The Effect of the Ketogenic Diet and Estrous Cycling on Inflammatory Pain in C57BL/6J Female Mice

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Introduction

- Chronic pain is defined as pain that persists beyond the normal timeframe of recovery, three to six months (Treede et al., 2015)
- It is estimated that 1 in 5 individuals over the age of 18 experience ongoing pain, with 8% of these individuals reporting a significant impairment in their ability to engage in everyday activities (Kuehn, 2018)
- Growing evidence suggests that inflammation underlies many chronic pain disorders, including multiple sclerosis, fibromyalgia, and inflammatory bowel disease (Marchand et al., 2005)
- Although chronic pain is common, current treatments remain inadequate, posing intolerable side effects or risk of addiction (Stucky et al., 2001)
- The ketogenic diet (KD), a low carbohydrate, moderate protein, high fat diet, may provide anti-inflammatory benefit, and thus be an effective pain management treatment (Ruskin et al., 2009)
- Ketone-based metabolism is known to produce fewer oxidative species than glucose-based metabolism (Veech, 2004)
- Estrous cycle refers to the 4-to-5-day reproductive cycle of rodents, involving fluctuating sex hormones, which has been suggested to affect pain sensitivity (Stoffel et al., 2003)
- This study aims to further characterize the effect of the ketogenic on tactile allodynia in female C57BL/6 mice, while monitoring the estrous cycle to determine possible hormonal influences

Methods

Rodent Population and Diet Treatment

- Female C57BL/6 mice were group-housed (3-5 per cage) and maintained under 12 h:12 h light:dark conditions
- At 6-8 weeks of age, mice were randomly assigned, by cage, to receive either the ketogenic diet (BioServ 3666) treatment or control diet (CD) treatment
- Dietary regiments were initiated three weeks prior to behavioral testing and maintained throughout; both groups were permitted to feed *ad libitum*

Procedures

Electronic von Frey and Complete Freund's Adjuvant Injection

- Plantar tactile sensitivity was assessed using the IITC Life Science Electronic von Frey Anesthesiometer equipped with a rigid tip
- Mice were placed individually within acrylic chambers (20 x 20 x 12 cm) above a gridded wire mesh and habituated for a period of 30 minutes prior to testing
- Rigid tip was slowly raised to contact the mid-plantar surface target of the hind paw; pressure required to elicit a withdraw reflex was recording (g)
- For each mouse, three trials were performed for both the left and right paw, with a minimum of two minutes between repeat measurements
- A minimum of two and maximum of four baselines for both the left and right hind paws were performed over a period of several days prior to Complete Freund's Adjuvant (CFA) injection—a final baseline was performed on the day of CFA injection
- An intraplantar injection (20 µl) of CFA (Thermo Scientific, 0.5 mg/ml) was administered to the right hind paw of each mouse on day 0 of behavioral testing
- Post-injection sactile sensitivity was assessed at 4h, 2d, 4d, 7d or 8d, and 11/12d

Collection and Analysis of Estrous Samples

- Estrous samples were collected from on the day of final baseline/CFA injection
- Vaginal lavage was performed while holding mouse in the scruff position
  - Tip of a 20 mL pipette containing a 0.9% saline solution was gently inserted into the base of the vaginal canal.
  - Fluid was ejected and recollected three times, and then transferred to a glass slide
- Wet mounts were viewed under a microscope to preliminarily determine the stage of estrous and then transferred to a hot plate to dry on low heat
- Estrous slides were stained according to the Shorr method and then analyzed under the 10x objective of a compound microscope (Paccola et al., 2013; Shorr, 1941).

Statistical Analyses

- Data was analyzed using JASP (Version 0.16.1) and expressed as mean ± SEM
- Left paw data for one animal, deemed to be an outlier due to measures of central tendency and variability yielding significant values, were excluded to meet parametric requirements
- A repeated measures ANOVA was conducted to analyze both within and between subject effects of dietary treatment, inflammatory paw condition, and stage of estrous cycle at day of injection
- Post hoc tests for significant factors and interactions were student t-tests protected for multiple comparisons
- Data were considered statistically significant when $p<0.05$

Results

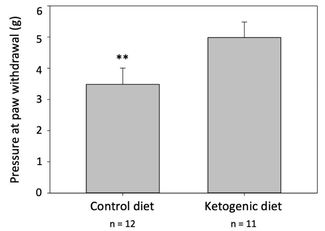

Figure 1. Effect of diet treatment on tactile sensitivity. Ketogenic diet treatment significantly increased the amount of pressure required to elicit a paw withdrawal. Data collapsed across paw and time. **$p < 0.01$

