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Modeling chronic bladder pain in male and female mice: Exploring the chronicity of repeated cyclophosphamide injections.

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INTRODUCTION

Chronic bladder pain syndromes such as interstitial cystitis/bladder pain syndrome (IC/BPS) and chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) affect patients with generalized pelvic pain, frequent urination, and nocturia in the absence of infection^{1,2}. The etiologies of these syndromes are unknown, thus the sufficiency of current therapy methods is hindered³. In order to learn more about chronic bladder pain and develop effective treatments, animal models are designed to recapitulate bladder pain symptoms in rodents⁴. In our studies, we focused on a specific model in which male and female mice are repeatedly administered a low dose injection of a chemotherapeutic agent, cyclophosphamide (CYP)^{3,5}. After cyclophosphamide is injected into the abdominal cavity it metabolizes into the bladder irritant, acrolein, and induces bladder nociception (i.e. pain)⁶.

In addition to bladder nociception, repeated cyclophosphamide injections have been shown to increase bladder weight, decrease body weight, and increase bladder tissue thickness^{3,7}. We do not know, however, how long these effects persist. It is important to fully understand the time parameters of this model so that we can establish one that is more representative of chronic bladder pain syndromes. Therefore we explored the chronicity of repeated cyclophosphamide injections in both male and female mice after injecting them with 100 mg/kg of cyclophosphamide every other day for five days. Throughout our experiments we measured the animals' referred abdominal hypersensitivity levels, body, bladder, and prostate weights, and tissue thicknesses. We hypothesized that the effects of this model would last for at least seven days¹⁰.

METHODS

Cyclophosphamide Administration

IC/BPS-like symptoms were induced by administering 100 mg/kg CYP (dissolved in 0.9% saline) intraperitoneally on days 1, 3, and 5. CYP was purchased from Sigma. Control animals received a 0.9% saline intraperitoneal injection on days 1, 3, and 5. Subsequent behavioral testing/tissue collection was completed on days 6 and 13.

Abdominal Hypersensitivity Test

Referred bladder pain was measured by assessing abdominal mechanical hypersensitivity. Two days prior to testing the mice were anesthetized with isoflurane and their abdominal regions were shaved to prevent hair from interfering with testing. On the days of testing, the animals habituated in plexiglass boxes that were separated with plastic dividers for approximately 90 minutes, and then for another 30 minutes with the experimenter in the room. Calibrated von Frey filaments were applied to the shaved abdomen in a graded fashion in order to determine the pressure at which the animal withdrew its abdomen 50% of the time (Figure 1.). The baseline 50% withdrawal threshold was determined on day 0 in both male and female mice (genders were tested on different days) using the previously published up/down method⁹. The abdominal region was probed approximately 0.5cm away from the urethra on both the left and right side, and two trials were performed on each side. This process was repeated one and seven days following the final injection of cyclophosphamide (days 6 and 13).



Figure 1. Mechanical hypersensitivity test set-up. Mechanical hypersensitivity tests were performed using von Frey filaments. The mice were kept in a cage with a wire floor to allow abdominal probing.

Body, Bladder, and Prostate Weights

The body weight of each animal was measured before von Frey testing on day 0, 6, and 13. On day 13 the animals were injected with Euthasol and were sacrificed. Their bladders were immediately harvested and weighed. Additionally, the males' prostates were harvested and weighed. The bladders and prostates were then fixed overnight in paraformaldehyde, cryopreserved in 20% sucrose for approximately two days, frozen in OCT, and stored at -80°C.

Bladder Histology

Bladder histology was performed to determine whether the repeated cyclophosphamide injections resulted in a change in bladder tissue morphology. To assess the effects, the bladders were cut into 30µm sections, mounted onto glass slides, and stained using an optimized hematoxylin and eosin staining procedure. In this procedure, the slides were stained with hematoxylin for four minutes, rinsed under tap water for five minutes, incubated in acid alcohol (95% ethanol, 10% acetic acid) for one minute, rinsed under tap water for one minute, and stained with eosin for five seconds. The slides were then dipped into deionized water to rinse off excess eosin, and finally they were dehydrated using 50%, 75%, 95%, and 100% ethanol.

