SKIN REGENERATION USING DERMAL SUBSTRATES THAT CONTAIN AUTOLOGOUS CELLS AND SILVER NANOPARTICLES TO PROMOTE ANTIBACTERIAL ACTIVITY- *IN VITRO* STUDIES

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ABSTRACT

We hypothesized that the addition of silver nanoparticles (AgNP) to a dermal substrate would impart anti-bacterial properties without inhibiting the proliferation of contained cells. Our *in vitro* model was based upon the commercial substrate, Integra®. The substrate was prepared by simple immersion into 0-1% suspension of AgNP (75 or 200 nm-diam.) followed by rinsing for 20 minutes and sterilization under a UV-C lamp. A total of 10^7 human adipose stem cells/cm^3 were injected and after 1 hour, $6x10^5$ keratinocytes/cm^2 were seeded and cultured for up to 14 days. Constructs were evaluated using a metabolic assay (WST-1), H&E and immunoperoxidase staining. Bactericidal activity was measured using a log reduction assay against bacteria that are prevalent in burns. The presence of AgNP did not significantly change the metabolic activity of constructs after 14 days of culture and the distribution of cells within the substrate was unchanged from the controls that did not have AgNP. Anti-bacterial activity of Integra® containing AgNP (75 nm-diam.) was concentration dependent. In conclusion, the addition of AgNP to the dermal substrate suppressed bacterial growth but did not significantly affect cell proliferation, and may represent an important property to incorporate into a future clinical skin regeneration system.

INTRODUCTION

Up to 20% of combat casualties suffer significant burn injuries[1]. The standard of care for deep

burn wounds is skin grafting, with a piece of split-thickness skin taken from a healthy donor site.

When burns cover a large area of the body, the area of healthy skin available for grafting is often

insufficient. Fortunately, skin can be harvested more than once from the same donor site

because the epidermis regrows over time from proliferating keratinocytes that migrate from the

hair follicles and sweat glands. The dermis, however, cannot regrow, so skin grafts are taken

with as little dermis as possible. Acellular dermal matrices (ADM) have been developed for use

with thin skin grafts to repair deep burn wounds. ADMs are placed onto the freshly excised

wounds and skin grafts are then applied on top two to three weeks later. This time interval

allows for migration of dermal cells into the matrix and generation of a vascular network before

the application of the skin graft.

A variation of the standard approach to repairing deep burn wounds could potentially accelerate

healing and involves seeding the ADM with autologous cells at the time of its surgical placement

onto the wound. Mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissues

(ASCs) have been shown to accelerate wound healing by secreting trophic factors into the

wound[2, 3] or by differentiating into epidermal and dermal cells[4-6]. Preclinical *in vivo* studies have

shown that treating cutaneous wounds with ASCs increases new blood vessel formation[7-9] and

accelerates re-epithelialization[8] and wound closure.[10, 11] ASCs could be easily isolated from

liposuction fat obtained from the patient at the time of the initial wound debridement surgery. In

addition to ASCs, the ADM could be seeded with autologous keratinocytes obtained by the

enzymatic digestion of cutaneous punch biopsies. This approach could potentially reconstitute

the epidermis over the wound using isolated keratinocytes rather than split-thickness skin

grafts[12]. It would maximize the epidermal coverage derived from limited donor sites, avoid the morbidity associated with skin grafting and eliminate the delays in waiting for the regrowth of donor sites before additional skin graft harvesting.

A major complication of deep burn wounds and large surface area wounds is the development of bacterial infections.[13] Wound infections lead to delayed healing, longer hospital stays and higher mortality.[14] Service members in particular have a higher rate of infection-associated mortality following combat-related burns because wound excision and skin grafting is often delayed until they are evacuated to a burn center.[15] The current methods used to control bacterial wound infections involve the use of topical antimicrobial agents such as silver nitrate soaks, silver sulfadiazine cream and silver ion-release dressings, which demonstrate rapid and broad-spectrum anti-bacterial activity.[16] Despite being clinically safe, silver anti-microbials are potentially cytotoxic, which may lead to impaired vascularization and epithelialization of the wound. In the present pilot study, we investigated the potential of regenerating skin using dermal substrates that contain human ASCs, keratinocytes and silver nanoparticles (AgNP). Integra® wound regeneration matrix was treated with AgNP and the resulting anti-bacterial properties of the construct and the effect of the AgNP on ASC and keratinocyte proliferation was measured *in vitro*.

