December 21, 2021

Analysis Report

Prepared by Nathan Cruz, M.S.
ndcruz@lanl.gov
B-10: Biosecurity and Public Health, Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico, 87544, USA

1. Statistical Analysis

1.1. R Version, Models, and Significance Level

Statistical analyses and figures were performed using R (ver: 3.6.2) and RStudio (ver. 1.2.5019). We created two-way ANOVA models with fixed-effects (treatment and time post-scratch; $p\text{-value}_{\text{treatment}} < 0.0001$; $p\text{-value}_{\text{time post-scratch}} < 0.0001$), accompanying Multiple Linear Regression (MLR) with interactions ($p\text{-value}_{\text{interaction}} < 0.0001$; $R^2_{\text{adj}} = 0.888$; RSE = 6.452; $F_{23,48} = 25.48$) to explain variation of the response variable "Percent Closure". Following rejected null hypotheses, we performed Tukey pairwise t-tests across levels of the discrete variable 'treatment' at both time intervals. Null hypotheses were tested against a predetermined significance threshold of α-level 0.05. Meanwhile, periodic checks of tenable assumptions for parametric statistics [i.e., $\sigma^2_{1,1} = \sigma^2_{2,1} = \cdots = \sigma^2_{i,j}$ and $\varepsilon \sim N(0, \sigma^2_{i,j})$] via diagnostic maps served to grade model conformity. The finalized MLR model, in which conclusions are drawn from, are displayed above their accompanying figures.

2. Results

We performed *in vitro* scratch assays on a representational wound healing model using hDFn cells. After monolayer scratching, we exposed cells to environmentally relevant levels of UN with or without the hypothesized therapeutic rhamnolipid at 6.25 µM and 50 µM. We then used pixel byte values of the images taken to quantify and discern areas void of cells. Conversely, pixel bytes of areas with cells were counted, and the ratio (normalized to initial scratch width ratio) used to model percent closure over 24 hours.

At 12 hours post-scratch, human dermal fibroblasts in media—free of toxicants, containing only UN, or UN supplemented with 6.25 µM of rhamnolipids—had negligible impact in percent closure response (Figures 1 and S1). Cultured fibroblasts exposed to 0.1 µM UN and 6.25 µM rhamnolipids had no significant increase in response compared to 0.1 µM UN group (p-value = 0.9474). These two groups had a 7.6% (95%CI, -25.7−10.5) magnitude of difference between mean percent closure (Figure 1A). Similarly, no differences in percent closure occurred when comparing 10 µM UN and 10 µM UN + 6.25 µM rhamnolipid groups (p-value = 0.8908). Twelve hours post scratch, the addition of rhamnolipids resulted in a non-significant 8.6% (95%CI, -26.7−9.5) elevation in closure percentage (Figure 1C). The most interesting result occurred with cells prone to 1.0 µM UN. In this group the co-existence of rhamnolipids at 6.25 µM brought about a significant increase in percent closure, in comparison to its untreated counterpart (Figure 1B; p-value = 0.0093). These samples treated with 6.25 µM of rhamnolipids and 1.0 µM UN had a relative 21.3% (95%CI, 3.2–39.4) increase in closure, with an average 12-hour recording of 59.8%. On the contrary, cells in the presence of 1.0 µM UN and free of rhamnolipids after 12-hours closed 38.5% of the initial scratch width. As expected, our statistical model denoted no differences in closure percentage over all UN levels, with or without 6.25 µM rhamnolipid treatment, after an elapse of 24 hours of growth. We found all UN groups, with/without 6.25 µM rhamnolipids, to have repopulated the initial scratch area completely.

Coinciding experiments implemented hDFn cells subjected to 0.1 µM−10 µM UN with or with the addition of 50 µM rhamnolipid treatment. Taken together, these data are first efforts in establishing a precursory interval of rhamnolipid treatment minimal- and maximal-effective doses (MinED; MaxED). Remarkably, dermal cells presented with rhamnolipids at 50 µM (regardless of UN level) reestablished a confluent monolayer 12 hours post-scratch (Figure D-F; p-value < 0.0001). Dermal cells contended with UN recovered confluence over the remaining twelve hours of the scratch

assay; thereupon, we no longer observed significant differences between interested groups, as expected (p-value > 0.05).

Table 1: Measurement of linear relationship in the data set.

Levels	Pearson's Correlation Coefficient ($r_{x,y}$)
Control	0.9605
6.25 µM R	0.9675
50 µM R	-0.4756
0.1 µM UN	0.8975
1.0 µM UN	0.9911
10 µM UN	0.9971
0.1 µM UN/6.25 µM R	0.8427
1.0 µM UN/6.25 µM R	0.9685
10 µM UN/6.25 µM R	0.8257
0.1 µM UN/50 µM R	-0.4080
1.0 µM UN/50 µM R	0
10 µM UN/50 µM R	0

$\hat{y}_{0.1\ \mathrm{UN}} = 32.5 + 2.63x$
$\hat{y}_{1.0\ \mathrm{UN}/6.25\ \mathrm{R}} = 44.5 + 2.27x$
$\hat{y}_{0.1\ \mathrm{UN}/50\ \mathrm{R}} = 101 - 0.14x$

