LOCALLY DELIVERED ADENOSINE AND GLUTATHIONE IMPROVE FIBROBLAST PROLIFERATION AND COLLAGEN PRODUCTION

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ABSTRACT—Diabetic foot ulcers are particularly difficult to treat due to neuropathy and healing deficiencies. Glutathione and adenosine have been studied as possible modulators of diabetic response and would healing. The objective of this *in vitro* study was to measure the effects of adenosine and glutathione on fibroblast proliferation and collagen synthesis. Further, methods were explored for delivering these compounds locally through chitosan sponges. Elution concentrations of adenosine and glutathione from chitosan sponges were evaluated through HPLC, and activity was evaluated for fibroblast proliferation and viability *in vitro*. Furthermore, collagen production of normal and hyperglycemic fibroblasts exposed to those compounds individually and in combination was evaluated using a colorimetric collagen assay. Results indicate a dose-dependent increase in fibroblast proliferation, with exposure to concentrations up to 133.6 μg/ml adenosine and 76.8 μg/ml glutathione. Total collagen concentration determination confirmed that adenosine alone at 133.6 μg/ml significantly increases collagen production in low glucose conditions, but not to the same extent in medium and diabetic glucose conditions. Glutathione at 76.8 μg/ml did not increase collagen production compared to control or adenosine alone, and the combination attenuated increases in collagen production in low glucose conditions. There were no additive or synergistic effects of combining these two small molecule mediators of growth on fibroblast growth or collagen production. Both adenosine and glutathione were released in a burst response from chitosan sponges at concentrations of 473±62 μM and 35±14 μM after 24 hours respectively. Adenosine eluates

increased cellular proliferation by 68±15% and 26±5% after 24 and 48 hours of elution, respectively. Glutathione eluates increased proliferation by 17±9% and 18±14, after 48 and 72 hours of elution, respectively. These preliminary *in vitro* evaluations demonstrate that the local delivery of adenosine and glutathione released from chitosan sponges may be a promising strategy for the treatment of diabetic foot ulcers under glucose conditions that are closely managed.

Keywords—chitosan, diabetes mellitus, diabetic foot ulcer, adenosine, glutathione, wound healing

1. Introduction

Diabetes mellitus affects approximately 20.8 million people annually in the United States alone (Wu et al. 2007; Singh, Armstrong, and Lipsky 2005). Nearly 15% of these diabetic patients will suffer from a diabetic foot ulcer with an estimated 20% of foot ulcers ending in amputation (Wu et al. 2007; Wu, Crews, and Armstrong 2005; Omar, El-Nahas, and Gray 2008; Lavery et al. 2006). For patients who suffer an amputation, the five-year survival rate is approximately 31% (Faglia et al. 2006; Brem, Sheehan, and Boulton 2004). Annual costs of healthcare for diabetic foot ulcer complications can be as high as $10.9 billion (Rathur and Boulton 2007; Shearer et al. 2003). Difficulties in treatment also arise due to the neuropathy and insufficient vascularization associated with the disease (Lippmann, Perotto, and Farrar 1976). Reduction of the fatty pad, in combination with a decreased perception of pain, may also impair weight-bearing, thereby causing excessive stresses on the foot and eventually ulceration or further injury to a previous ulcer (Lippmann, Perotto, and Farrar 1976; Bennett et al. 2003). These complications and a reduction in the presence of important natural growth promoting compounds due to impaired vascularization, such as vascular epithelial growth factors, platelet derived growth factors, adenosine, and glutathione, cause the healing of diabetic foot ulcers to be impaired (Kopal et al. 2007; Mudge et al. 2002; Papanas and Maltezos 2008; Boulton 2004).

The presence of hyperglycemia also increases the likelihood that an individual will develop a foot ulceration, due to impaired cellular functionality (Deveci et al. 2005; Lerman et al. 2003). Diabetic patients also suffer from poor vascularization, especially in the lower extremities (Omar, El-Nahas, and Gray 2008; Shearer et al. 2003; Veves and King 2001). This pathology leads to an inability for the proper growth factors and other growth promoting compounds to be transported to the wound site to assist in healing (Omar, El-Nahas, and Gray 2008; Shearer et al. 2003). As the disease progresses, these complications may lead to a loss of integrity of the fatty pad and skin on the bottom of the foot (Shearer et al. 2003; Lipsky et al. 2004). Successful healing requires adequate circulation, nutrition, oxygen level, immune response, and mechanical stress (Liu and Velazquez 2008). During wound healing, fibroblasts are responsible for wound repair activities throughout the sequence of overlapping inflammatory, proliferative, and remodeling phases of wound healing. During the initial stages of healing fibroblasts proliferate and migrate into the provisional wound matrix, depositing collagen, secreting growth factors, causing wound contraction, and signaling the migration of other important cells, such as keratinocytes and macrophages (Brem et al. 2008). Without the migration of these other important cells, the growth factors and cytokines they produce will not be available to maintain the healing process (Brem et al. 2008). In diabetic patients, fibroblast function is impaired due to hyperglycemic conditions, lack of nutrients, and oxidative damage (Deveci et al. 2005). Therefore, many new therapies are directed to target stimulation of fibroblasts at the wound site in diabetic foot ulcers in order to improve healing rate and quality.