MATERIALS AND METHODS

Isolation and Culture of Human ASCs

Human adipose stem cells were isolated from human adipose tissue samples obtained by liposuction. Briefly, samples were digested in 0.1% (w/v) collagenase P (Roche Diagnostics, Indianapolis, IN) for 25 minutes at 37°C. Cells were then freed by vigorous mixing and centrifuged at 300 xg for 5 minutes to separate the stromal vascular fraction (SVF) from the fat cells. The SVF pellet was resuspended in growth medium (EGM-2MV; Lonza, Walkersville, MD), pipetted sequentially through 500, 297 and 70 µm-sized mesh and cultured in T-75 flasks at 37°C in an atmosphere of 5% CO_2. The growth medium was changed every 48 hours and cells were passaged when 80-90% confluent. Cells were used for experiments between passages 3 and 6.

Isolation of Human Keratinocytes

Human keratinocytes were isolated from discarded bits of split-thickness skin used in skin grafting. The pieces (2-3 mm wide) were incubated overnight in 1 mg/ml Dipase II (PluriSTEM, EMD Millipore) at 4°C. The epidermis was then separated from the dermis, minced and incubated in 0.25% trypsin-EDTA (Gibco; Grand Island, NY) for 15 minutes at 37°C. The trypsin was neutralized with KGM-Gold medium (Lonza, Walkersville, MD) containing 10% fetal bovine serum. The pieces were gently triturated, pipetted through a series of meshes (500, 297 and 70 µm), resuspended in keratinocyte growth medium (KGM + 1.4 mM $CaCl_2$ + 10% fetal bovine serum[17]) and then used directly.

Culture of ASC/Keratinocytes in Integra®

Integra® wound matrix (Integra Life Sciences, Plainsboro, NJ) was cut into 8 mm-diameter discs using a cork bore and equilibrated with growth medium in a 48-well plate. Proliferating ASCs

were trypsinized, resuspended in growth medium and 5×10^5 cells per disc (50 µl of 10^7 cells/ml)

were injected using a 25-gauge needle. One hour was allowed for ASCs to attach to the matrix

and then 3×10^5 freshly isolated keratinocytes (6×10^5 cells/cm^2) were pipetted onto the surface.

Each construct was cultured in 1 ml of keratinocyte growth medium at 37°C with a medium change

every 48 h. After 7 days, the constructs were transferred to the 12 mm inserts of a 12-well

Transwell® plate (3.0 µm polycarbonate membrane; Costar®, Corning, NY) and cultured for an

additional 7 days in the same medium. Wells and inserts were filled with 0.8 and 0.4 ml of

medium, respectively, to maintain an air-liquid interface at the surface of the constructs to promote

keratinocyte differentiation.[18]

Measuring Cell Proliferation in Integra®

The proliferation of cells in the Integra® constructs was measured using the WST-1 assay

(Roche, Indianapolis, IN). Briefly, discs of Integra® were seeded with ASCs or keratinocytes

and cultured as previously described. The constructs were collected over time, washed with

Hanks Balanced Salt Solution containing calcium and magnesium (HBSS) and then incubated in

240 µl of the WST-1 reagent (1:10 dilution with HBSS) for 4 h at 37°C. Duplicate absorbance

readings of the reaction medium were made at a wavelength of 450 nm and were corrected with

background absorbance readings and a reference wavelength of 630 nm using the equation

$(A_{450nm} - A_{630nm})_{sample} - (A_{450nm} - A_{630nm})_{blank}$. In some experiments, the Integra® discs were pre-

loaded with 0, 0.25, 0.5 or 1.0% AgNP (200 nm diameter), seeded with 10^7 ASC/cm^3, cultured

for 14 days at 37°C and then measured with the WST-1 reagent.

Loading Integra with Silver Nanoparticles

The AgNPs (75 or 200 nm; aqueous suspensions) were purchased from nanoComposix, (San

Diego, CA). The particles were re-suspended by vigorous vortexing for 1 minute immediately

before use. One cm^2 pieces of Integra were inverted onto sterile gauze to remove free water and

then immersed into the AgNP suspension for 1 minute. Particles that remained unbound to the

matrix were washed free by vortexing the samples in Dulbecco's-Phosphate Buffered Saline for

20 minutes. Samples were then sterilized by exposure to a UV-C lamp and subsequently seeded

with ASCs or used to measure bactericidal activity.

