabolic activities, most notably increased bone metabolism, and has been incorporated into many biomaterials systems for local delivery [41]

The goal of this research is to evaluate the potential of raspberry ketone and simvastatin dual-loaded electrospun chitosan membranes for use in guided bone regeneration. Improving bone regeneration and wound healing in oral defects can lead to improvements in patient quality of life as well as a decrease in guided bone regeneration complication rate. Chitosan membranes offer several advantages in degradation kinetics and drug loading potential over commercially used guided bone regeneration membranes. The use of inexpensive, widely available, and multi-action compounds such as raspberry ketone and simvastatin could replace expensive growth factors like bone morphogenetic protein-2 and increase the availability and effectiveness of guided bone regeneration procedures.
Hypothesis and Rationale

This work aims to utilize the desirable biocompatibility, biodegradability, and nanofibrous structure of electrospun chitosan membranes in combination with potential osteoinductive wound healing properties of raspberry ketone and simvastatin to create a guided bone regeneration membrane that provides the traditional barrier function as well as enhances bone regeneration. The hypothesis of this work is that raspberry ketone and simvastatin dual-loaded electrospun chitosan membranes positively stimulate osteodifferentiation in preosteoblasts. The goals of the study are to determine the non-cytotoxic concentration combinations of raspberry ketone and simvastatin as well as raspberry ketone and simvastatin delivered from dual-loaded electrospun chitosan membranes and evaluate the osteogenic potential of raspberry ketone and simvastatin concentration combinations as well as raspberry ketone and simvastatin delivered from dual-loaded electrospun chitosan membranes.

Electrospun chitosan membranes will be used because they have desirable biocompatibility, biodegradability, and barrier functionality in vivo. The nonwoven, random nanofibrous structure of electrospun chitosan membranes allows for modification and drug loading for local delivery. Raspberry ketone and simvastatin have been shown to have potential positive effects on bone wound healing, and the co-incorporation of these compounds into an electrospun chitosan membrane may impart bioactivity that enhances bone regeneration.
Clinical Background

Tooth decay, bone loss, and dental implants

Tooth loss has many known causes, including dental caries, periodontal diseases, trauma, and orthodontic extraction. While tooth loss can affect all people, it has an increased tendency to develop in older adults [1]. Although tooth loss has generally declined in recent decades, those affected can experience additional health problems such as difficulty eating or communicating as well as problems with self-image [2]. Age, sex, socioeconomic status, diabetes, and many smaller factors have been associated with an increased risk of tooth loss [3]. While tooth loss has many causes, the two leading causes are untreated caries and periodontitis [1]. In both cases, tooth loss is caused by loss of supporting structure in tooth, alveolar bone, or both due to bacterial infection and degradation of adjacent tissue. If left untreated, supporting soft and hard tissue will continue to degrade until the tooth falls out or requires extraction [4]. Without the microforces and strains present on alveolar bone provided by lost teeth, bone begins resorbing, losing both mass and volume. If left untreated, bone resorption can worsen and affect the bone anchoring other teeth into place, causing further tooth loss and making replacement increasingly difficult. It has been estimated that over 38% of the population above 18 years old will lose at least one tooth in their lifetime [5]. Many different treatments for tooth loss exist based on degree and number of teeth lost, including dentures, crowns, bridges, and implants. Each option can replace the look and partial function of a lost tooth with varying degrees of longevity. In recent years, dental implants have become a common treatment to replace natural dentition for extended periods of time, and while they are notably more expensive, they have an estimated success rate of at least 90% over ten years [6]. It is estimated that over 5 million dental implants are placed each year worldwide, reaching a global market cost of nearly $5 billion [7]. However, due to the resorption of alveolar bone, dental implants often do not have sufficient bone to anchor to the jaw, which can lead to implant failure [8]. One of the most common methods to regrow alveolar bone is guided bone regeneration (GBR). In GBR, a
membrane is placed over a defect site to prevent invasion of soft tissue into the grafted space and maximize the amount of bone regeneration [9] (Figure 1). The goal of GBR is to regrow enough bone volume that will allow successful placement and anchorage of a dental implant in the mandible or maxilla bone.

Figure 1: Guided bone regeneration procedure using a bone graft material in combination with a space-maintaining membrane for dental application [10]

GBR and Problems with Current GBR Therapies

The initial GBR application of a barrier membrane was first proposed in 1959, but it was not until the 1980s that GBR was first applied to the regeneration of alveolar bone for periodontal applications. This initial application aimed to physically isolate the defect in the alveolar ridge from the surrounding soft tissue to prevent infiltration of fast-growing soft tissue before slow-growing bone tissue regenerates. Successful isolation requires several conditions: exclusion of epithelium and connective tissue, space maintenance, stability of fibrin clot, ample blood supply, and primary wound closure [11]. Successful bone growth after isolation requires several factors working together, including a blood clot
filling the alveolar defect space, attraction of neutrophils and macrophages, clot resorption and replacement with granulation tissue filled with blood vessels, transport of nutrients and stem cells, osteogenic differentiation of stem cells, and osteoid formation, mineralization, and maturation [11]. Use of GBR membranes is typically combined with a bone graft, usually an allograft, to supplement the bone growth process. Synthetic bone graft material may also be used [12]. If successful, GBR can replace lost bone in as little as 3-4 months [11]. GBR success rate and subsequent implant survival rate has been reported to be 65-99% depending on the defect size, defect location, membrane type, and time point observed [13], [14]. Furthermore, the soft tissue complication rate, including membrane exposure, soft tissue dehiscence, and acute infection, has been estimated to be approximately 17% [15].

Since the primary goal of initial GBR was physical isolation of the defect from the surrounding gum tissue, early GBR membranes were non-resorbable. These membranes were mostly polytetrafluoroethylene (PTFE) based, titanium meshes, or a combination of both [16]. However, the non-resorbable nature of these membranes requires a second surgery to remove, which brings an additional risk of failure, inflammation, and contamination of new tissue [17]. In more recent years, absorbable membranes have been developed and utilized for GBR applications to eliminate the need for a second surgery. Natural absorbable membranes have natural bioactivity for cellular interactions, but typically have undesirable biodegradation kinetics and require additional processing or chemical modification, which increases the risk of immune response [16]. Collagen membranes have been developed for clinical use, whereas gelatin, chitosan, and other polymer membranes are currently being tested for applications in GBR [16]. For each polymer, cross linking or hydrophobic modifications are required to achieve the desired mechanical and degradation properties for GBR applications. Synthetic absorbable membranes are more reproducible and require less modification compared to natural membranes [16]. Membranes made from polylactic acid (PLA) and polylactic-co-glycolic acid (PLGA) have been developed for clinical applications, but degradation of these materials causes acidic
byproducts, which can lead to undesired inflammation [16]. Polycaprolactone (PCL) and polyethylene glycol (PEG) have also been investigated but have undesirable degradation kinetics [16] Examples of commercially available GBR membranes can be found in Figure 2.

![Figure 2: Common commercially available GBR membranes, including titanium-reinforced e-PTFE (W.L Gore and Associates) (A), PGA (W.L Gore and Associates) (B), and porcine collagen (Geistlich) (C1, C2). Modified from [18]](image)

The autologous bone graft, the current gold standard therapy in combination with GBR membranes, provides three features to the alveolar defect site: osteoinduction, osteoconduction, and osteogenesis. Autologous bone grafts provide the growth factors and environment that aids osteoinduction, a structure mimetic of natural bone that aids osteoconduction, and the cells to undergo osteogenesis [19]. If an autologous bone graft is not possible due to insufficient donor site density, allografts, xenografts, or synthetic bone grafts may be used [19]. These alternative graft materials are sourced from human cadavers, other animals, or synthetic materials such as hydroxyapatite or tricalcium phosphate,
respectively [19]. Allografts and xenografts must be properly sterilized before implantation into a defect site, destroying the growth factors and cells present in an allograft sample. Similarly, synthetic materials do not contain these growth factors and cells that make autologous grafts so effective. As such, allografts, xenografts, and synthetic grafts are only osteoconductive in nature and must be further modified to become osteoinductive and osteogenic [19]. Bioactive GBR membranes may be used to potentially bridge this gap between autologous grafts and other grafting alternatives.