Adenosine is a purine nucleoside found in the DNA of almost all living organisms and serves as a building block for adenosine triphosphate (ATP), the energy source for cellular metabolism (Ohana et al. 2001; Abbracchio 1996). In the body it also acts at the A_1 and A_{2A} adenosine receptor sites to

promote wound healing and is generated at increased rates as a metabolic regulator during periods of high stress due to injury (Ohana et al. 2001). Several *in vitro* studies have found adenosine has a positive impact on fibroblast proliferation and wound healing (Valls, Cronstein, and Montesinos 2009; Montesinos et al. 2002). However, in diabetic patients, adenosine is not readily available because of inadequate vascularization in the lower extremities (Valls, Cronstein, and Montesinos 2009). *In vitro* studies have also found that adenosine is angiogenic, since it stimulates endothelial secretion of vascular endothelial growth factor, VEGF (Montesinos et al. 2002).

Glutathione is a tripeptide and a strong nucleophile that protects the cells in the body against oxidative damage when in the reduced form (Kopal et al. 2007; Boulton 2004; Rice et al. 1986; Marcelo et al. 2001). In diabetic patients there is an insufficient amount of reduced glutathione present to maintain the proper redox potential within the cells, which delays wound healing in diabetics (Mudge et al. 2002; Lavery et al. 2006). Several clinical studies have found that reduced glutathione is significantly decreased in diabetic patients (Kopal et al. 2007; Mudge et al. 2002). Previous studies have also shown that replacing the glutathione in diabetic models promotes wound healing by restoring the fibroblasts to their natural biological function (Mudge et al. 2002).

Adenosine and glutathione have very short half-lives in vivo of less than 10 seconds and 10 minutes, respectively (Moser, Schrader, and Deussen 1989; Klabunde 1983; Hong et al. 2005). In diabetic wounds poor vascularization also inhibits most intravenous drugs from reaching the wound site. Therefore, a local drug delivery system could achieve a more effective delivery of the adenosine and glutathione at therapeutically relevant local concentrations to improve wound healing (Noel et al. 2008; Roeder, Van Gils, and Maling 2000; Gentry 1997). In the diabetic foot ulcer, it is especially important to maintain therapeutically active levels of compounds from the local delivery vehicle for at least a three day time period, since the foot ulcer dressings are changed on a three to seven day cycle (Brem, Sheehan, and Boulton 2004). The proposed drug delivery mechanism must also allow for controlled drug loading and release (Brem et al. 2008; Lippmann, Perotto, and Farrar 1976; Faglia et al. 2006; Rees et al. 1995).

The natural polysaccharide biopolymer chitosan has advantages for local drug delivery to diabetic foot ulcers because of its biocompatibility and ability to be customized (Lu, Gao, and Gu 2008; Yilmaz 2004). Constructs can also be engineered as gel, bead, film, and sponge constructs which may be easily applied as a moist wound dressing (Noel et al. 2008; Montesinos et al. 2002; Brem, Sheehan, and Boulton 2004; Leffler and Muller 2000). A sponge construct was chosen for this study for its potential ability to achieve the targeted elution of adenosine and glutathione for local drug delivery to a diabetic wound site.

In this study, we hypothesized that two compounds, adenosine and glutathione, may be used to enhance fibroblastic response in a diabetic *in vitro* model. The purpose of this study was to investigate the co-delivery of both adenosine and glutathione from a chitosan-based local drug delivery system for their ability to enhance fibroblast proliferation and collagen production. Since adenosine promotes fibroblast proliferation and is an anti-inflammatory compound, it could be used in conjunction with glutathione to remove the source of

damaging oxidative species, allowing glutathione to bring the environment back to the proper redox state, and promote fibroblast proliferation and tissue repair. The sponge construct design of the system would allow for direct application of the treatment at the wound while still allowing for a moist wound dressing, thereby minimizing the risk of systemic toxicity while integrating with current diabetic wound dressing methods. The combination of the two compounds could have the additive benefits of decreasing the oxidative stress within the wound while improving the fibroblastic response, which could potentially decrease the recurrence of ulceration by improving the rate and quality of wound healing.

2. Materials and Methods

Adenosine was obtained from MP Biomedicals (Santa Ana, CA) and glutathione was obtained from Sigma-Aldrich (St. Louis, MO). Normal human dermal fibroblasts (NHDF) were purchased from (Cambrex BioScience; Walkersville, MD) and polystyrene tissue culture plates were purchased from (BD Falcon; Franklin Lake, NJ)., Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT) and Normocin^{TM} antibiotic was obtained from (Invivogen). Cell Titer-Glo Luminescence Cell Viability Assay was used (Promega Corp.; Madison, WI) with a 96 well plate reader (Bio-Tek Inst. Inc., Model No. FLx800; Ontario, Canada) and analyzed with data analysis software (kinecticalc KC4$^{®}$v3.4Rev.16; Ontario, Canada). Chondrex Sirius Red Total Collagen Assays (Chondrex; Redmond, WA) and Quant-iT PicoGreen kit (Invitrogen; Carlsbad, CA)were obtained and analyzed on a spectrophotometer (Biotek Synergy H1; Winooski, VT). Chitosan with degree of deacetylation (DDA) of 71% was purchased from Primex (Siglufjordur, Iceland), while lactic and acetic acid and a LabConco lyophilizer were purchased from Fischer Scientific (Pittsburgh, PA). High-Pressure Liquid Chromatography (HPLC) was a Varian Prostar (Palo Alto, CA) with data analysis using Galaxie Software. Glutathione assay kits were purchased from Sigma-Aldrich.