Assessment of AgNP Deposition by Scanning Electron Microscopy

Samples of Integra® (5 x 5 mm^2) were equilibrated in de-ionized water and upon removal, the

liquid in the matrix was wicked away using a laboratory wipe (Kimwipes). Next, samples were

immersed in a 0.5% solution of AgNP (200 nm-diam.) for 1 minute. Excess AgNP solution was

wicked away from the AgNP-loaded Integra sample and the sample was then allowed to dry

completely at room temperature. The Integra® was then imaged via scanning electron

microscopy (SEM). For comparison, a sample of Integra® without AgNP was also dried and

imaged in the same fashion.

Measuring Bactericidal Activity of Integra®-AgNP

The log reduction assay was used to measure the ability of Integra® containing AgNP to kill

bacteria. Bacterial cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (ATCC 25923

and 27853) were recovered from frozen stock by overnight growth in tryptic soy broth at 37°C and

Klebsiella pneumoniae (ATCC 31488) was recovered by overnight growth in nutrient broth #3.

Bacterial suspensions were diluted to an OD_{600nm} of about 0.2 immediately before use. Fifty

microliters of inoculum was injected per cm^2 of Integra-AgNP and incubated at 37°C for 30 min to

24 h in a humidified atmosphere. Controls consisted of using Integra without AgNP, 1 cm^2 pieces

of Acticoat® (Smith and Nephew), which is a silver-coated wound dressing, and 1 cm^2 pieces of

the Acticoat absorbant core (without the silver coating). At the appropriate times, the surviving

bacteria were recovered by immersing the samples into neutralizing solution (1:10 dilution of inoculum) containing 0.4% sodium thioglycolate and 1% TWEEN 20. Samples were vortexed and serially diluted in PBS. 100 µl of each dilution was plated on Mueller Hinton II Agar and colonies were grown at 37°C for 24 h and then counted. Log reductions were calculated as $\log_{10}$ (CFU/ml)$_{Integra}$ – $\log_{10}$ (CFU/ml)$_{Integra-AgNP}$. Similarly, log reduction for Acticoat was calculated as $\log_{10}$ (CFU/ml)$_{absorbant\ core}$ – $\log_{10}$ (CFU/ml)$_{Acticoat}$.

Histology/Immunoperoxidase Staining

Integra® constructs were washed two times in HBSS, embedded in Optimum Cutting Temperature compound (Tissue-Tek, Sakura-Finetek USA, Torrance CA) medium and flash-frozen in liquid nitrogen. Twenty micron thick sections were stained with hematoxylin and eosin (H&E) for light microscopy and 5 µm sections were processed using an immunoperoxidase stain against keratin AE1/AE3 by the Indiana University Health Immunohistochemical Lab.

Statistics

Linear regression analysis (Instat 3, Graphpad Software, La Jolla CA) was used to determine whether WST-1 absorbance of constructs containing ASC or keratinocytes increased with culture time to signify cell proliferation within the Integra matrix.

RESULTS AND DISCUSSION

Integra® Supports Adipose Stem Cell and Keratinocyte Proliferation

WST-1 absorbance readings are a measure of the amount of WST-1 reagent that is reduced by cell metabolism and therefore reflects the number of viable cells that are present in the Integra® matrix. The metabolic activity of constructs containing human ASC or keratinocytes increased over the time spent in culture (Figure 1). The number of ASCs that were injected into the Integra was about two-times the number of keratinocytes that were seeded on the surface. However, the finding that the absorbance readings were much higher for ASCs than for keratinocytes likely reflects a difference in plating efficiencies, which for freshly isolated keratinocytes on plastic is typically less than 5%.[19] For fresh ASCs, the plating efficiency has been reported at about 22%.[20]

