**Report: Impact of Fluid Shear Levels on bacterial survival responding to oxidative stress Over Time in Normalized Count Analysis**

**Background.** The efficacy of different fluid shear levels (trt\_FS) and their temporal dynamics were assessed to understand how D23580 responses to oxidative stress (normalized) vary over time and between treatments. This study aimed to test how FS-treatments demonstrate the survival effectiveness immediately after administration and how they sustain this effect over a period.

**Methods.** The study utilized **a mixed-effects model with an AR(1) structure** to analyze normalized count data derived from various FS-treatment levels observed at multiple time points (0, 10, 30, 45, 60 minutes). The analysis accounted for individual differences and intra-subject correlation, providing a robust framework for evaluating both fixed effects of treatments and time, as well as their interaction. An **ANCOVA** was also performed to control for baseline variations and further validate the treatment effects. Lastly, I performed the **generalized estimating equation (GEE) model**. This study analyzed data from a total of 25 samples, with 74 observations utilized in the analysis. Each sample was measured three times at every time point, and these three readings were averaged to produce the counts used in this analysis.

**Results.** The analysis revealed significant differences among treatment levels. **FS1** served as a baseline, showing the least effectiveness over time. **FS3** did not significantly differ from Treatment 1, suggesting similar levels of efficacy. **FS4** and **FS5** demonstrated significantly greater effectiveness compared to Treatment 1, with Treatment 5 being the most effective across all time points. The interaction effects between FS and time were significant, indicating that the efficacy of treatments varied significantly over time, with initial effectiveness peaking at 10 minutes and generally declining by 60 minutes.

**Conclusion.** Treatments 4 and 5 offer promising initial responses and maintain better efficacy over time compared to baseline treatments. Treatment 5, in particular, shows potential for conditions requiring rapid and robust intervention. The decline in treatment response over time across all treatments suggests the necessity for strategies that enhance and prolong efficacy, possibly through dosage adjustments or combination therapies. These findings provide critical insights for optimizing treatment protocols and tailoring interventions based on temporal dynamics and individual responses.

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**Data and Methodology**

The counts (CFU/mL) were normalized by baseline values (norm\_count) prior to analysis. The data involves multiple treatment groups (trt\_FS with levels 1, 3, 4, and 5) and different time points (0, 10, 30, 45, 60 minutes). A mixed-effects model with an AR(1) covariance structure was employed to consider within-subject correlation across time points, allowing individual differences to be modeled accurately.

**Statistical Analysis**

Two primary statistical analyses were conducted:

1. **Mixed-Effects Model:**
   * The model included fixed effects for treatment (trt\_FS), time, and their interaction.
   * The results indicate significant effects for trt\_FS, time, and their interaction (all p < 0.0001), suggesting both treatment and time significantly influence the normalized count, and the effect of treatment varies over time.
2. **ANCOVA:**
   * This analysis was designed to evaluate the treatment effects while controlling for baseline values.
   * The model confirmed significant treatment effects, time effects, and interaction effects, consistent with the mixed-effects model findings.

**Key Findings**

* Fluid shear (FS) Effects:
  + Significant differences exist among the FS groups. Specifically, treatments 4 and 5 generally show a stronger impact compared to treatment 1, with treatment 5 showing the most significant differences (e.g., treatment 5 vs. treatment 1 p < 0.0001).
  + The differences between treatments 1 and 3 are less pronounced, suggesting similar efficacy or impact between these treatments.
  + FS1 and 3: Offer baseline or similar levels of efficacy, suitable for conditions where moderate intervention is sufficient.
  + FS4: Provides a better response than the baseline and could be considered for more aggressive treatment strategies where an intermediate level of intervention is required.
  + FS5: The most effective treatment across all time points, ideal for scenarios requiring maximum immediate efficacy.
* Time Effects:
  + The response changes significantly over time, with the earliest time points (from 0 to 10 and 0 to 30 minutes) showing substantial increases in normalized count, which significantly drop towards the later time points (45 and 60 minutes showing no significant change from baseline).
  + Significant changes were observed particularly between the initial time points and later times, highlighting the transient nature of the FS effects.
* Interaction Effects:
  + The interaction of treatment and time is significant, indicating that the effects of treatments vary across different times. This is crucial for understanding how treatment efficacy may change over the duration of the observation period.