2.1. Seeding and analysis of cellular proliferation

Normal human dermal fibroblasts (NHDF) were plated at $1x10^4$ cells per cm^2 in 96 well polystyrene tissue culture plates to evaluate cell proliferation. NHDF cells were used from passages 3 through 8 and suspended in 25 mM glucose DMEM supplemented with 10% FBS and 100 μg/ml of the antimicrobial Normocin^{TM} for initial evaluation and proliferation. All fibroblasts in this proliferation study were seeded and allowed to proliferate in standard culture conditions (37^oC, 5% CO_2 atmosphere) overnight (n=4). Fibroblasts were then serum starved for 24 hours and then exposed to two-fold dilutions of either 2000 μM adenosine or 2000 μM glutathione, additionally a control group was similarly prepared with untreated fibroblasts. Fibroblast proliferation was analyzed after 24 hours of exposure to adenosine or glutathione solutions using the Cell Titer-Glo Luminescence Cell Viability Assay. Briefly, cells were exposed to the compound for 24 hours to elicit a response, and then culture media was removed from all wells and replaced with 100 μl of serum-free DMEM and an equal volume of reconstituted Cell Titer-Glo reagent solution. The luminescence signal corresponding to the amount of the metabolic molecule adenosine triphosphate (ATP) present was obtained and the emission was recorded using a 96-well plate

reader at 590 nm in conjunction with data analysis computer software. Results were reported as mean percentage luminescence compared to non-treated controls ± standard deviation. To calculate the percent luminescence, relative luminescence units (RLU) of the treated group was divided by the RLU value of the control group using Equation 1.

$$\frac{RLU\ treated\ group}{RLU\ control\ group} * 100 = \%\ luminescence$$

Equation 1

Preliminary testing confirmed that free base adenosine in solution did not contribute to noise in luminescence readings, since ATP is required to catalyze the luciferase reaction.

2.2. Seeding and analysis of fibroblast collagen production

To evaluate the effect of adenosine or glutathione on collagen production, fibroblasts were plated at $1x10^6$ cells per cm^2 in 24-well polystyrene tissue culture plates from passages 3 through 8. Fibroblasts were suspended in media containing varying concentrations of D-glucose to represent different degrees of diabetic and normal glucose conditions, with 5mM and 25mM being considered low and high normal glucose conditions, and 45mM glucose being considered diabetic cellular glucose levels based on a study by Deveci (Deveci et al. 2005). All media was supplemented with 10% FBS and 100 μg/ml of the antimicrobial Normocin™. Once plated, fibroblasts were allowed to grow to near confluence for 3 days and then exposed to 500 μM adenosine, 250 μM glutathione, or a combination of both over a 14 day time period (n=3).

Comparison collagen production evaluation of hyperglycemic fibroblasts versus normal fibroblasts was determined after the exposure experiments using the colorimetric Chondrex Sirius Red Total Collagen Assay using standard protocols. Absorbance measurements were taken at 530 nm using a spectrophotometer. Samples were compared to collagen Type I reference standards. DNA was also quantified for each sample after exposure experiments using the Quant-iT PicoGreen kit following standard protocols. Results of the collagen comparison evaluation were normalized to dsDNA and reported as amounts of collagen/dsDNA ([μg/ml]/[ng/ml]).

2.3. Chitosan sponge fabrication

Chitosan with 71% degree of deacetylation (DDA) was measured to 2.5g and dissolved in a 1% (v/v) acid solution (75/25 lactic/acetic acid). The chitosan sponges were cast in 5 cm diameter aluminum weigh dishes, placed in a -80°C freezer for 24 hours, and lyophilized in a LabConco freeze dryer. After 45 – 50 hours, the sponges were removed from the freeze dryer and neutralized by immersing in 0.5 M NaOH followed by washing in distilled water. Neutralized, saturated sponges were then placed back into the weigh dishes, re-frozen at -80°C for 12 hours, and re-lyophilized for 18 – 24 hours. Sponge constructs were packaged in sterilization pouches and sterilized using low-dose 25 kG gamma irradiation.

2.4. Elution study

Sterilized chitosan sponges (approximately 38mm diameter x 3mm thickness) were loaded by immersion in approximately 10 ml of 1000μM (267.2 μg/ml) adenosine or 500μM (153.7 μg/ml) glutathione for 1 minute. Immediately after uptake five replicates (n=5) of adenosine-loaded and four replicates (n=4) of glutathione-loaded sponges were completely submerged in 15 ml sterile filtered phosphate buffer saline (PBS) in sterile Nalgene containers. The loading concentrations used for the treatment solution were increased by a factor of two compared to optimal dose concentrations found in the preliminary cell proliferation studies. This two-fold increase was intended to account for the dilution of the compounds when submerged in PBS during elution as well as to allow for continued release of viable concentration over a three-day elution study. 1 ml samples were taken at 24-hour intervals (24, 48, and 72 hour sample time points). PBS was completely replaced after each sampling. All chitosan sponge samples were normalized to initial dry weight by dividing the detected adenosine or glutathione concentration by the initial dry weight of the associated chitosan sponge. Concentrations of adenosine in eluate solutions were determined using an isocratic HPLC technique adapted from Porkka-Heisanen *et al.* (Porkka-Heiskanen, Strecker, and McCarley 2000). Briefly, adenosine elution samples were injected into a Varian Microsorb-MV 100-5 C18 150 × 4.6 mm reverse phase column in a mobile phase of 0.01M dipotassium phosphate in 10% methanol adjusted to a pH of 4 using phosphoric acid and sodium hydroxide. Peaks of adenosine occurring at 8.25 minutes were detected using a Varian Prostar detector and chromatograms at a wavelength of 257 nm were obtained (Figure 1). Quantity was determined by area integration of the adenosine peak and comparing to a standard curve.