While a traditionally successful GBR membrane maintains space, it does not contribute to osteoinductivity, osteoconductivity, or osteogenic activity. Additional modification to make GBR membranes more bioactive regarding bone repair may have potential to make GBR therapy more effective and, depending on the efficacy of the modification, more cost-effective. An ideally bioactive GBR membrane would serve at least five functions: provide structural, compositional, and biochemical cues for formation of new tissue, promote recruitment, proliferation, and differentiation of progenitor cells, engage the resident immune cells to promote regeneration and recovery of adequate local blood supply, and provide antiseptic action in at-risk environments such as the oral cavity [20]. Additional modification to membrane properties such as biocompatibility, porosity, topography, asymmetry, mechanics, and resorption may also enhance regenerative properties [21]. The most promising method to achieve biological function is the incorporation and local delivery of drugs and bioactive molecules into GBR membranes for sustained and controlled release during degradation in an aqueous environment.

Need for local delivery mechanisms and targets for local delivery molecules

As opposed to systemic drug delivery, such as an oral administration or intravenous injection, local drug delivery administers a therapeutic agent into a wound site without reliance on systemic circulation. While systemic delivery cannot guarantee a therapeutically relevant dosing at a wound site, local delivery can result in a much higher, potentially more therapeutic drug concentration at a wound
site without risking adverse systemic effects [22]. Several local delivery systems exist for prevention and treatment of periodontitis in the oral cavity, including antibiotic-loaded fibers, gels, strips, films, microparticles, and nanoparticles [23]. However, these established systems are only designed to fight infection, and are not immediately translatable to the requirements for GBR delivery systems. Ideal GBR systems should have desirable modulatory effects on proliferation, osteodifferentiation, inflammation, regeneration, bacterial growth, and angiogenesis [23]. Of the delivery systems in development, very few are approved for clinical applications, and even fewer address more than one ideal GBR membrane function. For example, devices such as Actisite, Periochip, Arestin, Atridox, and Periocline are antibiotic delivery systems approved for the treatment of periodontal disease [23]. Preclinical studies are currently examining the ability of GBR devices to deliver inflammation-modulating therapeutics such as curcumin, atorvastatin, or aspirin as well as osteogenic therapeutics such as platelet-derived growth factor (PDGF), bone-morphogenetic protein 2 (BMP-2), simvastatin, or lovastatin [23]. However, it is unlikely that a single biomaterial and drug combination can address the many requirements of an ideal GBR membrane delivery system. Composite biomaterials in combination with multiple therapeutic delivery systems will likely be necessary to design the ideal macro and microenvironment for bone regeneration in the oral cavity.

**Chitosan Electrospinning, Raspberry Ketone, and Simvastatin**

Chitosan has gained popularity in recent years as a natural polymer derived primarily from chitin that can be modified for a variety of biomaterial applications. Many varieties of chitosan exist based on polymer chain length, molecular weight, and degree of deacetylation [24]. While each of these properties are modifiable based on the desired application, unmodified chitosan demonstrates desirable biocompatibility and biodegradability. Additional applications for chitosan are being studied based on innate properties of the molecule, including immunomodulatory activity, anticoagulant properties, antibacterial action, and wound healing properties [24]. Furthermore, chitosan can be chemically
modified via functional groups for applications in a wide variety of fields, including drug and gene delivery, cell culture, and tissue engineering [25]. Since chitosan is soluble in most acids, much research has investigated chitosan electrospinning, a common manufacturing technique to develop nanofibrous electrospun chitosan membranes (ESCM) [26]. Briefly, electrospinning applies a large voltage gradient to a collection surface and syringe needle containing a charged polymer solution. The electrical force applied on the polymer solution pulls the solution from the needle tip towards the collection plate. When exposed to air, the solvent evaporates and leaves behind a fibrous polymer strand that is deposited on the collection plate as a membrane. The properties of the resultant membrane can be modified based on electrospinning parameters, electrospinning solvent, chitosan concentration, cosolvent ratios, copolymer blends, and post-spinning modifications [26]. One application that has received much attention in recent years is the potential to modify membranes for GBR applications [27]–[29]. For example, ESCMs spun in a TFA solvent are very hydrophilic and will almost instantly dissolve in an aqueous environment such as the oral cavity. To prevent immediate breakdown and loss of barrier capabilities of ESCM, they require further modification to retain nanofibrous structure, such as cross-linking or increased hydrophobicity [28].

Beyond the barrier function of traditional GBR membranes, new membranes may be further modified to impart additional biological function. The most common cause for GBR failure is wound dehiscence, resulting in membrane exposure to the oral cavity and leading to premature degradation and potential infection [30]. Membrane exposure following wound dehiscence has been estimated to reduce bone formation as much as six times compared to non-exposed membranes, with membrane type having no difference on dehiscence rate [15],[31]. Imparting more desired biological function onto GBR membranes has potential to benefit GBR and implant success rate by increasing bone volume or regeneration timeline. Shortening the timeline for bone regeneration could reduce GBR complication rate by achieving adequate bone regeneration before membrane degradation or premature exposure. The
typical bone regeneration timeline is divided into the inflammatory, repair, and remodeling phases, which take months to properly heal damaged bone. Wound healing consists of pro-inflammatory and anti-inflammatory (pro-healing) cytokines, released from immune cells such as macrophages and neutrophils, which affect the overall bone remodeling microenvironment. Much is known about bone remodeling and wound healing separately, but their interactions are not as well understood [31]. Pro-inflammatory cytokines such as TNF-α, PGE-2, IL-6, IL-8, and IL-17F are necessary for bone healing to begin and finalize. Anti-inflammatory cytokines such as IL-4 and IL-13 have also been shown to enhance osteogenic markers, but the optimal transition between a pro-inflammatory to pro-healing microenvironment is unknown [32]. Therefore, the two biological functions our lab has chosen to pursue for GBR development are the enhancement of wound healing and osteogenesis.

Raspberry ketone is a small, natural phenol found in raspberries and other fruits that has seen widespread use in nutraceutical, food, and cosmetic industries. Although raspberry ketone has not been widely or appropriately studied, it has been associated with improved skin elasticity, hair growth, weight loss, and obesity. It has also been associated with changes is blood glucose, insulin production, insulin sensitivity, inflammation, and lipolysis [33]. Most research of raspberry ketone has focused on its potential anti-obesity effects for weight loss supplements. In both in vitro and in vivo studies, raspberry ketone increases fat metabolism using peroxisome proliferator-activated receptor α and low-density lipoprotein receptor dependent pathways [34]. Very limited studies exist evaluating the effect of raspberry ketone on bone growth or wound healing. In one study, Takata et al reported raspberry ketone to have a large positive effect on stem cell osteodifferentiation in vitro. [35]. Further in vitro testing within our lab group seemed to indicate that RK loaded culture medium produces an alkaline phosphatase (ALP) response similar to rhBMP-2 in W-20-17 cells. However, this did not translate to ESCM-loaded delivery of RK, in which RK produced ALP and calcium responses one hundred and ten times smaller than the BMP-2 response, respectively[36]. However, this is likely due to inappropriate
release kinetics that do not mimic RK exposure in media [36]. Additional testing seems to indicate that RK has little to no effect on in vitro osteodifferentiation, both by itself and when supplemented with rhBMP-2.

