Improved Sensitivity of Continuous Animal Monitoring Increases Power and Reduces Numbers of Experimental Animals Required for Statistical Significance

Laura Schaevitz and Erwin Defensor

Vium, San Mateo, CA, USA

INTRODUCTION

Reducing animal numbers used in research must be balanced with generating experimental designs possessing sufficient statistical power. We hypothesize that continuous monitoring of behavioral, and physiological conditions (motion, breathing, etc.) would provide valuable insight into disease processes and increase statistical power, such that the number of animals required to detect significant relationships could be reduced. Retrospective power analyses were conducted across a number of experimental models representing liver and lung injuries in order to determine the animal numbers needed to observe statistical significance, thereby contrasting traditional measures with continuously recorded breathing and motion metrics. Analyses were conducted using G*Power with the criteria of power=0.90 and alpha<=0.05. In a Concanavalin A-induced liver toxicity model, effect sizes were determined to be 1.40 and 1.31 for liver enzyme elevations (ALT and AST, respectively), when sample sizes of n=15 and 17 were employed. In contrast, continuous animal monitoring resulted in effect sizes of 4.01 and 1.67 for motion and breathing metrics respectively, with sample sizes of n=4 and n=11. In a paraquat-induced lung injury model, effect sizes were determined to be 2.23, 1.40 and 3.94 for temperature, body weight, and lung weight when sample sizes of n=7, 15 and 4 were employed. In contrast, continuous animal monitoring resulted in effect sizes of 5.33 and 9.48 for motion and breathing metrics respectively, with sample sizes of n=3 and n=2. In a bleomycin-induced lung-injury model, an effect size of 1.47 was determined for hydroxyproline elevations when a sample size of n=11 was employed. In contrast, continuous animal monitoring resulted in an effect size of 4.13 for breathing, with sample size of n=3. Overall, we demonstrate that continuously monitored motion and breathing rates were able to reduce the overall number of animals needed for an experiment without compromising statistical conclusions.

METHODS

Experiments were conducted in Vium's AAALAC-accredited Digital Vivarium in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Vium's Institutional Animal Care and Use Committee. All experiments employed single housed male subjects. Animals were allowed to acclimate to the vivarium and Smart Housing for at least seven days after arrival before initiating experiments. Six-week old BALB/c mice, 11-14 week old Lewis rats, and eight-week old C57BL/6 mice were used for the **Liver Toxicity, Lung Injury, and Pulmonary Fibrosis (Bleomycin: BLM)** experiments, respectively. BALB/c mice and Lewis rats were obtained from Charles River Labs (Hollister, CA), and C57Bl/6 mice were obtained from Envigo (Livermore, CA).

Disease Induction:
Liver Toxicity: Mice received intravenous (IV) administrations of Concanavalin A (Con-A: 15 or 25 mg/kg) in sterile phosphate buffered saline (PBS). Control mice received sterile PBS alone.
Lung Injury: Rats were anesthetized using isoflurane, followed by intratracheal administration of paraquat (0.02 mg) in sterile saline (300 uL). Control rats received sterile saline alone.
Pulmonary Fibrosis (BLM): Mice were anesthetized using both an injection of a ketamine/xylazine and isoflurane to achieve the desired anesthetic plane, followed by oropharyngeal administration of bleomycin sulfate (3 U/kg) in sterile water (50 uL). Control mice received sterile water alone.

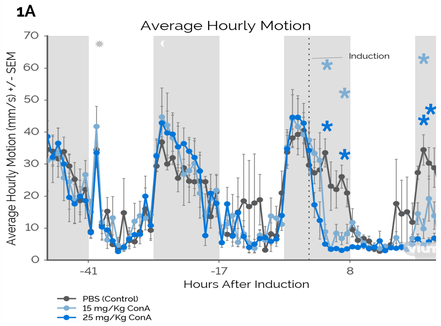
Experimental Monitoring:
Using the online Research Suite, remote clinical observations were performed.
Liver Toxicity: Observations were conducted hourly from 5-8 hrs post-dose and every 30 mins thereafter until endpoint. If remote clinical observations suggested an animal was nearing humane endpoint or in distress, a cage- side observation was conducted. Mice found to be at humane endpoint were euthanized.
Lung Injury & Pulmonary Fibrosis (BLM): In additional to remote monitoring, cage side observations were conducted at least daily for 1 week following paraquat exposure or every other day for 3 weeks following bleomycin exposure.
Breathing Rate and Motion: The Vium Digital Vivarium is comprised of intelligent sensors and HD cameras, allowing for continuous and minimally invasive monitoring of animals, as well as collection of automated metrics (e.g. motion and breathing rate), **in the home cage**. Study data is available in real-time and accessible via the online Research Suite.
Additional Monitoring: Throughout all studies, body weights and body condition scores were assessed in individual animals to monitor disease progression. Daily Body Temperature was measured rectally using a digital thermometer during the Lung Injury study.