A light microscope was used to take pictures of the bladder sections at 40x magnification so that the thickness of the various tissue layers could be measured using imaging analysis. Four bladders from each treatment group and three sections from each bladder were used to acquire an average thickness of each tissue layer. Image J software was used to measure the thickness of the urothelium, submucosa, and muscularis layers in pixels (Figure 2.). The measurements were converted to micrometers.

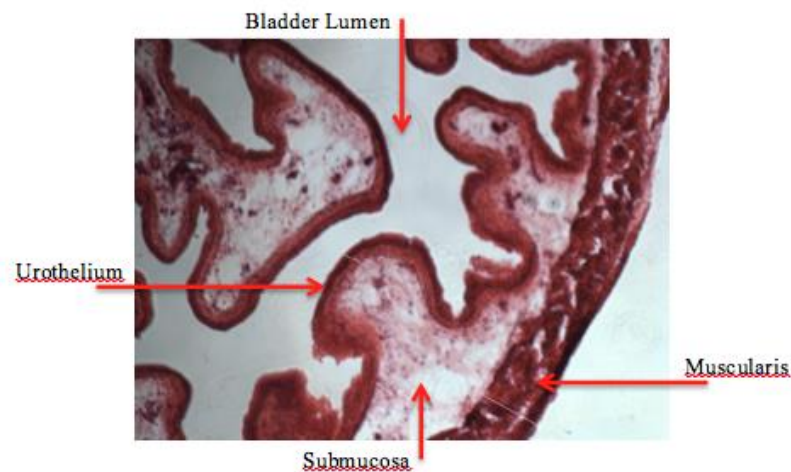


Figure 2. Bladder Tissue Anatomy. Hematoxylin and eosin staining exposed the various tissue layers within the bladder. The white region is the hollow bladder lumen. The three main layers of the bladder include (listed from deep to superficial) the urothelium, submucosa, and the muscularis.

RESULTS

Repeated CYP Injections Induce Referred Abdominal Hypersensitivity in Females for at Least Seven Days.

In both male and female mice, abdominal von Frey tests were performed on day 0, 6, and 13 to assess the degree of referred bladder pain. CYP-treated females exhibited significant increases in referred bladder pain (i.e. decreases in 50% withdrawal thresholds) when compared to saline-treated females (Figure 3; Two-way ANOVA, main effect of treatment $p < 0.0001$, main effect of interaction $p = 0.0202$). Bonferroni's posttest revealed that the withdrawal threshold on day 6 was significantly lower in the CYP-treated females than in the saline-treated females ($p < 0.001$). Thus, this data suggests that repeated CYP injections induce referred abdominal hypersensitivity in female mice for at least seven days.

CYP treatment failed to induce the same increases in referred bladder pain in male mice (Figure 4.; Two-way ANOVA, effect of treatment $p = 0.1558$) Therefore, in this experiment repeated CYP injections did not induce referred abdominal hypersensitivity.