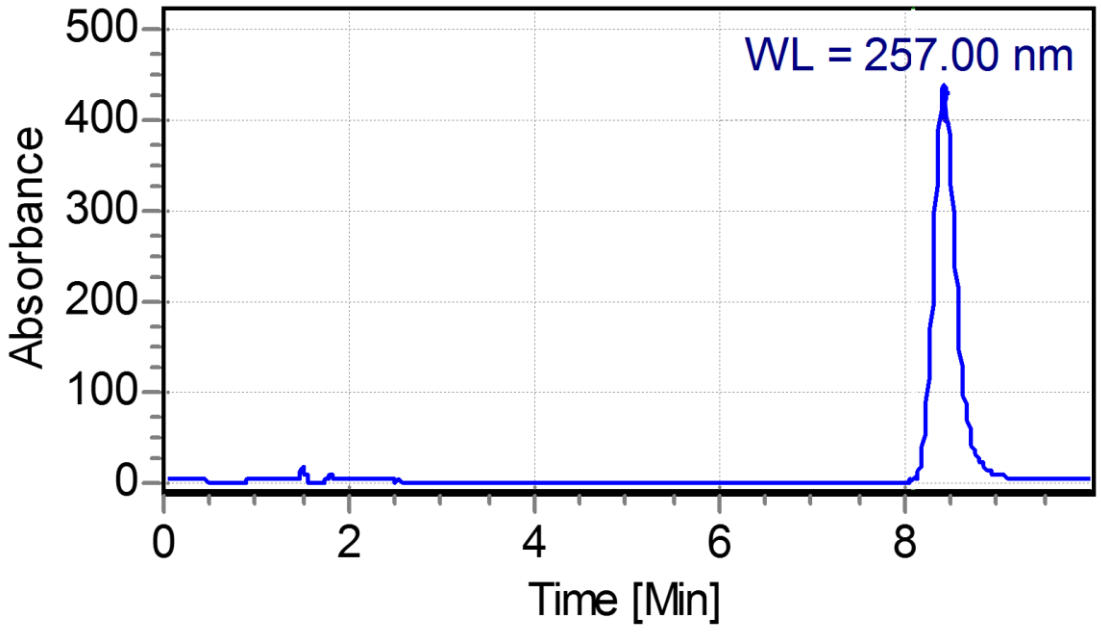

Figure 1. Representative HPLC chromatograph of adenosine showing absorbance units over time with peak adenosine concentration measured at 8.25 min.

Concentrations of glutathione were determined using a Glutathione assay kit. Briefly, monochlorobimane, a thiol probe, was used to bind to reduced glutathione. This reaction, catalyzed by glutathione-S-transferase, can be used to detect the amount of reduced glutathione present by measuring fluorescence.

The effect of eluates on proliferation of fibroblasts was evaluated using the Cell Titer-Glo assay as a measure of biologic activity of adenosine or glutathione eluates. Analysis was performed similar to the initial proliferation testing described in section 2.1, however eluate samples were used in place of the prepared concentrations of adenosine and glutathione. The results were compared to a negative control group prepared of fibroblasts exposed to DMEM alone.

2.5. Statistical analysis

Quantitative data were expressed as the mean ± standard deviation ($n \geq 3$ for all groups). Statistical differences in proliferation and collagen concentration were determined using analysis of variance (ANOVA) with Holm-Sidak post-tests. A p-value < 0.05 was considered statistically significant for all experiments.

3. Results

Initial proliferative activity showed a dose-dependent increase in fibroblast response to the addition of adenosine or glutathione at concentrations up to 500 μM (133.6 μg/ml) or 250 μM (76.8 μg/ml), respectively (Figure 2). However, the results suggest that levels of glutathione higher than 500 μM (153.7 μg/ml) may have adverse effects on fibroblast proliferation, since concentrations above this level were significantly lower than controls.