Constructs containing both ASC and keratinocytes were examined histologically after 14 days, with the final 7 days of growth occurring at the air-liquid interface. The micrographs show that the ASCs were scattered throughout the matrix while the keratinocytes formed multiple layers at the surface (Figures 2A and 2B). This matched the method of delivery used for each cell type, which was injecting a small volume of concentrated ASCs into the Integra versus pipetting a dilute suspension of keratinocytes onto its surface. Although our usual culture medium for ASCs is endothelial growth medium, the constructs containing both ASCs and keratinocytes were cultured in keratinocyte growth medium to support the proliferation of keratinocytes, which we have found to be the more difficult. The keratinocyte medium contained added serum and calcium to promote the differentiation of keratinocytes as previously reported.[17] The micrographs show the presence of a dense surface layer that stained strongly for keratin, but it is

not clear if this represents differentiated or cornified cells or if it is an effect of dehydration due to the proximity of the water-air interface.

Integra® Retains AgNP Following Immersion

Immersion of Integra® into a 0.5% (w/v) AgNP suspension changed the Integra® from colorless to a dark brown or black coloration (Figure 3). Integra® is made of type I collagen and chondroitin 6-sulphate in a 92/8 ratio.[21] Ninety-eight percent of Integra® by volume consists of fluid-filled pores that are 30-120 μm in diameter. When some of this large volume of fluid is wicked out of the matrix, its subsequent immersion into the AgNP suspension causes rapid imbibition of the particles. Scanning electron microscopy shows that the short exposure time was sufficient for the uptake and adherence of the particles to the collagen matrix (Figures 3C-3E). Drobota et al. studied AgNP in collagen solutions and found that AgNP bind to collagen mainly through strong electrostatic interactions with the carboxylate (COO-) and amine (NH_2+) groups of the collagen.[22] Moreover, particle-induced changes in collagen secondary structure occurred, from α-helix to the more compact β-sheet.[22] In the Integra®, the collagens are cross-liked by a gluteraldehyde treatment, which potentially prevents similar AgNP-induced changes to the collagen structure.

Integra®-AgNP Demonstrates Antibacterial Activity

Figure 4 shows logarithmic reductions in the number of bacterial colonies that followed exposure of bacteria to Integra-AgNP. *S. aureus* and *P. aeruginosa* were injected into Integra® that was pretreated with a 1% (w/v) suspension of 200 nm-diameter AgNP and incubated for 2 hours. Log reductions were less than 1 (0.10 and 0.44, respectively) and corresponded to decreases in colony forming units of about 20% and 64%, respectively, when compared to Integra® that did not contain AgNP (Figure 4A). *K. pneumoniae* exposure to Integra®-AgNP for 24 hours

produced a log reduction of 0.33 or a decrease in colony forming units of 54%. In comparison, a 30 minute exposure of *K. pneumoniae* to Acticoat wound dressing resulted in a log reduction of 3.1 (over a thousand-fold decrease) when compared to a similar exposure to the Acticoat absorbant core that lacked the nanocrystaline silver sheet. Behm et al. exposed *S. aureus*, *P. aeruginosa* and *K. pneumoniae* to Acticoat for 30 minutes and measured reductions in colony-forming units of 3.7-4.7 logs,[16] which is comparable to our measurements using Acticoat. Some Integra® was pretreated with 75 nm-diameter AgNP, injected with *K. pneumoniae* and incubated at 37°C for 24 hours. Figure 4B shows that there was an increasing trend in log reduction as the AgNP concentration increased between 0.1 and 1.0%. A reduction of 3 or more logs is considered *bactericidal*.[16] Our Integra®-AgNP constructs, on the other hand, may be defined more appropriately as *bacteriostatic*.

The antimicrobial activity of silver is due to its ionic form (Ag+) and has been attributed to several mechanisms, including the blocking of cellular respiration,[23] binding to bacterial membranes to disrupt membrane function[24] and binding to and condensing bacterial DNA to inhibit cell replication.[25] AgNP are composed of metallic silver, which is neutral, and the associated antimicrobial activity has been attributed to its release of ionic silver.[26] However, some studies have implicated the particles themselves as the source of these toxic effects.[27, 28] The activity of AgNP is inversely proportional to their diameter as the surface to volume ratio of the particles increases with decreasing radius, thereby increasing the release of Ag+ ions. In our studies, the log reduction following exposure to 75 nm diameter AgNP was about 2 times that of the 200 nm-diam. particles (0.66 vs. 0.33 CFU/ml; Figure 4).