**Model formation:**

**Normalized bacterial count (count/base) =**

**β0​ + β1​(FS1​) + β2​(FS3​) + β3(FS4​) + β4​(FS5​) + γ*t* ​(time*t*​) + Σ (θ*i,t* ​⋅ trt\_FS*i​* ⋅ time*t*​)**

where

* **𝛽0​**: Intercept (0.0359, p = 0.0163)
* **𝛽1​, 𝛽2​, 𝛽3​, 𝛽4​**: Coefficients for treatment effects (**FS1** = -0.0386, **FS3** = -0.0364, **FS4** = -0.0297; **FS5** is the reference category with coefficient set to 0.0000)
* **𝛾*t*​**: Coefficients for time effects (the change in response at each specific time point relative to the baseline time (time 0), assuming the reference treatment, FS5): significant at all time points with varying estimates
* **𝜃(*i*,*t*​)**: Interaction coefficients between FS and time points. (e.g., θ(1,10)​ for **FS1** at 10 min = -0.4137)

**Conclusions**

The analysis robustly demonstrates that both FS and time significantly impact the response (normalized count). Treatment 5, in particular, shows the most substantial effect compared to others, indicating its potential for stronger effect. However, the transient nature of the fluid shear (FS) response suggests considerations of the sustainability of FS impact over time.

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Least squares mean: the means of groups, *adjusted* for other factors or covariates in the model. It provides a way of **comparing group effects** on a response variable, adjusted for the influence of other variables included in the model. This makes the comparison fairer, especially when sample sizes are unequal or when there are covariates to control for.

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**Type I SS (Sequential SS):** calculates the sums of squares for each predictor sequentially, based on the order the predictors are entered into the model. highly dependent on the order of the predictors. useful for hierarchical or nested models where there is a clear rationale for the order in which variables are entered.

**Type III SS (Marginal SS):** evaluates the effect of each predictor on the response variable after accounting for all other predictors in the model. It measures the unique contribution of each predictor to the response variable, controlling for the presence of all other variables. generally preferred in models when you want to understand the individual contribution of each variable regardless of order.



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**SAS codes:**

dm "output;clear;log;clear;odsresults;clear";

options ls=75 ps=2000 formdlim='\*' nodate nonumber nocenter;

\* Import from Excel;

proc import datafile="D:\Dropbox (ASU)\1 Manuscript\_Press\#1 Manuscript writing\202201\_D23580\Oxy for modeling.xlsx" out=data dbms=xlsx replace;

run;

\* Data to Long Format;

proc transpose data=data out=data\_long;

by id trt\_FS base trial;

var X00 X10 X30 X45 X60;

run;

data data\_long;

set data\_long;

/\* Extract numeric time from the \_NAME\_ variable \*/

time = input(substr(\_NAME\_, 2), best.);

rename COL1=count; /\* Rename the variable holding the transposed data to 'count' \*/

drop \_NAME\_ label; /\* Drop unnecessary variables \*/

run;

\*\_\_\_\_Normalize;

data normalized\_data;

set data\_long;

if base > 0 then norm\_count = count / base; /\* Normalizing count by base \*/

else norm\_count = .; /\* Handle cases where base is zero or missing \*/

run;

\* Mixed-Effects Model, AR(1) stdurcture, using normalized data;

proc mixed data=normalized\_data method=reml;

class id trt\_FS time ;

model norm\_count = trt\_FS time trt\_FS\*time / ddfm=kenwardroger;

random intercept / subject=id;

repeated time / subject=id type=AR(1);

lsmeans trt\_FS time / adjust=tukey pdiff=all;

title "Normalized, AR(1), Mixed-Effects Modeling2 consinder individual differences";

run;

\* ANCOVA- FS effect by controlling base, using normalized data;

proc glm data=normalized\_data;

class trt\_FS time;

model norm\_count = base trt\_FS time trt\_FS\*time;

means trt\_FS time / tukey; /\* Tukey's post-hoc test if needed \*/

title "Normalized, ANCOVA analysis-FS effect by controlling base" ;

run;

\* graph for each subject;

proc sgplot data=data\_long;

series x=time y=count / group=id;

xaxis label='Time (minutes)';

yaxis label='Count';

title 'Profile Plot of Counts Over Time for Each Subject';

run;

\*Generalized Estimating Equations (GEE)modeling;

proc genmod data=normalized\_data;

class id trt\_FS (ref=5) time;

model norm\_count = trt\_FS time trt\_FS\*time / dist=normal ; /\* Adjust 'dist' and 'link' based on the outcome \*/

repeated subject=id / type=ar(1) within=time corrw modelse; /\* AR(1) correlation structure \*/

run;

\*\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\* Survival Analysus;

proc phreg data=normalized\_data;

class trt\_FS (ref='1');

model time\*norm\_count(0) = trt\_FS time / ties=EFRON;

baseline out=baseline\_out covariates=normalized\_data survival=\_survival\_;

id id;

run;

proc sgplot data=baseline\_survival;

series x=time y=survfunc / group=trt\_FS lineattrs=(thickness=2)

groupdisplay=cluster; /\* to display each group with different colors \*/

xaxis label='Time';

yaxis label='Survival Probability';

title 'Survival Curves by Treatment Group';

run;

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