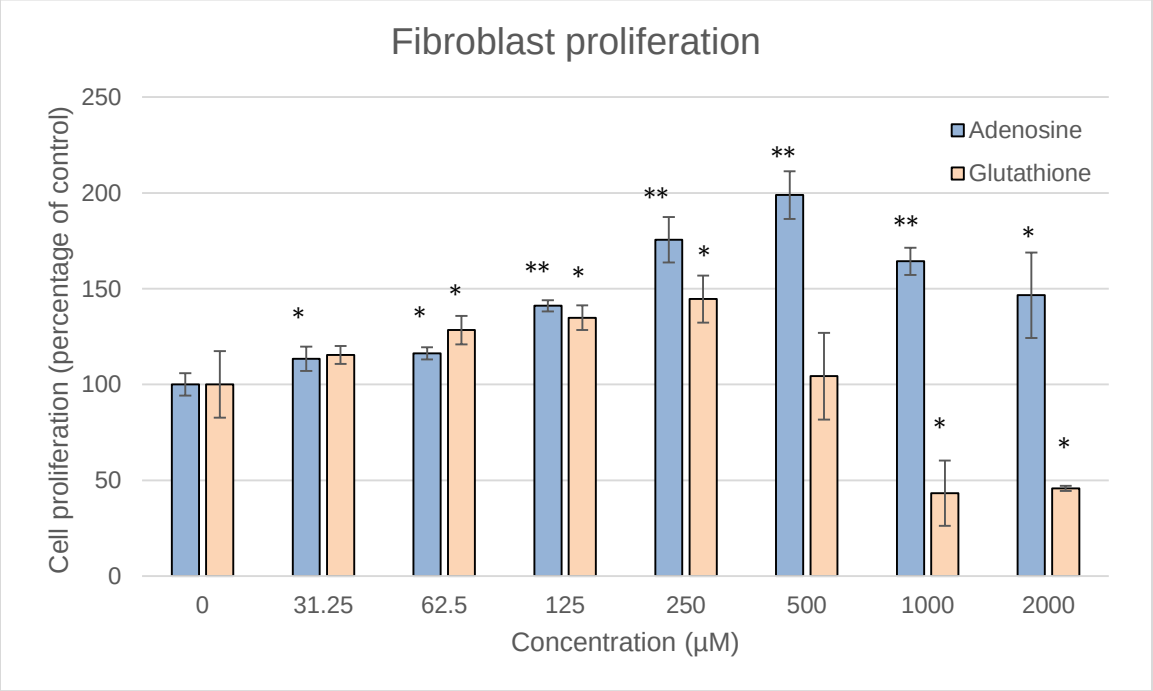

Figure 2. Graphical representation of fibroblast proliferation in response to varying concentrations of adenosine and glutathione. Data is expressed as mean percentage of non-treated control values with error bars representing standard deviation. Cell proliferation is presented as a percentage of the control (0 µM) RLU from the Cell Titer-Glo Luminescence Cell Viability Assay. * represents statistical difference between group and non-treated controls with $p<0.05$, ** represents $p<0.001$ as determined by ANOVA with Holm-Sidak post-hoc tests (n=4).

Adenosine, glutathione, and a combination treatment were evaluated as additives to increase fibroblast collagen production in order to identify potential additive or synergistic effects in fibroblast stimulation (Figure 3). Increasing levels of glucose in the media reduced total collagen formation at day 14. There were no detectable differences in early collagen production on day 3 or day 7 (not shown). On day 14, adding adenosine alone to the media significantly increased collagen production over the control in the low glucose group only. Glutathione alone did not increase collagen production in any glucose condition, and the addition of glutathione to adenosine attenuated the increase in collagen production observed with adenosine alone. Because cells were allowed to reach near confluence before exposure to additives, there were no significant differences in cell number determined by picogreen analysis of DNA content/well between groups or days.

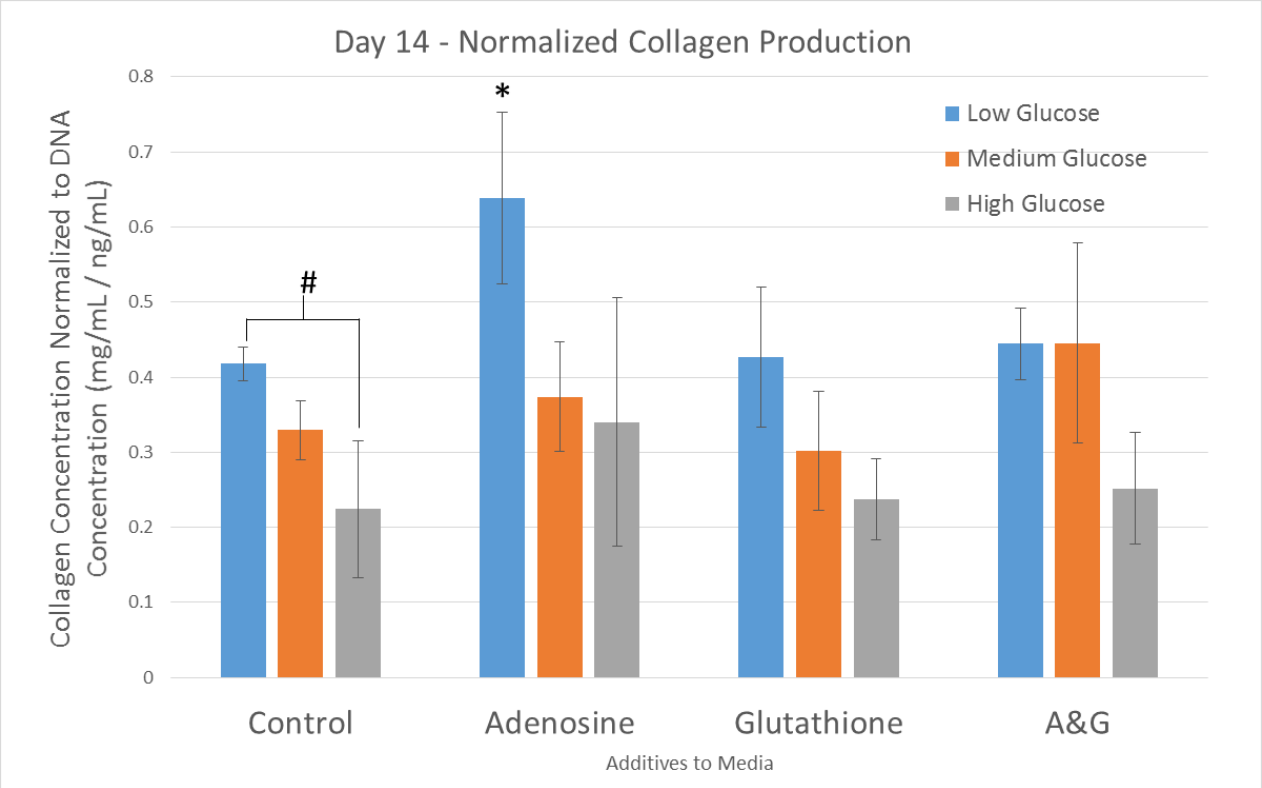

Figure 3. Graphical representation of total collagen production determined by Sirius Red assay and normalized to cell number by normalizing to total DNA in each well. Data represented is mean ± standard deviation. # represents difference detected between glucose levels in control groups and * represents difference detected between additive group and corresponding control at $p<0.05$ using two-way ANOVA with Holm-Sidak post hoc tests (n=3).

Elution of adenosine from sponge constructs (Figure 4) showed an initial burst release of adenosine at 24 hours, followed by significantly lower release concentrations after 48 hours. Adenosine eluted from chitosan sponges appeared to maintain significant levels of biological activity at 24 and 48 hours (Figure 5).