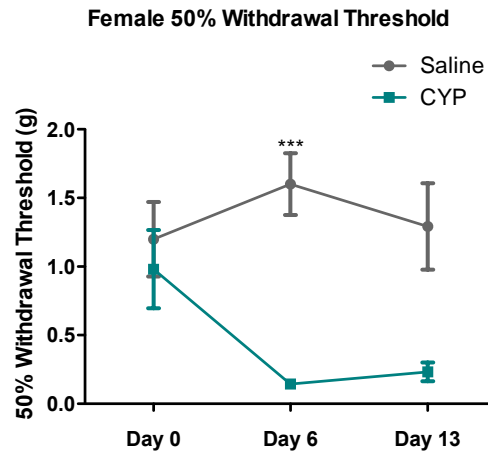


Figure 3. Repeated CYP injections induce referred abdominal hypersensitivity in females for at least seven days. Female mice underwent abdominal von Frey tests on days 0, 6, and 13. A two-way ANOVA showed a main effect of treatment ($p < 0.0001$) and interaction ($p = 0.0202$). Bonferroni's posttest showed a significantly lower 50% withdrawal threshold in CYP-treated mice compared to saline-treated mice on day 6 ($p < 0.001$). A similar trend was seen on day 13 (without significance) (day 0 and 6 $n = 12$, day 13 $n = 6$).

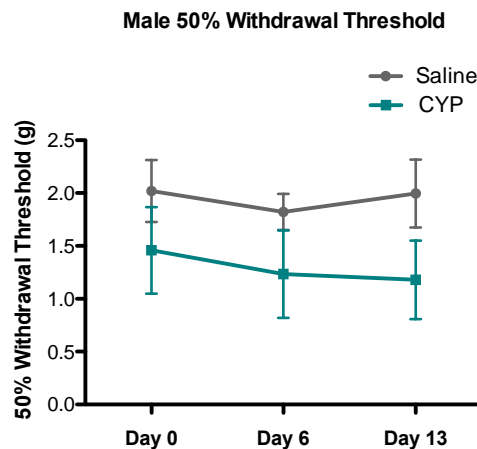


Figure 4. Repeated CYP injections did not induce referred abdominal hypersensitivity in male mice. Male mice underwent abdominal von Frey on days 0, 6, and 13. A two-way ANOVA showed that there was no change in 50% withdrawal threshold in either group ($p = 0.1558$). Bonferroni's posttest revealed that there was no statistical difference between the 50% withdrawal thresholds in the CYP and saline-treated mice on days 0, 6, and 13 (day 0, 6, and 13 $n = 12$).

Repeated CYP Injections Cause the Changes in Body, Bladder, and Prostate Weights to Last for at Least Seven Days in Both Genders

The animals' body weights were measured on day 0, 6, and 13. In both genders, CYP treatment significantly decreased body weight 6 and 13 days following the start of treatment (Figure 5.; Females:

Two-way ANOVA, effect of treatment $p < 0.0001$, Bonferroni's posttest on day 6 and 13 $p < 0.001$; Males: Two-way ANOVA, effect of treatment $p < 0.0001$, Bonferroni's posttest on day 6 and 13 $p < 0.001$). On day 13 the bladders were harvested and weighed. The CYP-treated females had significantly larger bladder weights than the saline-treated females (Figure 6.; unpaired t-test $p = 0.0007$), while the males did not show changes in bladder weight (data not shown). The males' prostates were also collected and weighed on day 13, and the CYP-treated males had significantly lower prostate weights than those of the saline-treated males (Figure 7.; unpaired t-test $p = 0.0304$). This data suggests that the changes observed in body, bladder, and prostate weights are maintained in both male and female mice for at least seven days.