Raspberry ketone is also currently being studied for anti-inflammatory properties [37]. Within our lab group, in vitro testing indicates that RK upregulates expression of anti-inflammatory cytokines like IL-10 while downregulating pro-inflammatory cytokines like TNF-α and IL-1β in RAW 264.7 cells. Additionally, in vivo testing of RK loaded ESCMs on a rat calvarial defect model indicates that RK has a strong dose-dependent effect on facilitating the transition of macrophages from the M1 to M2 phenotype, which correlated to an enhanced bone formation in the calvarial defect [38].

Simvastatin is a commonly used anti-cholesterol medication that has received increased attention for its pleiotropic effect on improving bone metabolism. It has also been associated with positive effects in inflammation and angiogenesis. Simvastatin traditionally acts as an inhibitor of HMG-CoA reductase, the rate-limiting step in the cholesterol synthesis pathway. However, it has also been shown to influence many metabolic pathways, most notably the antagonism of TNF-α and subsequent upregulation in BMP-Smad signaling [39], [40]. Simvastatin has become increasingly incorporated in biomaterials-based local drug delivery systems for bone regeneration. Such systems include simvastatin coated scaffolds, simvastatin-embedded scaffolds, simvastatin microspheres, and simvastatin nanoparticles. Despite the large magnitude of work done to develop simvastatin-loaded delivery systems, much work remains to be done to fully understand the cellular pathways involved in simvastatin-mediated bone regeneration, optimize simvastatin dosing and delivery mechanisms for efficient osteogenesis, and improve and standardize in vitro and in vivo analytical techniques to more closely mimic the dynamic bone regeneration environment [41]. Within our lab, extensive in vitro testing has been performed to determine the ideal loading and release conditions for SMV on ESCMs to induce osteodifferentiation. Results concur with the larger body of literature that at higher concentrations SMV can elicit a
significantly increased ALP and calcium response compared to a negative control, with an increased response when combined with BMP-2. These effects were seen in both media and ESCM loading [42]. Further *in vivo* testing on a rat calvarial model indicated that higher concentrations of SMV loaded onto membranes with longer chain fatty acids produced significantly more bone volume than lower concentrations of SMV loaded onto ESCMs with shorter chain fatty acids. This is most likely due to a more sustained SMV release caused by increasingly stronger interaction between hydrophobic SMV molecules and the increasing nonpolar regions of the fatty acid chains [28].

The goal of this study is to determine the ability of dual-loading and delivery of raspberry ketone and simvastatin from fatty acid modified electrospun chitosan membranes on osteoblast differentiation *in vitro*. The first aim of this study was to examine the effect of RK+SMV combinations in media on osteogenic response. The second aim was to evaluate the effect of RK+SMV combinations delivered from electrospun chitosan membranes on osteogenic response.
Research Goal and Hypothesis

The goal of this work is to determine the effects of raspberry ketone and simvastatin dual-loaded electrospun chitosan membranes on osteodifferentiation in a pre-osteoblast cell line. It is hypothesized that electrospun chitosan membranes loaded with raspberry ketone and simvastatin will affect osteoinductivity in vitro. The specific objectives are to determine:

- Non-cytotoxic combinations of raspberry ketone and simvastatin
- The osteoinductive capabilities of raspberry ketone and simvastatin combinations
- The osteoinductive ability of raspberry ketone and simvastatin delivered from electrospun chitosan membranes

Chapter 2: Manuscript Submission

Introduction

Guided bone regeneration (GBR) membranes are used at bone defect sites treated with bone graft to prevent infiltration of soft tissues into the defect site and maximize bone healing response [1]. The concept of GBR membranes was first proposed in 1959 but was not applied to periodontal applications until the 1980s [2]. The initial purpose of dental GBR membranes was to physically isolate an alveolar defect from the surrounding soft tissue and passively promote bone regeneration. GBR membranes were initially made from expanded polytetrafluoroethylene (ePTFE), titanium mesh, or a combination of the two materials [3]. While these GBR membrane materials are effective, they are non-biodegradable and non-resorbable, and require a second surgery for removal, increasing the cost, risk of new tissue damage, and risk of infection [4]. Resorbable membranes were developed and utilized for GBR applications to eliminate the need for a second surgery. Collagen GBR membranes have desirable biocompatibility, but rapid degradation kinetics resulting in loss of barrier functionality [3]. Synthetic polymers such as PLLA, PLGA, PLA, and PGA are biodegradable, but have acidic degradation products, potentially leading to increased inflammation and inhibiting bone regeneration [3]. GBR
success and subsequent implant survival rate has been reported to be 65-99% depending on defect size, defect location, membrane type, and time point observed [5], [6]. The soft tissue complication rate during GBR therapy, including membrane exposure, soft tissue dehiscence, and acute infection, has been estimated to be 17% [7]. Therefore, there is still a clinical need to improve GBR membranes to both increase the rate of bone regeneration and decrease the complication rate of GBR therapy.

Chitosan has gained popularity in recent years as a natural polymer that can be modified for a variety of biomaterials applications. Chitosan has demonstrated desirable biocompatibility, biodegradability, and non-toxic degradation products [8]-[10]. Chitosan also has been investigated for innate properties such as immunomodulation, anticoagulation, antibacterial, and wound healing [10]. Additionally, chitosan can be electrospun into nanofibrous membranes. These electrospun chitosan membranes (ESCM) have been investigated for their potential as a GBR material, showing desirable barrier properties, nanofiber size, and high surface area to volume ratio [11]. Wu et al., developed a post-spinning process using short chain fatty acid anhydrides that prevents fiber swelling and helps retain the biomimetic nanofiber structure. [12]. In vivo rat calvarial models have shown these modified ESCMs to be biocompatible, have good barrier functionality, and regenerate bone in a comparable manner to commercial GBR membranes [11], [13].

More recently, attempts have been made to give GBR membranes a more active role in bone healing by imparting bioactivity. One method of imparting biological activity onto GBR membranes is the incorporation of drugs or growth factors into the membrane for local delivery at the defect site. Bone morphogenetic protein-2 (BMP-2), a potent osteoinductive growth factor, has been used clinically with GBR therapy to enhance bone formation [13]. However, recombinant BMP-2 is very expensive, has undesirable elution kinetics from GBR membranes, and can cause several adverse reactions such as ectopic bone formation [14]. Other compounds, such as curcumin, statins, and platelet-derived growth factor, are currently being investigated for their ability to positively influence bone regeneration, both
directly through osteogenic mechanisms and indirectly through angiogenic, immunomodulatory, or
wound healing mechanisms [15].

Raspberry ketone (RK) is a small, natural phenol found in raspberries and other fruits that has
been widely used as a nutraceutical in the food and cosmetic industries. RK has been studied in vitro and
in vivo for its reported anti-obesity effects [16], [17]. RK has also been associated with other metabolic
changes such as inflammation regulation and osteogenesis, although these effects have not been
thoroughly investigated [16]. In one study, RK was reported to promote osteodifferentiation
commitment of C3H10T1/2 murine stem cells through the increased expression of TGF-β, alkaline
phosphatase, osteocalcin, and collagen [18]. RK also was reported to reduce inflammatory mediators
such as NO and PGE-2 and block LPS-induced iNOS and COX-2 gene expression [19]. In a rat calvarial
defect model, RK loaded ESCMs caused increased transition of macrophages from the M1 to M2
phenotype, resulting in higher bone volume regeneration [20]. The multi-modal potential of RK on bone
wound healing as well as its affordability makes RK a good candidate for incorporation into GBR
membranes, once its effects are more thoroughly investigated.