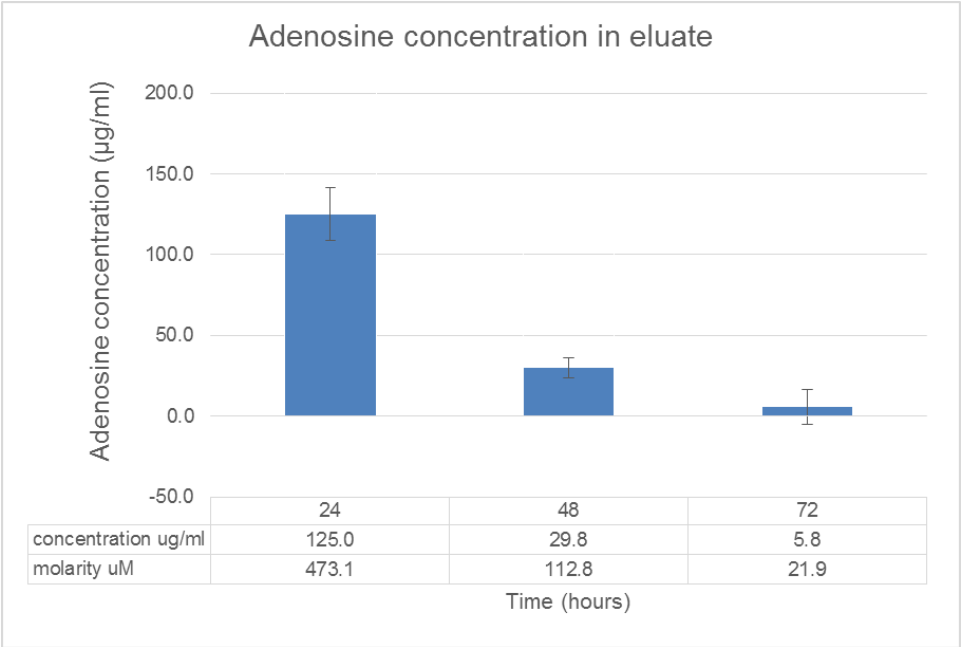

Figure 4. Graphical representation of adenosine concentration in PBS eluates. Data represented is mean ± standard deviation (n=9).

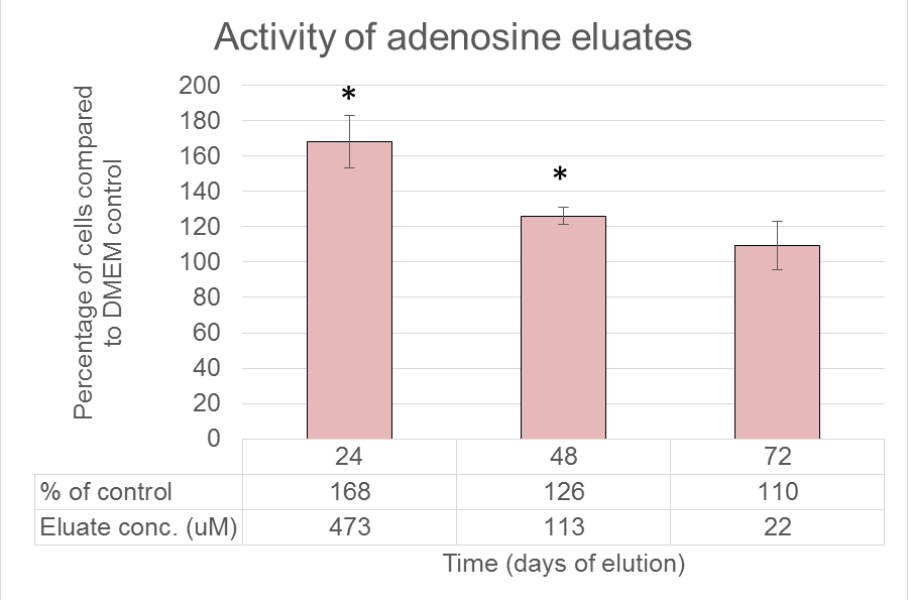

Figure 5. Graphical representation of proliferation of fibroblasts compared to controls after exposure to eluates at 24, 48, and 72 hours of adenosine from chitosan sponges. Data represented is mean ± standard deviation (n=5). * represents difference detected between additive group and corresponding DMEM only control at $p<0.05$ using ANOVA with Holm-Sidak post hoc tests (n=5).

Elution of glutathione from chitosan sponge constructs (Figure 6) also showed an initial burst release of glutathione at 24 hours, followed by significantly lower release concentrations after 48 hours. Glutathione eluted from chitosan sponge constructs also had small increases in fibroblast proliferation at the 48 and 72 hour tie points, however these increases were smaller than those seen with adenosine eluates (Figure 7).

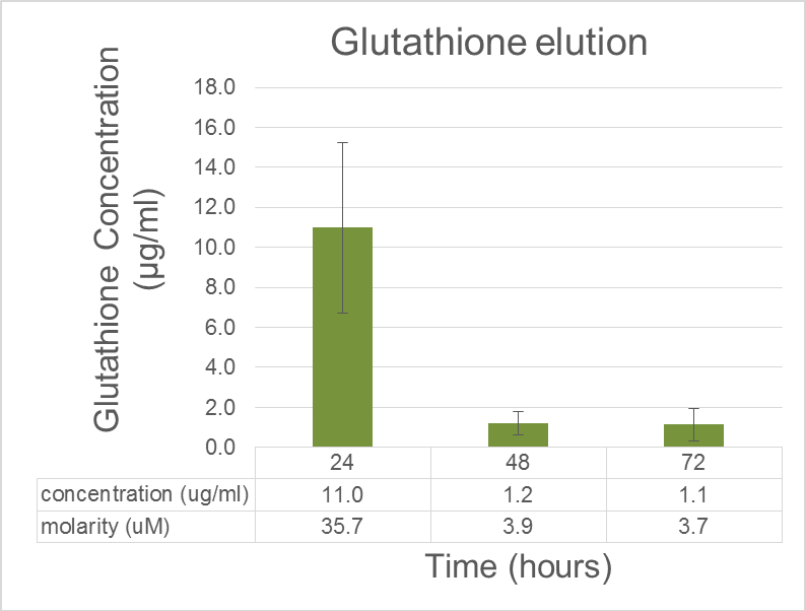

Figure 6. Graphical representation of glutathione concentration in PBS eluates. Data represented is mean ± standard deviation (n=4).