$\hat{y}_{1.0\ \mathrm{UN}} = (-14.9) + 4.45x$
$\hat{y}_{1.0\ \mathrm{UN}/6.25\ \mathrm{R}} = 20.4 + 3.28x$
$\hat{y}_{1.0\ \mathrm{UN}/50\ \mathrm{R}} = 99.5$

$\hat{y}_{10\ \mathrm{UN}} = 23.8 + 3.14x$
$\hat{y}_{10\ \mathrm{UN}/6.25\ \mathrm{R}} = 40.9 + 3.63x$
$\hat{y}_{10\ \mathrm{UN}/50\ \mathrm{R}} = 101$

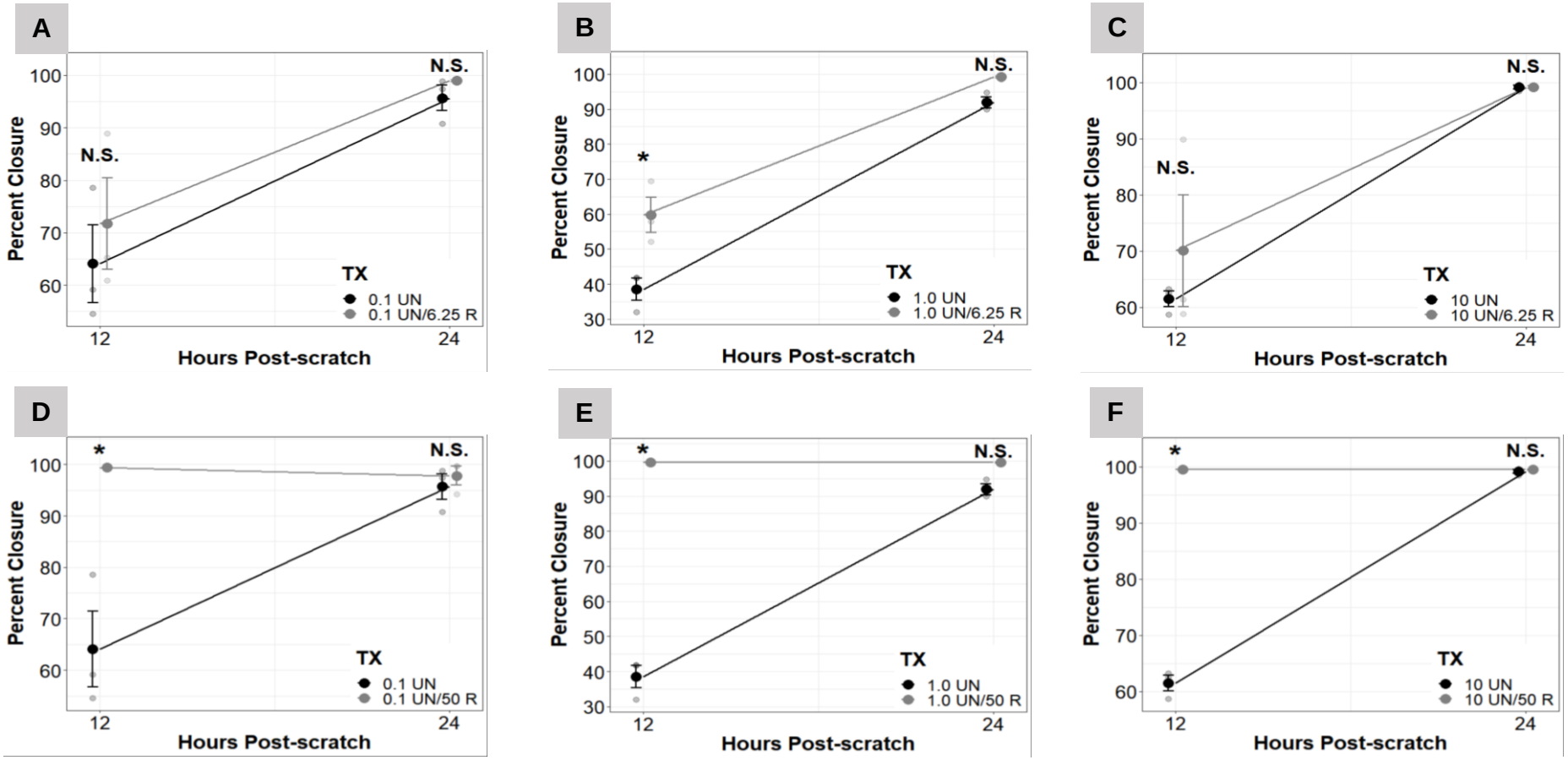
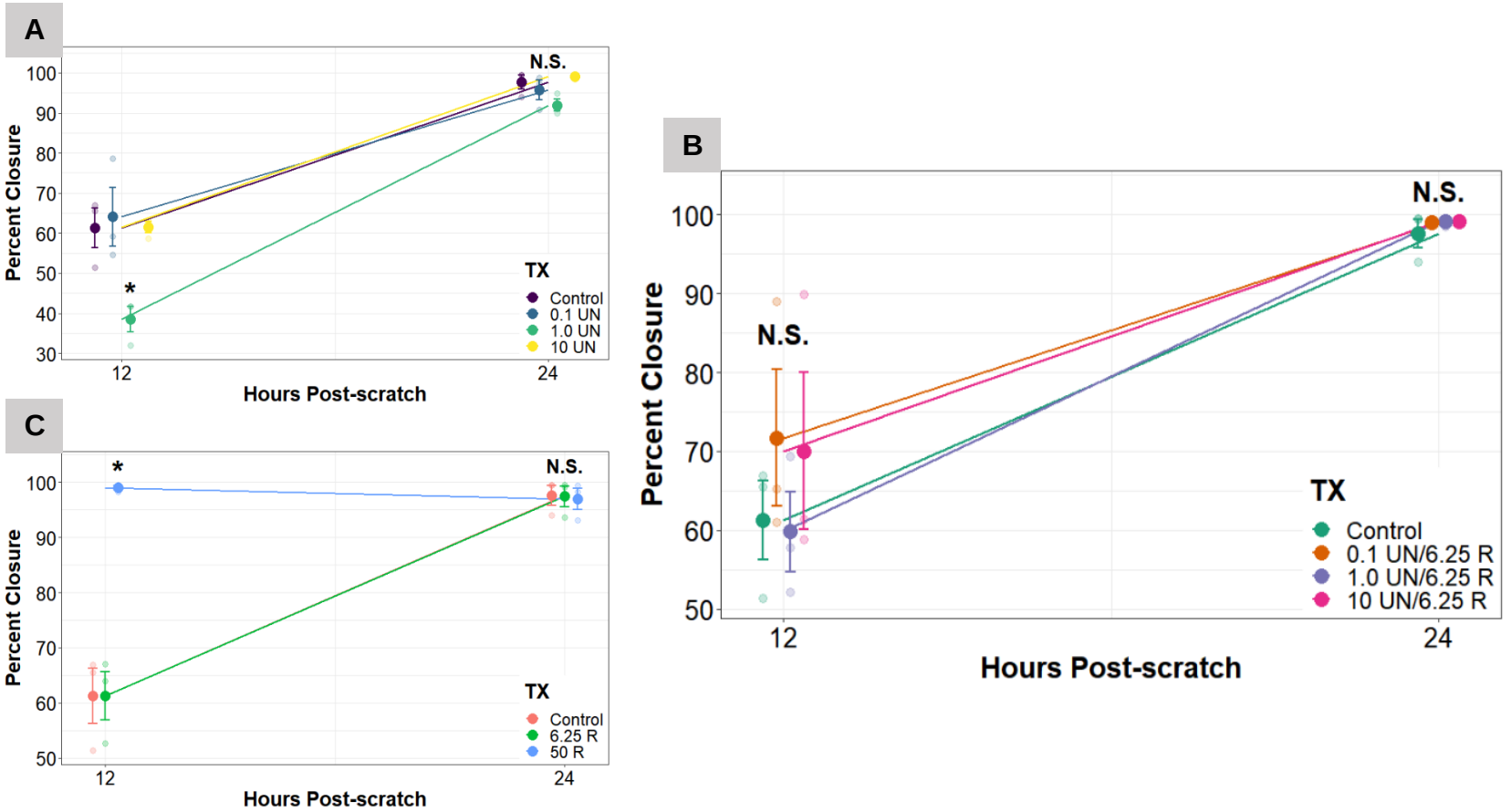