Integra®-AgNP Does Not Affect ASC Proliferation

Integra® samples were preloaded with 200 nm-diameter AgNP. Unattached particles that filled the pores of the matrix were washed out by vortexing the samples in D-PBS. Samples were then injected with $10^7/cm^3$ ASC and cultured in EGM-2MV for 14 days. The metabolic activity of the constructs was measured using the WST-1 assay. The absorbance readings were not significantly different when the Integra® was preloaded with 0, 0.25, 0.5 or 1% AgNP (Figure 5), hence cell proliferation was not affected by the use of up to 1% (w/v) AgNP. The H&E staining of the constructs after 14 days shows that ASCs were attached to the matrix and evenly distributed throughout for all concentrations of AgNP (Figure 6). No AgNP or AgNP aggregates were present in the pores and demonstrates that the wash-out step was effective. However, the eosine staining of the collagen is slightly darker or appears to have a slight "dusty" appearance in samples that were treated with 1% AgNP (Figure 6D). The wash-out step eliminated a significant source of toxicity (data not shown) and therefore is an important part of the sample preparation.

Cytotoxicity generally correlates with silver dissociation.[29] Le Duc et al. measured the effect of topical antiseptics on human skin substitutes *in vitro* and found that Acticoat decreased metabolic activity to about 60% of untreated control samples.[30] However, decreases in metabolic activity due to the presence of AgNP did not equate with a loss of cell viability *in vitro* and did not inhibit wound healing,[31] despite the fact that aggregates of AgNP became localized in the cytoplasm of dermal fibroblasts of healed wounds.[31] The implications of these reports and our findings are that higher concentrations of AgNP in Integra® and increased anti-bacterial activity may be achievable before metabolic suppression, loss of cell viability and impairment of wound healing are likely to occur.