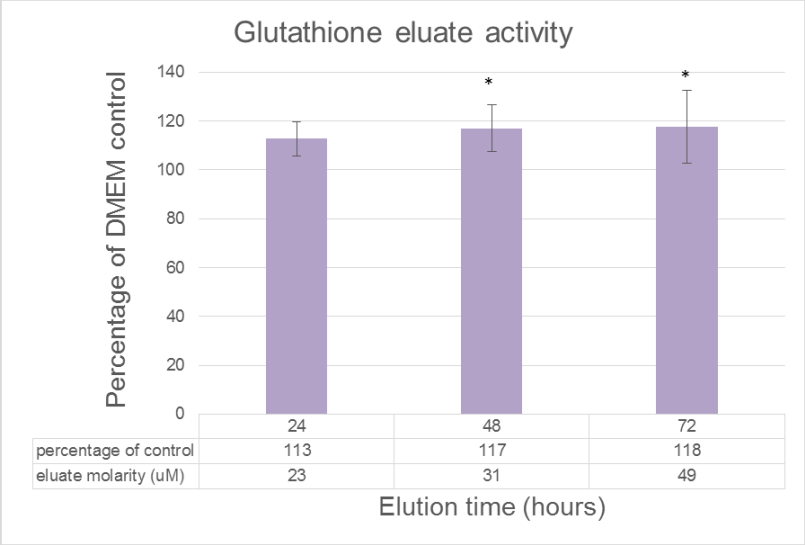

Figure 7. Graphical representation of proliferation of fibroblasts compared to controls after exposure to eluates at 24, 48, and 72 hours of glutathione from chitosan sponges. Data represented is mean ± standard deviation. * represents difference detected between additive

group and corresponding control at $p<0.05$ using two-way ANOVA with Holm-Sidak post hoc tests (n=5).

4. Discussion

The objective of this preliminary *in vitro* study was to determine if local delivery of adenosine and glutathione could be considered as a therapeutic approach to improve the treatment and healing of diabetic foot ulcers. Many current treatments used to improve the impaired healing of diabetic ulcerations require the use of growth factors, which are expensive and can be relatively ineffective in the overall healing of these type of wounds (Papanas and Maltezos 2008; Lavery et al. 2006; Rao and Lipsky 2007; Victor-Vega et al. 2002). Based on these preliminary results, the use of adenosine, a common nucleoside, and the tripeptide glutathione, may potentially promote the wound healing process. However, combining the two does not show additive or synergistic effects in this in vitro model. In foot ulcers with controlled glucose levels, the use of a chitosan sponge construct could provide the local delivery vehicle for active healing agents and the moist environment needed for proper healing of these healing impaired wounds.

Our study found adenosine or glutathione alone to effectively increase fibroblast proliferation response within a range of concentrations. However, at high levels of glutathione, this study suggests that glutathione may in fact have adverse effects on fibroblast proliferation. In a study by Victor-Vega highly selective adenosine receptor agonists were effective at all concentrations tested (Victor-Vega et al. 2002). However, the less selective receptor agonist lost its capacity to promote rapid wound closure at higher and lower end dosage concentrations (10 and 0.5 μg/wound) (Victor-Vega et al. 2002). This study partially confirms the results found in the Victor-Vega study since short-term dosage of adenosine showed significant activity in fibroblast proliferation. A study by Ohana also confirmed that adenosine regulates cellular proliferation of many cell lines at low concentrations (4 μM or 1.07 μg/ml) (Ohana et al. 2001). Additionally, similar findings by Shimegi showed that at a concentration of 100 μM adenosine (26.7 μg/ml), MC3T3-E1 osteoblast-like cell proliferation and DNA synthesis were stimulated in a dose dependent manner (Shimegi 1998). Since many foot ulcers include infection and osteolysis of the larger bones in the foot, these findings hold significant implications for the potential for future studies on diabetic wound healing (Lippmann, Perotto, and Farrar 1976).

The balance of proliferation and collagen production is shifted by several different factors in different cell types (Yang, Crawford, and Wang 2004; Peplow and Baxter 2012). Often there is an inverse relationship between proliferation and matrix production or differentiation activities (Quarles et al. 1992; Ishaug et al. 1994). In fibroblasts derived from diabetic patients, increased glucose levels inhibited proliferation (Hehenberger et al. 1998). The results of this study did not agree with such findings, however it should be noted that this study explores normal dermal fibroblasts contrary to the Hehenberger study. Short-term hyperglycemia has been found to enhance the function of cardiac fibroblasts (Shamhart et al. 2014), though collagen was found to be increased by the hyperglycemic condition in this study. In this study, while increased glucose levels increased proliferation, total collagen