Simvastatin (SMV) is a very commonly used lipid-lowering medication used to treat high
cholesterol and has been investigated for pleiotropic effects on improving bone metabolism [21].
Simvastatin also has been reported to positively change inflammation and angiogenesis, but these effects
have not been thoroughly investigated [21]. The osteogenic effects of SMV as well as SMV-
incorporated biomaterials have been widely studied, and while the biological, dosing, and delivery
mechanisms have not been fully optimized, there is consensus that SMV produces notable positive
effects on osteogenesis [21]. In one study, SMV nanoparticles loaded into a chitosan scaffold enhanced
alkaline phosphatase expression, collagen production, and faster bone regeneration in vitro and in vivo
[22]. Additionally, ESCMs loaded with SMV regenerated bone equal to or better than blank membranes
depending on length of the fatty acid acylated in rat calvarial defect models [11] [23].
The goal of this study was to determine the ability of dual-loading and delivery of raspberry ketone and simvastatin from fatty acid modified electrospun chitosan membranes on pre-osteoblast differentiation *in vitro*. The rationale for dual loading is to combine the immunomodulatory effects of RK to enhance macrophage polarization and the osteogenic effects of SMV to enhance bone healing, to lead to a bioactive membrane that stimulates bone regeneration. The first aim of this study was to examine the effect of RK+SMV combinations in media on osteogenic response. The second aim was to evaluate the effect of RK+SMV combinations delivered from electrospun chitosan membranes on osteogenic response.

**Materials & Methods**

Delbecco’s modified Eagle’s medium, high glucose (DMEM), heat-inactivated qualified fetal bovine serum, and penicillin-streptomycin-neomycin antibiotic mixture, and cell culture plasticware were obtained from ThermoFisher (Waltham, US). Cell culture transwell inserts were purchased from Corning Life Sciences (Durham, US). Raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) was purchased from Sigma-Aldrich (St. Louis, MO). Simvastatin lactone was purchased from Cayman Chemicals (Ann Arbor, US). Chitosan was purchased from Primex (Siglufjordur, Iceland).

**Raspberry Ketone and Simvastatin Cytotoxicity Testing**

Cytotoxicity of RK, SMV, and RK+SMV combinations was determined using W-20-17 (ATCC-CRL-2623) mouse preosteoblast stromal cells in complete DMEM (Dulbeccos Modification of Eagles Medium (ThermoFisher) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and a 50 µg/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL neomycin cocktail (ThermoFisher). Cells were exposed over three days to SMV, RK, or combinations of RK+SMV (n=4) and viability was measured using CellTiter-Glo (Promega). RK (10 mg/ml) and SMV (1 mg/ml) stocks were dissolved in 200 proof ethanol and filtered using a 0.22 µm syringe filter. From these stocks, RK and SMV were serially diluted in culture medium to make RK+SMV combinations containing 0, 12.5, 25, 50, or 100 µg/mL RK with 0, 75, 150, 300, or 600 ng/mL SMV.
W-20-17 cells were seeded at the bottom of 96-well cell culture plates at 10,000 cells/well and allowed to attach overnight. Medium was then removed and replaced with 200 µL experimental media containing test RK+SMVMV concentrations. A control group was treated with complete DMEM without further supplementation. At 1 and 3 days after adding experimental media, the plates were tested for cell viability according to the Cell Titer Glo assay kit protocol, which uses ATP-dependent luciferase-oxyluciferin luminescence to determine a relative proportion of viable cells in culture. In brief, plates were removed from the incubator and allowed to equilibrate at room temperature for about 30 minutes. 100 µL media was removed from the wells to make the final well volume 100 µL before adding 100 µL Cell Titer Glo reagent to each well before incubating at room temperature for 10 minutes. Luminescence was measured using a BioTek Synergy H1 plate reader. The resulting data were used to evaluate concentration combinations of RK and SMV that were toxic to the cells by comparing viability of experimental groups to viability of the control group.

RK+SMV Mineralization Dosing
To reduce the total number of combinations that need to be tested for osteoinductive potential, the non-cytotoxic combinatory concentrations’ ability to induce alkaline phosphatase production was tested over 5 days (n=4). Combinations of 75, 150, 300, and 600 ng/mL SMV with 12.5, 25, 50, and 100 µg/mL RK were tested. SMV and RK stocks and media were created as previously described. W-20-17 cells were seeded at the bottom of 48-well cell culture plates in 200 µL DMEM at 30,000 cells/well and allowed to attach overnight. After 24 hours, complete DMEM was removed and replaced with 400 µL mineralization medium (complete DMEM supplemented with 50 µg/mL 2-phospho-L-ascorbic acid trisodium salt (MilliporeSigma) and 5 mM β-glycerophosphate disodium salt pentahydrate (MilliporeSigma) containing experimental RK and SMV concentrations. A negative control group was treated with only mineralization medium, while a positive control group was treated with mineralization medium further supplemented with 25 ng/mL BMP-2. Media were changed on day 3. At 3 and 5 days after adding experimental media, plates were prepped for DNA and ALP assays by removing the plate
from the incubator, removing all media from each well, washing each well with 200 µL sterile 1x PBS, adding 200 µL distilled deionized (DDI) water to each well, and freezing at -20 °C. The cells were lysed by freeze-thawing plates and shaking plates on a plate shaker while the cells were thawed. DNA was measured using the Picogreen DNA Kit. Twenty microliters cell lysate and 80 µL 1x TE buffer (10mM Tris-HCl, 1mM EDTA in DDI) was added to wells in an opaque 96-well plate. One hundred microliters Picogreen reagent was added to each well, and the plate was covered from light for 5 minutes before reading fluorescence at 498 nm transmission, 528 nm emission. Sample fluorescence was compared to a calibration curve created from DNA standards ranging from 0-2,000 ng/mL. ALP production was assessed using the QuantiChrom Alkaline Phosphatase Assay Kit. Briefly, 50 µL cell lysate and 150 µL working solution containing magnesium acetate and pNPP were added to clear 96-well plates and the resulting absorbance was read at 485 nm in 2-minute intervals for 6 minutes. ALP concentration was calculated using the formula supplied by the assay kit. ALP data were normalized to DNA content to account for variation in cell number in individual wells. All assays were measured using a BioTek Synergy H1 plate reader.

Osteoinductive Potential of RK+SMV Loaded in Medium

RK+SMV concentration combinations that did not cause an cytotoxic effect were further evaluated for the ability to induce osteoblast differentiation in vitro. RK and SMV stocks were created as previously described. W-20-17 cells were seeded at the bottom of 48-well cell culture plates in 200 µL DMEM at 30,000 cells/well and allowed to attach overnight. After approximately 48 hours, medium was replaced with 300 µL mineralization medium containing experimental RK and SMV concentrations. A negative control group was treated with only mineralization medium, while a positive control group was treated with mineralization medium further supplemented with 25 ng/mL BMP-2. Media was changed every 2-3 days. At 3, 7, 10, 14, and 21 days after adding experimental media, plates were prepped for DNA and ALP assays as previously described and the assays were performed as previously described.
Additional plates were prepared for OCN evaluation by immunohistochemistry. At days 14 and 21, cells were fixed in 4% ultrapure formalin for 20 minutes before washing three times with 1x PBS. Cells were permeabilized with a 0.1% NP-40 detergent solution before addition of the primary antibody. Rabbit anti-mouse osteocalcin/GLAP Antibody Picoband (BosterBio) was diluted at a 1:500 ratio, added to cells, and incubated for at least one hour. After incubation, cells were washed three more times with 1x PBS before adding the secondary antibody. Goat anti-rabbit AlexaFlour antibody (BosterBio) was diluted at a 1:100 ratio, added to cells, and incubated for at least 30 minutes. During the final 10 minutes of the incubation, a DAPI/anti-photobleaching solution was added to the cells. After incubation, cells were washed and stored in 1x PBS for approximately 24 hours before imaging on a confocal microscope (Nikon). Fluorescence between groups was compared to qualitatively determine relative osteocalcin production.