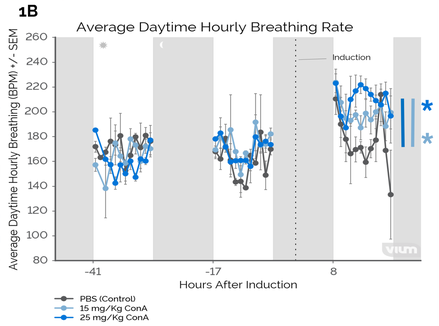
Tissue Collection: Animals were euthanized by isoflurane inhalation.
Liver Toxicity: Blood was collected via submandibular vein (Hr 8 or Hr 24) for determination of liver transaminase levels (ALT: alanine transaminase and AST: aspartate transaminase) using a Piccolo Xpress.
Lung Injury: Lung tissues were harvested and weighed (Day 2 or Day 6)
Pulmonary Fibrosis (BLM): Lung tissues were harvested, weighed and photographed (Day 21). The left lobe was insufflated and placed into 10% neutral buffered formalin for histological analysis, while the right lobes were snap frozen for hydroxyproline quantification by ELISA (Quickzyme).

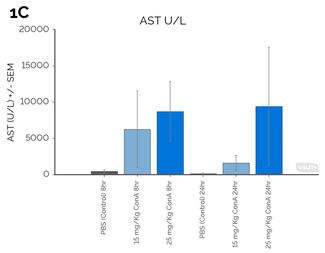
Statistical Analyses:
Two-way ANOVAs with adjustments for multiple comparisons (Lung Injury & Pulmonary Fibrosis: Bonferroni's; Liver Toxicity: Tukey's) were employed to assess differences among groups over time. Kruskal-Wallis tests were used to assess liver transaminase activity levels among groups, while unpaired t-tests were used to assess tissue weights. Correlations were assessed using Pearson tests. Two tailed test were used for all analyses with P values less than 0.05 considered statistically significant. Retrospective power analyses were calculated using G*Power (Heinrich-Heine-University Düsseldorf, Germany).

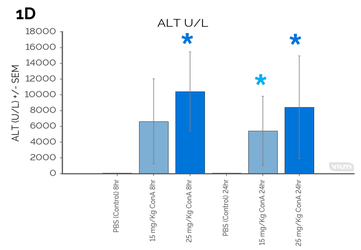
RESULTS

LIVER TOXICITY





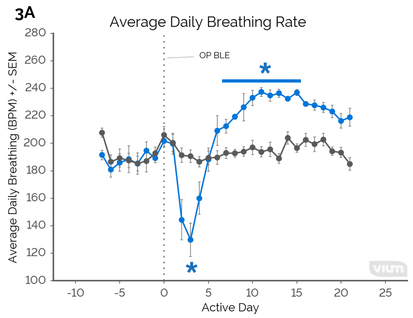


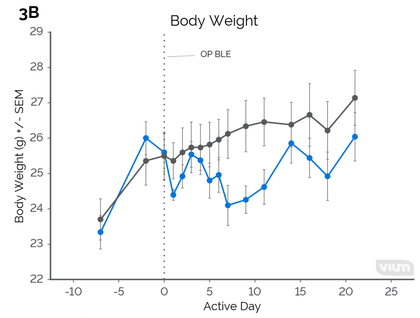


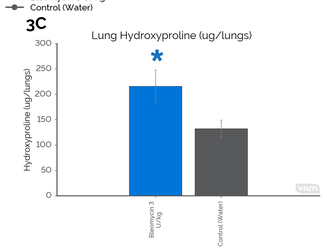
1E	Night-Time Motion Night 0 Post-Induction	Breathing Rate Day 1 Post-Induction	AST 8-hr Post-Induction	ALT 8-hr Post-Induction
Effect Size	4.01	1.67	1.31	1.40
Sample Size (N)	4	11	17	15