production was decreased at high normal and hyperglycemic conditions. The pattern of increased proliferation with decreasing collagen production in hyperglycemic conditions may hinder the function of fibroblasts to deposit collagen needed for defect filling in the wound healing process and lead to common dysfunctional healing patterns in diabetics such as healing delays, hypertrophic scarring, and poor mechanical integrity of healed tissue (Epstein, Singer, and Clark 1999; Demidova-Rice, Hamblin, and Herman 2012). This suggests that treatment with adenosine may be most successful if glucose levels are closely managed. A study by Deveci *et al* suggested that the addition of glutathione is effective in restoring the collagen contractility of fibroblasts that were previously grown in high glucose conditions representative of hyperglycemia similar to the conditions in this study (Deveci et al. 2005). However, it should be noted that our preliminary study evaluates the production of collagen necessary for matrix formation rather than the contractility of collagen after healing begins, which is the focus of the Deveci study. Although in this study adenosine alone induced a higher proliferative and matrix production response in dermal fibroblasts than the combination with glutathione, the addition of glutathione may assist in alleviating oxidative stress present due to hyperglycemia intrinsic in diabetes mellitus (Camera and Picardo 2002; Edwards et al. 2008). Further evaluation in different in diabetic-specific in vitro and in vivo models, including studies of other cell types, growth factor signaling, and oxidative stress are needed to completely understand the effects of adenosine on impaired healing conditions.

The most common local drug delivery system currently in use clinically is the incorporation of antibiotics into polymethylmethacrylate (PMMA) beads (Roeder, Van Gils, and Maling 2000). However, one major disadvantage of PMMA, which is non-degradable, is the necessity of removal after the initial wound has healed (Roeder, Van Gils, and Maling 2000). In diabetic foot ulceration therapy, the growth factor-containing hydrogels, such as Becaplermin (Regranex, Johnson & Johnson, New Brunswick, NJ), are available for topical treatment of ulcerations (Wu et al. 2007; Bennett et al. 2003; Papanas and Maltezos 2008). However, growth factors may not result in sufficient healing to justify the high annual cost of treatment and possibility of side effects (Ma et al. 2015) (Wu et al. 2007; Bennett et al. 2003; Papanas and Maltezos 2008). The increased proliferative activity of fibroblasts exposed to eluates of adenosine and glutathione from chitosan sponges observed in this preliminary study suggest chitosan, a biodegradable bipolymer, may be an effective local drug delivery vehicle for adenosine and/or glutathione (Noel et al. 2008; Parker et al. 2013; Aimin et al. 1999; Smith et al. 2013; Noel et al. 2010; Parker et al. 2014; Khor and Lim 2003). Proliferative increases for adenosine at measured concentrations are similar to those observed in the initial study of proliferation, and suggest that clinical application would release therapeutic doses of adenosine to foot ulcers. Glutathione may interact with the chitosan matrix, which may have resulted in lower cumulative daily release compared to adenosine-loaded sponges. By modifying loading amounts and sponge properties, release profile may be controlled or extended. Studies have demonstrated that degradable chitosan sponges can be engineered to locally release an antibiotic over a period of 72 hours and that the released compound remains active, similar to the activity results observed in the present study (Noel et al. 2010; Parker et al. 2013).

Similarly, chitosan constructs released active amounts of gentamycin over a 2 week period in a study by Aimin *et al* (Aimin et al. 1999). A study by Parker *et al* also suggest that cross-linked chitosan may be a solution for a more predictable elution profile with minimal burst release (Parker et al. 2013). Results of this preliminary study indicate that the biological activity of adenosine and glutathione incorporated into chitosan sponges is maintained at 24 and 48 hour time points. Improved control of the burst release could result in higher amounts of active compound available over the course of treatment. A study by Kirk *et al* also suggests that crosslinking within a collagen local drug delivery material minimizes the burst release of eluted compound (Kirk et al. 2013). Similar chitosan studies found the material to have minimal cytotoxic effects on normal human dermal fibroblasts as well as healing properties during wound repair (Reves, Bumgardner, and Haggard 2013; Khor and Lim 2003; Ueno et al. 1999) . Ueno *et al* found that the application of chitosan in an *in vivo* wound model resulted in improved healing over the 15 day study (Ueno et al. 1999). Since current foot dressings require a clean, moist environment to assist in healing, the chitosan sponge construct would provide an environment conducive to wound healing while still maintaining a desirable elution profile, since a sponge will retain water thereby keeping the wound moist.

5. Conclusions

This preliminary *in vitro* study found that normal human dermal fibroblasts exposed to adenosine or glutathione exhibited improved proliferation over a 24-hour exposure. Furthermore, adenosine showed improved collagen production in fibroblasts exposed to low and high glucose conditions over a 14-day exposure. However, the glutathione exposure did not have any significant effect on collagen production. Fibroblast exposure to a combination of adenosine and glutathione did not result in a significant increase of collagen production compared to the DMEM control group. The elution study results found that biological activity of adenosine and glutathione incorporated into chitosan sponge constructs was maintained in elution. Such an application could be used as a drug delivery vehicle for local drug delivery at a foot ulceration site. The use of a chitosan sponge would also have the added benefit of maintaining a moist and clean environment necessary to the wound healing process.

Based on the findings in this preliminary *in vitro* study, adenosine and glutathione show promise as compounds to improve healing of impaired wounds under conditions in which glucose levels are closely monitored and controlled. Further experimentation may be beneficial to explore the potential of adenosine to improve the bone healing response, as might be needed in an infected diabetic foot ulcer (Sia and Berbari 2006; Ramsey et al. 1999). Future experimentation should include *in vivo* evaluations of other possible applications of adenosine and glutathione and local drug delivery with other delivery systems tailored to specific applications such as bone repair or soft tissue regeneration. Furthermore, diabetic preclinical models might reveal the full potential of adenosine and glutathione therapy for use as a clinical treatment for healing-impaired injuries.

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