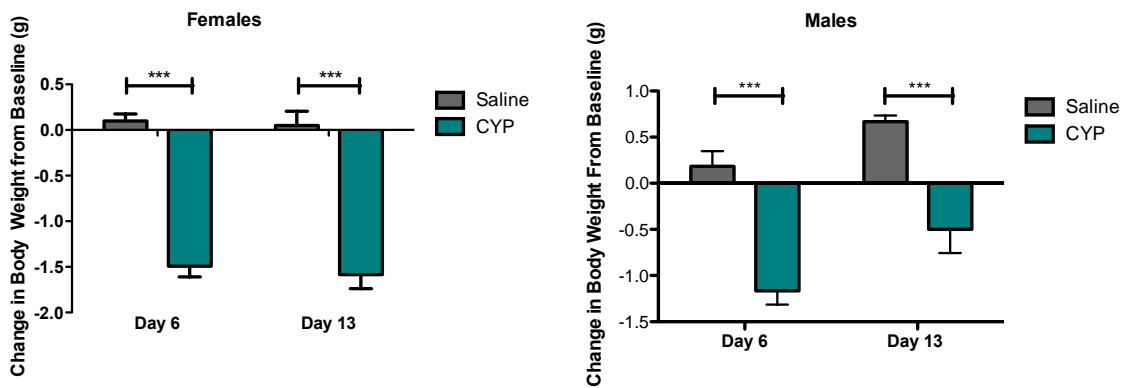


Figure 5. Decreases in body weights were maintained for at least seven days. The male and female body weights were measured on days 6 and 13. Females: A two-way ANOVA showed an effect of treatment ($p < 0.0001$). Bonferroni's posttest showed that the CYP-treated females had significantly larger decreases in body weight than the saline-treated females on day 6 and 13 ($p < 0.0001$). Males: A two-way ANOVA showed an effect of treatment ($p < 0.0001$). Bonferroni's posttest showed that CYP-treated males had significantly larger decreases in body weight than saline-treated males on day 6 and 13 ($p < 0.0001$) (day 6 $n = 12$, day 13 male $n = 12$, day 13 female $n = 6$).

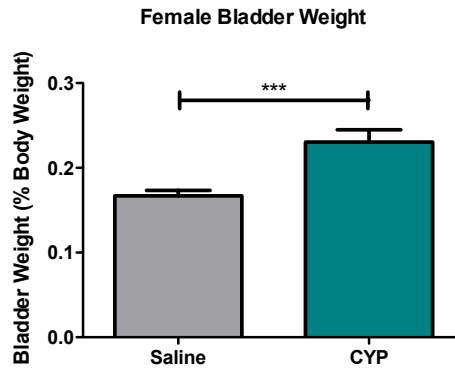


Figure 6. Female bladder weight increases were maintained for at least seven days. Female bladders were harvested and weighed on day 13. An unpaired t-test showed that the CYP-treated bladders had significantly larger weights than the saline-treated bladders ($p = 0.0007$) ($n=6$).

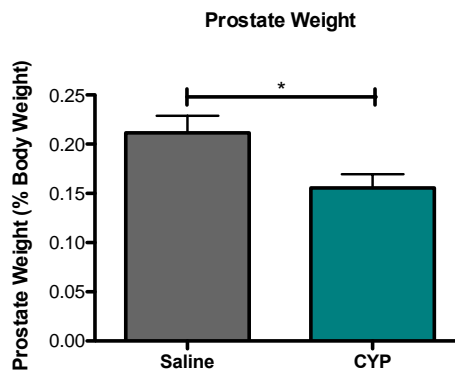


Figure 7. Decreases in prostate weights were maintained for at least seven days. The male prostates were harvested and weighed on day 13. An unpaired t-test revealed that CYP-treated males had prostates with significantly lower weights compared to the saline-treated males ($p = 0.0304$).

Repeated CYP Injections Cause Increases in Bladder Tissue Thickness that Lasts for at Least Seven Days

After the female bladders were harvested and preserved, they were sectioned, stained, and analyzed to explore changes in bladder tissue thicknesses. CYP treatment increased urothelium thickness (Figure 8.; One-way ANOVA $p=0.0047$, Bonferroni's posttest on day 6 $p<0.01$, on day 13 $p<0.05$), submucosa thickness (Figure 8.; One-way ANOVA $p=0.0004$, Bonferroni's posttest on day 13 $p<0.0001$), and muscularis thickness (Figure 8.; One-way ANOVA $p=0.0013$, Bonferroni's posttest on day 6 $p<0.01$, on day 13 $p<0.05$) for at least seven days following the start of drug treatment. These procedures have not

yet been completed in the male mice bladders, but they suggest that repeated low-dose administration of CYP induces long lasting changes in bladder morphology.