Figure 1: Analysis of *in vitro* wound closure of hDFn cells in the presence of UN or UN + rhamnolipids.
(A-C) Comparison of scratch assay cellular percent closure of 0.1 µM UN (A), 1.0 µM UN (B), and 10 µM UN (C) with and without 6.25 µM Rhamnolipids.
(D-F) Comparison of scratch assay cellular percent closure of 0.1 µM UN (D), 1.0 µM UN (E), and 10 µM UN (F) with and without 50 µM Rhamnolipids.
Bars indicates $SE(\hat{\mu})^2$ for each treatment group where n = 3
* p-value < 0.05
N.S. Not Significant



Supplementary Figure 1: Accessorial analyses of *in vitro* percent closure regression models: control, UN, UN + rhamnolipids, and rhamnolipids.
(A) Contrasts performed 12- and 24-hours post-scratch across control, 0.1 µM UN, 1.0 µM UN, and 10 µM UN.
(B) No differences observed in combination treatment of UN and 6.25 µM rhamnolipids when compared to control after 12 or 24 hours of growth.
(C) Significant increase of average cellular percent closure in samples treated with 50 µM rhamnolipids 12 hours post-scratch.
Bars indicates $SE(\hat{\mu})^2$ for each treatment group where n = 3
* p-value < 0.05 from all groups within the same time post-scratch
N.S. Not Significant
$\hat{y}_{\text{Control}} = 24.9 + 3.03\text{x}$
$\hat{y}_{6.25\text{ R}} = 25.0 + 3.02\text{x}$
$\hat{y}_{50\text{ R}} = 101 - 0.18\text{x}$