At days 14 and 21, additional plates were prepared for analysis of calcium deposition using an Alizarin Red stain (MPBio) and Calcium Assay Kit (Ponte Scientific). For the calcium assay (n=3), wells were washed with 200 μL sterile 1x PBS before adding 150 μL of 0.5 N glacial acetic acid and freezing at -20 °C. For analysis, 5μL of the calcium lysate was added to 250 μL calcium reagent containing o-cresolphthalein complexone and the resulting absorbance was read at 570 nm. For Alizarin Red stain (n=1), a 2% Alizarin Red stain solution was prepared by adding 2 g of Alizarin Red S in 100 mL DDI water and pH-adjusted to 4.2. Wells designated for the Alizarin Red stain were rinsed with 1x PBS and fixed with 3.7% formalin for 10-15 minutes. Wells were washed three times with 1x PBS before adding 100 μL of 2% Alizarin Red solution and incubating for 15 minutes. After incubation, the staining solution was removed, and wells were washed with DDI water until all unreacted staining solution is removed. Staining intensity between groups was observed on an OSTEOIMAGER scanning microscope (Bioquant) and qualitatively compared to determine calcium deposition.
Electrospinning Chitosan Membranes

Chitosan was electrospun into membranes as previously described [11], [12]. Briefly, chitosan (71% DDA, Primex) at 5.5% w/v in 70% v/v trifluoracetic acid (TFA) - 30% v/v dichloromethane was electrospun at 26 kV onto a 38 cm diameter collection plate approximately 15 cm away from the spinning needle. The collection plate was covered in non-stick aluminum foil and rotated at approximately 8 rpm for uniform fiber distribution. The resulting electrospun chitosan membranes (ESCM) were removed from the collection plate for post-spinning processing.

Modification and Treatments

As previously reported, ESCMs were treated to remove residual TFA salts from the spinning process via a fatty acid modification using hexanoic anhydride (HA) [11]. Briefly, ESCMs were punched into 1 cm diameter disks and immersed in a magnetically stirred solution consisting of 50% v/v pyridine and 50% v/v hexanoic anhydride at 5 mg/mL for approximately 1.5 hours. To remove excess pyridine/hexanoic anhydride, the resulting membranes were washed in 1 L magnetically stirred DDI water for 72 hours with water replacement every 24 hours. After 72 hours of washing, the membranes were removed from the water, placed on the interior walls of a glass beaker, placed in a -80°C freezer overnight, and lyophilized for 48 hours.

Membrane Characterization

Fourier transform infrared spectroscopy was performed on unmodified and HA modified ESCMs to verify the removal of signal peaks associated with TFA as well as the addition of methyl group peaks due to the HA-fatty acid modification. Scanning electron microscopy was used to visually verify the fiber size and structure of both untreated and treated ESCM.

Cytotoxicity Testing of RK+SMV Loaded Electrospun Chitosan Membranes

Based on cytotoxicity testing and previously established RK and SMV loaded ESCM elution profiles, ESCM-delivered RK+SMV cytotoxicity was investigated. Hexanoic anhydride modified ESCMs (HA-ESCM) were fabricated, treated, and characterized as previously described. Membranes were punched into 1 cm disks before HA treatment and gas sterilized using ethylene oxide.
Combinations of 100 µg RK with 25, 50 or 100 µg SMV were loaded onto ESCMs for cytotoxicity testing. To load the membranes with RK and SMV, 20 mg/mL stock solutions of each compound were made in 200 proof ethanol and filtered using a 0.22 µm syringe filter. A 10 mg/mL dilution of SMV was made by combining equal parts of 20 mg/mL stock and filtered 200 proof ethanol. The RK and SMV stocks were then combined at a 1:1 dilution and 10 µL of these stocks were then added directly onto HA-ESCMs. The HA-ESCMs were placed on the cross-section of a syringe plunger to minimize contact surface area and maximize stock absorption. Loaded HA-ESCMs were allowed to air-dry before collection and placement with cells. W-20-17 cells were seeded at the bottom of 24-well plates in 500 µL complete DMEM at 50,000 cells/well and allowed to attach. After approximately 24 hours, the RK+SMV loaded HA-ESCMs were placed in cell culture transwells (Corning Costar), the trans wells were placed in the 24-well plates, and an additional 200 µL complete DMEM was added into the trans well. Positive control groups included cells grown on TCP with no trans wells added as well as a trans well with unloaded HA-ESCMs. At 1, 3, or 7 days after adding the membranes, the plates were tested for cell viability according to the Cell Titer Glo assay kit protocol as previously described.

Osteoinductive Potential of RK+SMV Loaded onto Electrospun Chitosan Membranes

Based on previously established elution profiles, previously described studies on the mineralization capabilities of RK+SMV combinations, and cytotoxicity testing, the test combinations of RK+SMV were loaded onto gas sterilized sterile 1 cm diameter HA-ESCM. W-20-17 cells were seeded at the bottom of 24-well plates in 500 µL DMEM at 50,000 cells/well and allowed to attach. After approximately 24 hours, medium was replaced with 500µL mineralization medium. Membranes were loaded as previously described and introduced to the cells using 24-well cell culture transwells (Corning Costar). 200 µL of additional mineralization medium was added to the cell culture inserts on top of the membranes. A negative control group was treated with only mineralization medium and without a cell culture insert, while a positive control group was treated with mineralization medium loaded with 25 ng/mL BMP-2 and without a cell culture insert. Media was changed daily. At 3, 7, 10, 14, and 21 days
after adding experimental media, plates were prepped for DNA and ALP assays. At days 14 and 21, additional plates were prepped for OCN analysis and OCN immunohistochemistry. At days 14 and 21, additional plates were prepared for calcium analysis via Alizarin Red and Calcium Assay. All plates, assays, and analysis were performed as previously described.

**Statistical Analysis**

For DNA, ALP, and calcium data, ANOVA and Tukey HSD post-hoc testing was used to test for differences between groups. Statistical significance was set when \( p < 0.05 \).

**Results**

**RK+SMV Cytotoxicity Testing**

On day 1, significant reductions in cell viability were observed among many concentration combinations of RK+SMV, but no combination group resulted in a viability less than 75% of the TCP control group (Figure 1A). By day 3, several significant declines in cell viability were observed in combination groups. Most notably, all combination groups containing 600 ng/ml SMV had significantly lower viability than the TCP control, and all these groups exhibited viability of less than 50%. Similarly, all groups containing 300 ng/ml SMV had significantly lower viability compared to the TCP control, but the viability ranged from approximately 60-80% of the control (Figure 1B). It is also of note that the viability of combinations groups with 300 ng/ml and 600 ng/ml SMV decreased as RK concentration increased, although this effect was not significant.
Figure 1: Graph shows viability of W-20-17 cells exposed to different concentration combinations of RK+SMV loaded in cell culture medium after 1 (A) and 3 (B) days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)
RK+SMV Mineralization Dosing

Results of the induction of ALP by W-20-17 cells exposed RK+SMV combinations are shown in Figure 2. On day 3, only the 12 µg/ml RK+75 ng/ml SMV, 100 µg/ml RK+150 ng/ml SMV, and 600 ng/ml SMV produced significantly more ALP compared to the TCP negative control, and each of these groups produced ALP at levels comparable to the 25 ng/ml BMP-2 positive control (Figure 2). By day 5, only the 100 µg/ml RK, 75 ng/ml SMV, 100 µg/ml RK+75 ng/ml SMV, and 150 ng/ml SMV combinations showed an increased ALP production compared to the negative control, and of these only the 150ng/ml SMV group was comparable to the BMP-2 control (Figure 2). Although some combination groups had large standard deviations, no combination groups containing SMV at or above 300 ng/ml showed significantly different ALP production compared to the negative control.