CONCLUSIONS

Integra® wound matrix was made bacteriostatic by the addition of silver nanoparticles. The new antibacterial properties did not affect the proliferation of ASCs or keratinocytes that were cultured in the matrix. An expanded study of these properties at higher AgNP concentrations and their effects on oxidative stress, inflammation, vascularization and epithelialization of wounds is expected to further enhance the performance of this matrix and increase its potential utility in current and future treatments of deep burn injuries.
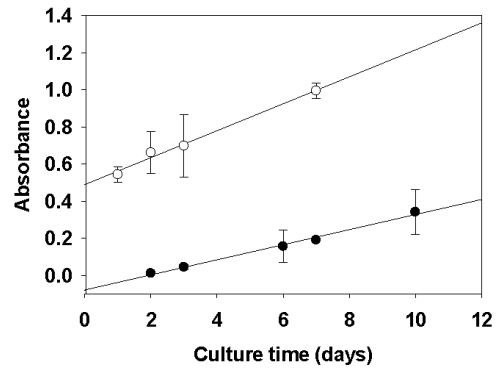
Figure 1. Metabolic activity of Integra constructs containing adipose stem cells ($\circ$) or keratinocytes ($\bullet$). Absorbance was calculated as $(A_{450} - A_{630})_{construct} - (A_{450} - A_{630})_{blank}$. The plotted values are the mean $\pm$ standard deviation of 2-4 replicate constructs. Slopes of regression lines are significantly different from 0 ($\bullet$: p=0.003; $\circ$: p=0.005).
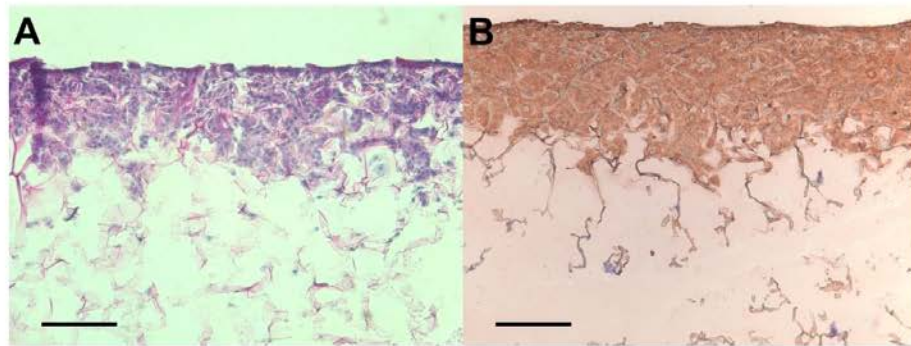
Figure 2. (A) H&E staining of Integra® cross-sections shows the presence of adipose tissues (ASCs) and keratinocytes after 14 days. (B) Peroxidase staining with anti-cytokeratin AE1/AE3 shows the presence of keratinocytes. The ASCs were injected into the matrix and keratinocytes were pipetted onto the surface after 1 hour. The constructs were cultured at the air-liquid interface for the final 7 days. Bar = 200 μm.
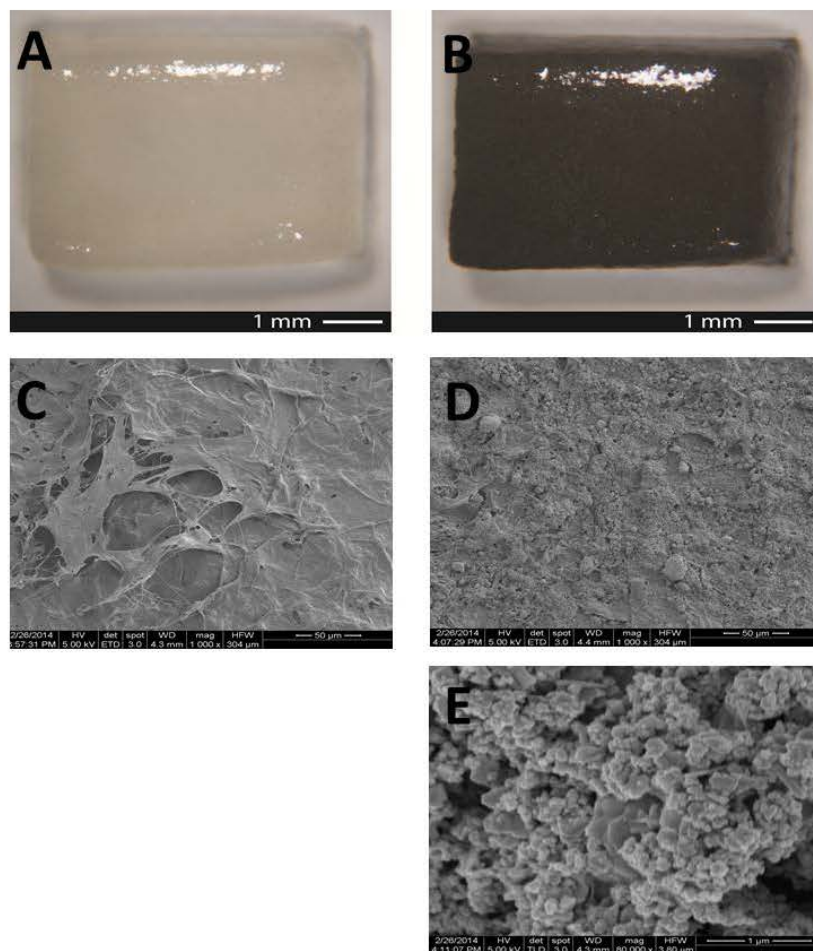
Figure 3. Integra® (A) was loaded with 200 nm-diameter AgNP by simple immersion into a 0.5% (w/v) suspension for 1 minute. (B) Representative scanning electron microscopy images of untreated Integra® (C) and Integra®-AgNP at low (D; 1000x) and high magnification (E; 80,000x).