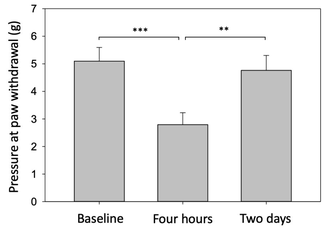

Figure 2. Effect of time on tactile sensitivity. Consistent with the expectation that CFA injection would induce local inflammation of the hindpaw, tactile sensitivity significantly increased 4 hours post-injection. There was no significant difference between baseline and 2-day post-injection sensitivity. **$p < 0.01$

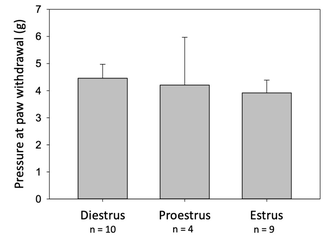

Figure 3. Effect of estrous stage on day of CFA injection on tactile sensitivity. No significant effect of estrous stage on tactile sensitivity was observed. Sample size limited and may have constrained results. Data collapsed across paw, time, and diet treatment.

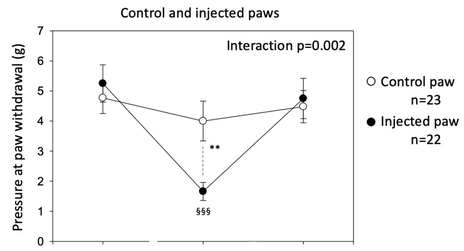

Figure 4. Effect of CFA injection on tactile sensitivity over time. ***$p < 0.001$, significant difference between control and injected paws., §§§$p<0.001$, significant differences between injected paw 4 hours post-CFA injection and injected paw at other time points.

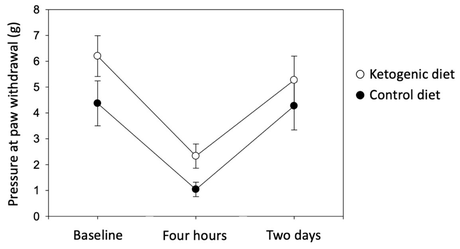

Figure 5. Interaction between diet treatment, paw condition, and time was not found to be significant. KD significantly increased tolerance for hindpaw stimulation across all timepoints, consistent with the main effect of diet treatment, but did not significantly reduce inflammatory pain. The main effect of time is demonstrated by the tactile hypersensitivity 4 hours post-CFA injection, regardless of diet treatment or estrous stage at day of injection. Data collapsed across stage.

Discussion

- KD female mice exhibited a significantly higher paw withdraw latency than CD female mice (Figure 1)
- Although the effect of KD treatment on tactile sensitivity was not significant across time, the KD curve appears to be shifted upward relative to the CD curve (Figure 5)
  - Finding is contrast to the results of Ruskin et al., who found no significant difference in the tactile sensitivity of KD and CD male mice at baseline (2021). KD male mice exhibited a significantly higher paw withdraw latency than CD male mice at post-CFA injection timepoints (4h, 2d, 4d, and 7d)
- Female mice had a much shorter recovery period than male mice
  - All mice (male and female, regardless of diet treatment) exhibited hyperalgesia 4 hours post-CFA injection
  - Within the female mice study, both KD and CD groups recovered to baseline by the 2d timepoint (Figure 2)
  - In contrast, KD male mice did not achieve full recovery until 7d post-CFA injection and CD male mice exhibited only partial recovered by the 7d timepoint (Ruskin et al., 2021)
- In female mice, it is unclear what to term the generalized effect of KD on increased paw withdraw latency. If the von Frey probe is perceived by mice as an innocuous stimulus, causing annoyance or unpleasantness rather than pain, it could be argued that the effect of KD is to increase tolerance or permissiveness
- Study conducted in an osteoarthritic mouse model found parallel sex differences in pain: male, but not female mice had a significant effect of treatment on reducing tactile sensitivity and males experienced a longer duration of recovery (Hwang et al., 2021)

Future work

- We hope to increase sample size to increased statistical power and develop a better understanding of which factors significantly impact tactile sensitivity
- We plan to collect additional physiological data and measurements of inflammatory pain (assessment of paw volume) to supplement our existing work
- To better assess the effect of estrous cycling and sex hormones on inflammatory pain, an ovariectomy and hormone replacement procedure could be down to control for fluctuation
- A future study should test females alongside males to allow for decreased experimental variability and allow for comparisons between baseline tactile sensitivity, recovery duration, and sex-specific effect of treatment on inflammatory pain
- To better characterize the difference in pain perception and behavior between male and female mice additional measures of pain should be implemented, such as computerized analysis of mice facial expressions (Tuttle et al., 2018) or a test of tolerance, such as reaction to innocuous touch stimulation

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