Figure 2: Graph shows DNA-normalized alkaline phosphatase production of W-20-17 cells exposed to different concentration combinations of RK+SMV at 3 and 5 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)
Osteoinductive Potential of RK+SMV Loaded Media

In general, all combination groups and most individual RK or SMV groups yielded significantly less DNA compared to the TCP negative control up to the day 10 time point, with the only exception being 12 µg/ml RK. After day 10, only groups containing 100 µg/ml RK consistently yielded less DNA than the TCP control (Figure 3).

**Figure 3**: Graph shows DNA content of W-20-17 cells exposed to different concentration combinations of RK+SMV over 21 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)

Only the 25 ng/ml BMP-2 control significantly produced more ALP than the TCP control at all time points. The 150 ng/ml SMV and 12 µg/ml RK+150 ng/ml SMV produced significantly more ALP than the TCP control up to day 10. The 100 µg/ml RK+75 ng/ml SMV and 100 µg/ml RK+150ng/ml SMV groups appear to produce significantly more ALP than the negative control after 10 days in culture. Additionally, while both individual SMV groups upregulated ALP expression during at least one time point, neither individual RK group had significant effect on ALP expression at any time point (Figure 4).
Figure 4: Graph shows DNA-normalized ALP production of W-20-17 cells exposed to different concentration combinations of RK+SMV over 21 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)

Results of the immunostaining for osteocalcin are shown in Figure 5. The 25 ng/ml BMP-2 control group showed clusters of cells staining positively for osteocalcin. All other groups showed little to no positive staining for osteocalcin.
Figure 5: Microscopic images of W-20-17 cells exposed to TCP only (A, F), 25 ng/ml BMP-2 (B,G), 100 μg/ml RK (C,H), 75 ng/ml SMV (D,I), or 100 μg/ml RK+75 ng/ml SMV (E,J) and fluorescently stained for osteocalcin (green) and DAPI (blue) at 14 (A-E) and 21 (F-J) days (10x magnification, n=3).

Results from Ca assay showed that only the BMP-2 control deposited significantly more calcium than the negative control. At day 14, no experimental groups appear to deposit notable levels of calcium. By day 21, some SMV-containing experimental groups show increased calcium deposition compared to the negative control, but this increase is not statistically significant due to the large standard deviation (Figure 6).
**Figure 6:** Graph shows calcium deposition by W-20-17 cells exposed to different concentration combinations of RK+SMV over 21 days. Bars represent average ± standard deviation (n=4)

*Statistically significant difference compared to TCP control (p<0.05)*

The Alizarin Red staining results also showed minimal positive staining in the experimental RK+SMV groups compared to the BMP-2 control at both 14 and 21 days. However, compared to the negative control, the some SMV-containing groups appeared to have a slightly more intense red stain (Figure 7).
Figure 7: Microscopic images of W-20-17 cells exposed to TCP only (A, F), 25ng/ml BMP-2 (B,G), 12µg/ml RK (C,H), 75ng/ml SMV (D,I), or 12µg/ml RK+75ng/ml SMV (E,J) and stained with alizarin red for calcium deposition at 14 (A-E) and 21 (F-J) days (4x magnification, n=1)

SEM

SEM imaging confirmed the retention of the nanofibrous structure of the ESCM after hexanoic anhydride treatment. The HA-ESCM showed little fiber swelling after multiple washes in an aqueous solution (Figure 8).

Figure 8: Graph depicts SEM images of unmodified (A) and hexanoic anhydride modified (B) electrospun chitosan membranes (1000x magnification)

FTIR

FTIR data confirmed the removal of harmful TFA salts from the HA-ESCM following fatty-acid pyridine treatment. The peaks present around 700-800 cm⁻¹ correspond to TFA salts remaining in
unmodified ESCM, but these peaks are no longer present after the hexanoic anhydride treatment. The peaks observed around 2900-3000 cm\(^{-1}\) in the HA-ESCM group correspond to the increase in methyl groups associate with the fatty acid chain (Figure 7).

![FTIR data for unmodified and hexanoic anhydride modified electrospun chitosan membranes](image)

**Figure 9:** FTIR data for unmodified and hexanoic anhydride modified electrospun chitosan membranes (n=1)

Cytotoxicity Testing of RK+SMV Loaded Electrospun Chitosan Membranes.

Blank HA-ESCMs had no significant effect on cell viability compared to the TCP control. Membranes loaded with 100 µg RK did show statistically lower viability than the TCP group at days 1 and 3. Membranes loaded with 50 µg or 100 µg SMV individually and in combination with 100 µg RK displayed significantly lowered viability compared to the TCP control after 3 days in culture and changing the culture medium every 2-3 days. By day 7, all these groups demonstrated significant cytotoxicity to W-20-17 cells, with individual SMV membranes having less than 60% cell viability and RK+SMV combination membranes have less than 10% viability (Figure 10A). When changing the culture medium daily, the SMV becomes noticeably less cytotoxic. Membranes loaded with 50 µg SMV displayed over 85% viability compared to the TCP control, and there was notably less error in most
membrane groups. Additionally, membranes loaded with 100 µg RK+25 µg SMV had statistically similar viability compared to the TCP control after 3 days in culture (Figure 10B).

**Figure 10:** Graph shows viability of W-20-17 cells exposed to RK+SMV loaded HA-ESCMs over 7 or 3 days with media changed every 2-3 days (A) or daily (B). Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)
At day 3 and day 7, the RK+SMV membrane groups resulted in significantly less DNA content compared to the TCP control. The BMP-2 group contained similar DNA content to the TCP control at days 3, 7 and 21, but significantly lower DNA content at days 10 and 14. Except for the day 21 time point, only the 100 µg RK+100 µg SMV group regularly appears to be cytotoxic. The 100 µg RK+25 µg SMV and 100 µg RK+50 µg SMV groups appear to be slightly antiproliferative during the first 7 days, but for later time points are no longer significantly different from the TCP control (Figure 11). By day 21, the 100 µg RK+50 µg SMV and 100 µg RK+100 µg SMV groups have increased DNA content, but this increase is not statistically different from any other group.

**Figure 11**: Graph shows DNA content of W-20-17 cells exposed to RK+SMV loaded HA-ESCMs over 21 days (n=4) *Statistically significant difference from TCP control. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)

At day 3, 7, 10, and 14, only the BMP-2 group produced significantly more ALP compared to the TCP control. At day 14 and 21, only the 100 µg RK+100 µg SMV group showed significantly lower
ALP production compared to the TCP group. By day 21, there was no detectable difference in ALP production between any groups (Figure 12).

![Graph showing DNA normalized ALP production of W-20-17 cells exposed to RK+SMV loaded HA-ESCMs over 21 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)]

**Figure 12:** Graph shows DNA normalized ALP production of W-20-17 cells exposed to RK+SMV loaded HA-ESCMs over 21 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)

On day 14, minimal osteocalcin staining was observed at any group. By day 21, all groups appeared to have increased OCN production compared to day 14, but the BMP-2, 100 µg RK+25 µg SMV, and 100 µg RK+50 µg SMV groups exhibited consistently increased staining intensity and frequency compared to the TCP control, with the BMP-2 groups exhibiting the most staining (Figure 13).
Figure 13: Figure depicts representative images of W-20-17 cells exposed to TCP only (A, F), 25 ng/ml BMP-2 (B,G), 100 µg RK+25 µg SMV (C,H), 100 µg RK+100 µg SMV (D,I), or 100 µg RK+100 µg SMV (E, J) and fluorescently stained for osteocalcin (green) and DAPI (blue) at 14 (A-E) and 21 (F-J) days (10x magnification, n=4).