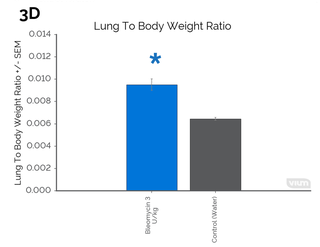
Figure 1: Mice receiving 15 or 25 mg/kg of Con-A (n=5 per group) demonstrated significantly reduced Motion as early as 3 hrs post-dose (A), increased Breathing rates (B), and elevated AST (C) and ALT (D) levels compared to mice receiving PBS (n=5). Effect Sizes were determined from means and standard deviations followed by calculations of required group sizes to achieve Power of 0.90 (E). Values represent means ± SEM. * P≤0.05

PULMONARY FIBROSIS





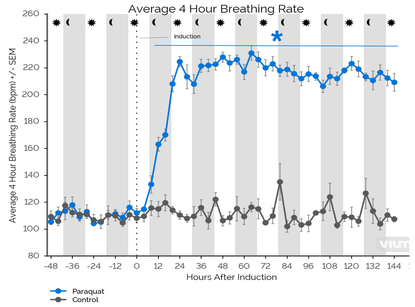


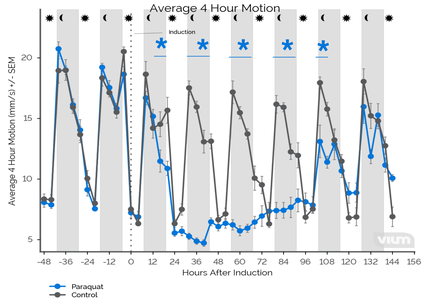


3E	Max Breathing Rate Post-Induction	Min Body Weight Post-Induction	Lung to Body Weight Ratio	Lung Hydroxyproline (µg/lungs)
Effect Size	5.06	1.05	3.70	1.48
Sample Size (N)	3	25	4	13

Figure 3: Mice receiving BLM (n=5) demonstrated significantly reduced Breathing rates on Day 3 post-dose and a significantly increased Breathing rates from Day 7 to Day 16 post-dose (A). Mice receiving BLM demonstrated overall weight loss during the study (B), significantly increased Hydroxyproline levels in lung homogenates (C) and increased Lung to Body Weight ratios (D) compared to mice receiving water (n=5). Effect Sizes were determined from means and standard deviations followed by calculations of required group sizes to achieve Power of 0.90 (E). Values represent means ± SEM. * P≤0.05

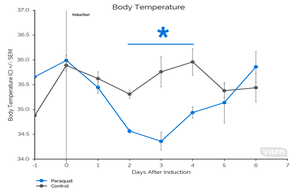
LUNG INJURY

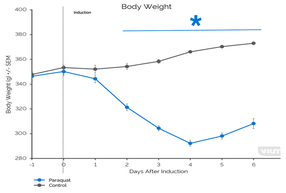


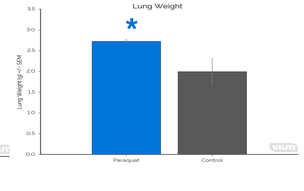


2F	Max Breathing Rate Post- Induction	Min Motion Post-Induction	Min Body Temperature Post-Induction	Min Body Weight Post- Induction	Lung Weights
Effect Size	9.48	5.33	2.23	1.40	3.94
Sample Size (N)	2	3	7	15	4

Figure 2: Rats receiving paraquat (n=10) demonstrated significantly elevated Breathing rates (A) and reduced Nighttime Motion (B), Body Temperature (C), and Body Weight (D), as well as increased Lung weights (E) compared to rats receiving saline (n=10). Effect Sizes were determined from means and standard deviations followed by calculations of required group sizes to achieve Power of 0.90 (F). Values represent means ± SEM. * P≤0.05







CONCLUSION

VIUM Digital Biomarkers result in more powerful experiments:

The VIUM Digital Vivarium, facilitates experiments that fulfill the "**REDUCTION**" principle, utilizing fewer experimental subjects without sacrificing the ability to provide statistical confidence around data interpretation.

- Continuous digital monitoring provided behavioral and physiological indicators of disease severity and progression.
- VIUM Digital Biomarkers provided increased sensitivity over traditional biomarkers, such as:
  - In-life measures (body weight and temperature), enzyme activity (ALT and AST), and protein levels (hydroxyproline)
- Improved sensitivity was generalizable across three disease models involving different organ systems and study durations.

Due to the refined sensitivity of Vium Digital Biomakers, there is greater potential to detect more subtle differences among compounds or dose levels when traditional group sizes are employed.

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