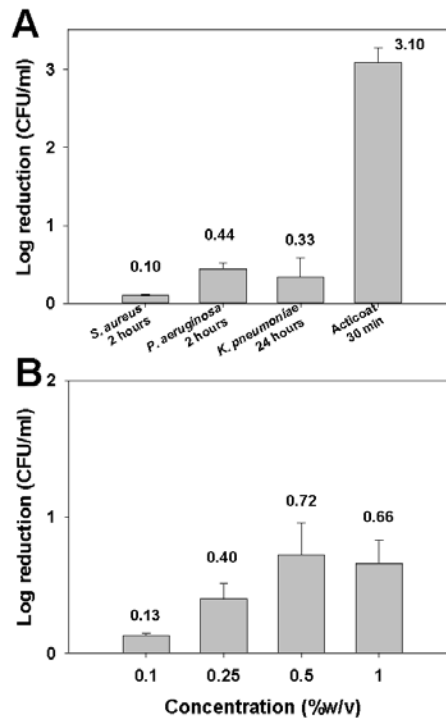
Figure 4. Integra[®] with silver nanoparticles demonstrates antibacterial activity against 3 bacterial species prevalent in burn wounds. (A) Log reduction of *S. aureus*, *P. aeruginosa* or *K. pneumoniae* exposed to Integra[®]-AgNP (1% suspension; 200 nm-diam.) for 2 or 24 h. Data are compared to the log reduction of *K. pneumoniae* following 30 min exposure to Acticoat. (B) Log reduction of *K. pneumoniae* exposed to Integra[®]-AgNP for 24 h. Integra[®] was pre-equilibrated in 0.1, 0.25, 0.5 or 1% AgNP (75 nm-diam.). Values are mean ± standard deviation of 2-4 constructs. There were significant differences in mean values (one-sided ANOVA; p=0.004) and the increase in log reduction with increasing AgNP concentration was significant (test for linear trend, p=0.0008). CFU = colony forming units.
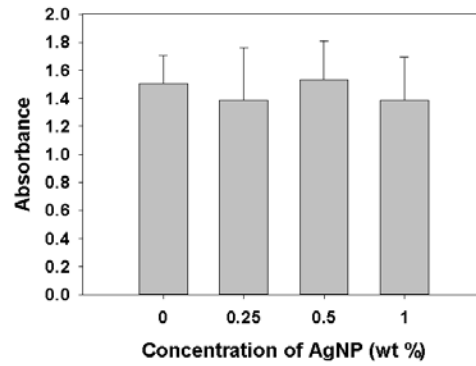
Figure 5. Metabolic activity of Integra[®] constructs that were pre-equilibrated in AgNP suspensions (200 nm-diam.; 0, 0.25, 0.5, or 1% w/v), injected with 10^7 adipose tissue/cm^3 and cultured for 14 days. Absorbance values (mean ± standard deviation) did not vary significantly between the different AgNP concentrations (n=4 constructs).
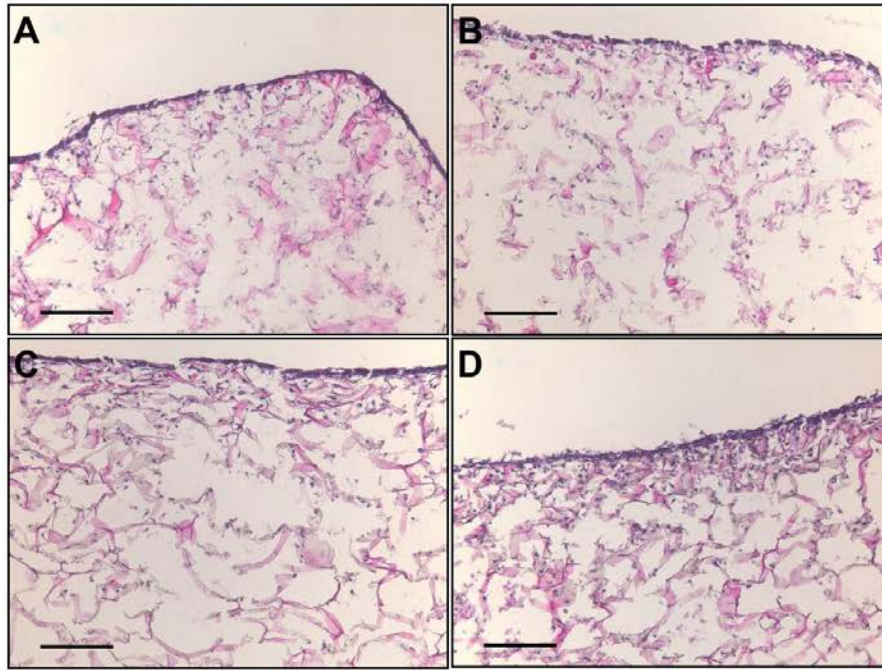
Figure 6. H&E staining of cross sections of Integra® that was preloaded with 0 (A), 0.25 (B), 0.5 (C), or 1.0% AgNP (D), injected with 10^7 ASCs/cm^3 and cultured for 14 days. Bar = 200 µm.

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