Similar to ALP production, only the BMP-2 groups showed significantly higher calcium deposition at day 14 and 21 compared to the TCP control. At day 21, the 100 µg RK+50 µg SMV and 100 µg RK+100 µg SMV groups showed significantly lower calcium deposition compared to the TCP control, whereas the 100 µg RK+25 µg SMV showed no difference (Figure 14).
**Figure 14:** Graph shows calcium deposition by W-20-17 cells exposed to RK+SMV loaded HA-ESCMs over 21 days. Bars represent average ± standard deviation (n=4) *Statistically significant difference compared to TCP control (p<0.05)

At both days 14 and 21, the Alizarin Red stain showed some positive calcium staining in the TCP, 100 µg RK+25 µg SMV, and 100 µg RK+50 µg SMV groups, although the BMP-2 group had much more intense staining. The 100 µg RK+100 µg SMV appeared to show minimal staining at both time points (Figure 15).
**Figure 15:** Figure depicts full-well images of W-20-17 cells exposed TCP only (A, F), 25 ng/ml BMP-2 (B,G), 100 µg RK+25 µg SMV (C,H), 100 µg RK+100 µg SMV (D,I), or 100 µg RK+100 µg SMV (E,J) and stained with alizarin red for calcium deposition at 14 (A-E) and 21 (F-J) days (4x magnification, n=1)

**Discussion**

GBR membranes traditionally act as a physical barrier to isolate a bone defect site from soft tissue infiltration and passively aid in bone regeneration during GBR therapy. Fatty-acid modified electrospun chitosan membranes have been investigated as a potential improvement to clinical GBR membranes due to their desirable biocompatibility, degradation rate, and nanofibrous structure that prevents cell infiltration but allows for drug loading and signal diffusion across the GBR site. RK is a natural phenol that has been investigated for innate immunomodulatory and anti-obesity properties but also has been shown to have osteogenic properties under certain conditions [18]. Previous studies showed hexanoic anhydride modified ESCMs have an initial burst release of RK and then continue to release RK for up to 14 days, although the RK elution had minimal effect on *in vitro* osteodifferentiation [24]. SMV is traditionally a lipid-lowering drug that has been shown to have pleotropic osteogenic properties [21]. Previous studies have shown that hexanoic anhydride modified ESCMs release SMV in a sustained pattern for at least 30 days [25]. The combination, loading, and delivery of these two drugs
with multimodal actions from modified electrospun chitosan membranes may result in a bioactive GBR membrane that will more actively contribute to and enhance GBR wound healing.

While some combinations of RK+SMV lowered cell viability at day 1, cytotoxicity was not observed until day 3. The significant differences in viability observed on day 1 are most likely due to small variations in cell seeding or proliferation. Additionally, 3-day viability data indicates that constant exposure to SMV concentrations at and above 300ng/ml SMV are cytotoxic to W-20-17 cells, and that combining RK+SMV may have a combinative cytotoxic effect compared to equivalent individual groups. According to ISO standard 10993-5, a substance is considered cytotoxic if it causes the reduction of cell viability by 30% or more [26]. Based on this definition, 600 ng/ml SMV alone and 300 ng/ml SMV in combination with some RK concentrations are cytotoxic and any biomaterial delivery system should aim to keep chronic exposure of RK+SMV concentrations below these levels. However, lower concentrations of SMV have been reported to be angiogenic as well as osteogenic, so dosing and delivery of SMV must be carefully balanced to achieve the greatest osteogenic response [21],[27].

In one study, 4.2 ng/mL (10nM) SMV reduced peroxide-induced apoptosis and increased vascularization in MSCs and a mouse hindlimb ischemia model [27]. Since no RK+SMV combination tested showed a viability reduction larger than 30% after 24 hours, it is possible that brief exposure to higher concentrations of SMV could begin an osteogenic response without harmful effect on long-term proliferation and differentiation. Additionally, W-20-17 cells may be more sensitive to SMV cytotoxicity than other cell lines. While W-20-17 cells exhibited cytotoxicity at 600 ng/ml SMV, SMV IC\textsubscript{50} values for MCF-7 and MDA-MB-231 human breast cancer cell lines were reported to be 3.7 µg/mL (8.9 µM) and 1.9 µg/mL (4.5 µM), respectively [28]. SMV exposed to primary human skeletal muscle cells was had an IC\textsubscript{50} of around 21 µg/mL (50 µM) [29]. The differences in reported cytotoxicity may be attributed to the sensitivities of different cell lines, levels of protein binding, or type of SMV used. SMV binding to plasma proteins, which has been reported to be 95-98%, would reduce concentration of SMV.
exposed to the cells below cytotoxic amounts [29]. Simvastatin lactone, the prodrug that is bioconverted in situ, has been reported to be 37 times more potent at inducing cytotoxicity of muscle cells compared to its active form, simvastatin acid [29].

RK has also been reported for cytotoxicity at different concentrations for different cell lines. While W-20-17 cells have exhibited cytotoxic effects above 100 μg/mL (608 μM) RK, normal human melanocytes, A375 human melanoma cells, HaCaT human keratinocytes, and HS68 human fibroblasts have reported RK IC₅₀ values of 230 μg/mL (1.4 mM), 130 μg/mL (790 μM), 1.59 mg/mL (9.71 mM), and 1.29 mg/mL (7.83 mM) RK, respectively [30].

RK+SMV combinations in media produced ALP levels similar to rhBMP-2 at day 3, but reduced levels by day 5, indicating that combination groups may have enhanced very early osteodifferentiation, but not for a prolonged duration. However, in the mineralization study no group produced ALP at levels similar to the BMP-2 control at the day 3 time point or later time points. This agrees with previous studies, in which W-20-17 cells treated with 600 ng/ml SMV over 21 days had increased ALP production compared to blank medium, but not compared to BMP-2 [25]. Similarly, RK has been reported to enhance ALP production in combination with other osteogenic factors such as BMP-2 or all-trans-retinoic acid (ATRA) but causes no significant changes to ALP expression alone [18]. While W-20-17 ALP expression in response to rhBMP-2 is well documented, terminal differentiation behavior of the cell line is not commonly reported [31]. It is possible that W-20-17 cells are a good model for rhBMP-2 mediated differentiation, but not with respect to RK and SMV stimulation.

RK+SMV combinations in media did not exhibit mineralization capabilities comparable to BMP-2 at any time point, but at day 21 some SMV containing combinations exhibited non-significantly elevated calcium deposition. These results are similar to other studies in which only medium containing 600ng/ml SMV induced increased calcium deposition compared to blank medium. There are currently no published results evaluating mineralization capabilities of RK-loaded media, but based on the results
of this study, continuous exposure to high concentrations of RK over 21 days does not have a significant effect on mineralization.

RK+SMV loaded ESCMs resulted in significant cytotoxicity when media was not changed daily. Since blank HA-ESCMs and membranes loaded with 100 µg RK exhibited no cytotoxic effect, the cytotoxicity seen in combination groups was likely caused by a high concentration of SMV released by the membranes. Both changing media daily and loading with less SMV resulted in minimal cytotoxicity after 3 days. Significant differences in viability with daily media change is likely not indicative of cytotoxicity, as cell viability remains above 75% in all groups. Based on previously reported elution profiles, HA-ESCMs loaded with 50 and 100 µg SMV had release profiles in 1x PBS of less than 5 µg/mL SMV in the first 24 hours and approximately 1 µg/mL per day in the following 1-4 weeks [25]. While these SMV concentrations were above the cytotoxic levels, they were not cytotoxic when exposed to W-20-17 cells in culture, likely due to SMV binding of FBS proteins in culture medium and reducing the effective SMV concentration below cytotoxic levels [25]. HA-ESCMs loaded with 100 µg RK released 70% of total RK delivered within the first 3 days, with the remaining 30% delivered over 11 days in 1x PBS [24]. The difference in cytotoxic response of RK+SMV delivered from ESCMs observed in this study could be attributed to altered elution kinetics or competitive protein binding from dual delivery of RK+SMV at early time points. Unpublished RK+SMV elution data have shown that, when loaded onto ESCMs in combination with RK, about 7µg (14 µg/mL) SMV is released within the first 24 hours, compared to 1-5µg (2-10 µg/mL) SMV when loaded individually. Dual-loading potentially reduced hydrophobic SMV interactions with the ESCMs, resulting in higher SMV release into the medium and thus increased cytotoxicity.

RK+SMV loaded ESCMs did not induce higher ALP production or calcium deposition compared to the negative control at any time point throughout the mineralization study. These results generally agree with previous ESCM mineralization studies in which HA-ESCMs loaded with 50 µg
SMV produced minimal effect on ALP production or calcium deposition in W-20-17 cells [25]. Additionally, butyric anhydride modified ESCMs loaded with 250 µg SMV stimulated similar bone volume to unloaded membranes in a rat calvarial defect model [23]. However, in another rat calvarial defect model, HA-ESCMs loaded with 50 µg SMV stimulated significantly more bone volume compared to blank membranes after 8 weeks [11]. HA-ESCMs loaded with 100 µg RK resulted in minimal effect on ALP production or calcium deposition in W-20-17 cells, although this could be due to the burst release kinetics in which RK is not maintained at therapeutically relevant concentrations for the duration of the study [24]. It is more likely that RK exerts effects on bone healing through macrophage polarization. In a rat calvarial study, HA-ESCMs loaded with 100 µg RK resulted in increased transition from M1 to M2 phenotype and a subsequent increase in bone regeneration after 4 weeks [20]. Since RK and SMV delivered from HA-ESCMs have been separately shown to enhance bone regeneration in vivo while having more subtle effects on in vitro osteodifferentiation, it is possible that the RK+SMV ESCM conditions tested in vitro did not appropriately model in situ conditions.

Since both RK and SMV have been reported to have multiple metabolic effects related to wound healing and bone regeneration, the ideal concentrations for each compound for use in GBR is unknown, and as such, the appropriate loading and delivery mechanisms of each compound cannot be known. Since RK exerts effects on macrophage polarization, the burst release from ESCMs may be more beneficial than sustained release, as the inflammatory phase of bone healing is largest immediately after injury. Since SMV directly stimulates osteodifferentiation, a more sustained release may be desirable throughout the bone healing timeline. Investigating different release profiles, bone cell lines, ESCM modification, and drug loading may lead to a more complete understanding of the osteogenic potential of RK+SMV loaded ESCMs.
Conclusion

Raspberry ketone and simvastatin have shown promise for use in wound healing and bone regeneration, and combining the two compounds has also shown some potential for use in guided bone regeneration therapy. Electrospun chitosan membranes were fabricated and modified with hexanoic anhydride-pyridine solution to stabilize the nanofibrous structure, which imparted hydrophobicity onto the membranes and allowed for dual-loading of RK+SMV solutions using absolute ethanol. There was a dose-dependent reduction in cell viability when exposed to RK+SMV combinations versus the equivalent RK or SMV concentrations alone, suggesting a slightly increased cytotoxic effect when using the two compounds in combination. RK+SMV concentration combinations loaded into media resulted in increased ALP production at lower doses, but decreased cell viability and ALP production at higher concentration combinations. However, the increased ALP production did not translate to increased bone cell differentiation, potentially due to the combinatorial cytotoxic effects being exacerbated when cells were exposed to continuous RK+SMV combinations over multiple weeks. Similarly, RK+SMV loaded membranes did not enhance bone cell differentiation, and when loaded with higher concentrations of SMV, resulted in decreased bone cell differentiation, likely due to cytotoxic levels of SMV being released from the membranes for several days. Although the full effects of RK+SMV loaded electrospun chitosan membranes require further investigation and development, they have shown some potential for use in bone regeneration.
References


Chapter 3: Conclusions

Raspberry ketone and simvastatin have shown promise for use in wound healing and bone regeneration, and combining the two compounds may have some potential for use in guided bone regeneration therapy. Electrospun chitosan membranes were fabricated and modified with hexanoic anhydride-pyridine solution to impart hydrophobicity and allow for dual-loading of RK+SMV solutions using absolute ethanol. There was a dose-dependent reduction in cell viability when exposed to RK+SMV combinations versus the equivalent RK or SMV concentrations alone, suggesting a slightly increased cytotoxic effect when using the two compounds in combination. RK+SMV concentration combinations loaded into media resulted in increased ALP production at lower doses, but decreased cell viability and ALP production at higher concentration combinations. However, the increased ALP production did not translate to increased bone cell differentiation, potentially due to the combinatorial cytotoxic effects being exacerbated when cells were exposed to continuous RK+SMV combinations over multiple weeks. Similarly, RK+SMV loaded membranes did not enhance bone cell differentiation, and when loaded with higher concentrations of SMV, resulted in decreased bone cell differentiation, likely due to cytotoxic levels of SMV being released from the membranes for several days. Although the full effects of RK+SMV loaded electrospun chitosan membranes require further investigation and development, they have shown some potential for use in bone regeneration.
Chapter 4: Future Work

Additional studies are needed to further explore lower levels of RK+SMV combination to induce osteodifferentiation. Since RK appeared to have minimal effect on differentiation of preosteoblasts, studies need to be performed on cells at other stages in the osteoblast differentiation pathway to determine if RK effects osteogenesis at earlier stages. W-20-17 cells have a well-established ALP response to rhBMP-2, but more terminal differentiation markers are not as well studied, so other preosteoblast cell lines could have a different response to RK+SMV combinations.

Unpublished data from RK+SMV elution studies in our lab have shown that, when loaded onto ESCMs in combination with RK, about 7µg SMV is released within the first 24 hours, compared to 1-5µg SMV when loaded individually. Furthermore, the SMV release profile in the first few days was not dependent on initial SMV loading concentration. Further RK+SMV elution testing may be beneficial in determining the effect of dual-loading the drugs onto ESCMs and the corresponding changes in the drug-membrane interactions.

The mechanism of action of RK on osteoblast differentiation, or lack thereof, needs to be determined. The presented results show that RK has minimal direct effect on preosteoblast differentiation, but unpublished data from our lab show that RK-loaded ESCMs have a positive effect on bone regeneration in vivo in rat calvaria defects. It is possible that RK indirectly contributes to bone regeneration through effects on precursor cells or inflammation modulation.

In the broader context of wound healing, the potential multimodal effects of RK and SMV need to be further studied. In addition to their osteogenic effects, these compounds have been investigated for their effects on angiogenesis, adipogenesis, and inflammation. If relevant, these additional biological effects may impact the osteogenic ability of RK and SMV and the effectiveness of membranes loaded with these compounds to enhance bone regeneration. Furthermore, if these multimodal actions are observed in co-culture or in vivo models, the dosing and elution properties of RK+SMV loaded
membranes will need to be further studied to maximize wound healing response in addition to bone regeneration.
References