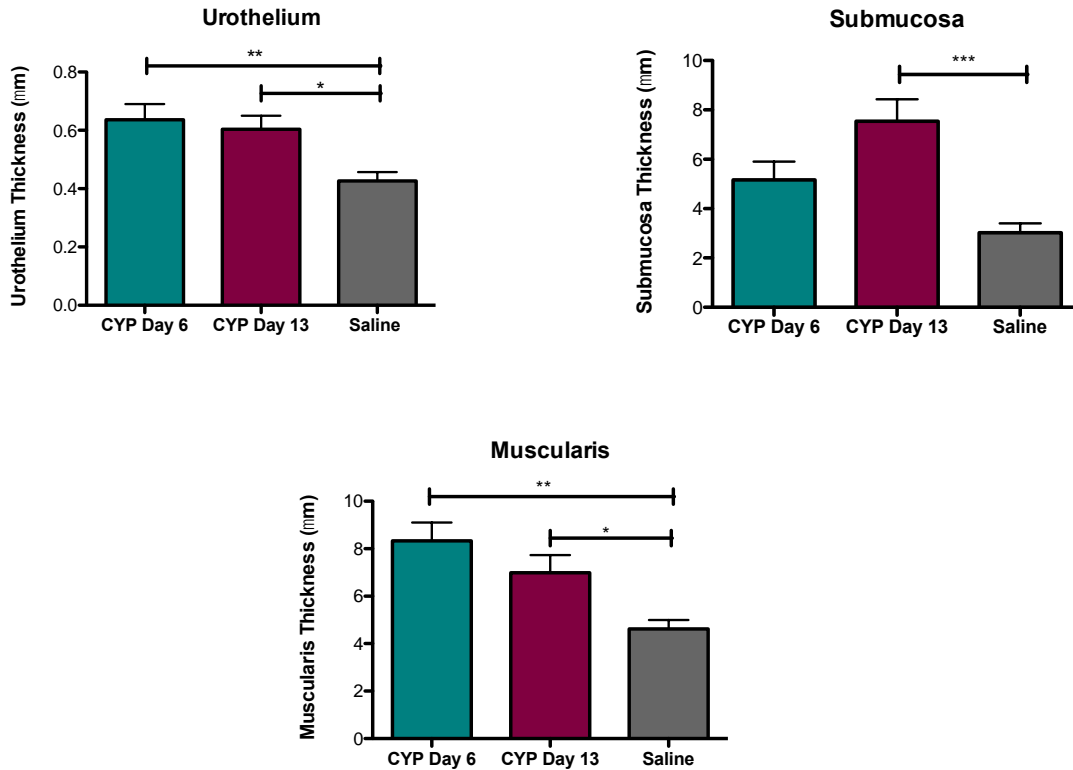


Figure 8. Repeated CYP injections induced an increase in bladder tissue thickness for at least seven days in females. Female bladders were cut into 30 μ m sections and stained. The thickness of the bladder tissue layers (the urothelium, submucosa, and muscularis) was measured. A one-way ANOVA revealed that each layer increased in thickness for at least seven days following CYP treatment (urothelium $p=0.0047$; submucosa $p=0.0004$; muscularis $p=0.0013$) Bonferroni's posttests showed that these tissue layers in CYP-treated mice were significantly thicker on both day 6 and 13 ($p<0.05$, $p<0.01$, $p<0.0001$).

DISCUSSION

In these experiments we investigated how long the effects of repeated CYP injections persist in both male and female mice. We found that the effects, such as increases in referred abdominal hypersensitivity, changes in body, bladder, and prostate weights, and increases in bladder tissue thickness last in both genders for at least seven days. In the male mice, however, we did not see an effect of repeated CYP injections in the context of referred abdominal hypersensitivity, even though there was a decrease in body and prostate weight. One potential explanation for this inconsistency is that the male

mice displayed baseline withdrawal thresholds that were higher than the von Frey filaments used in the experiments. To further explain the effects that repeated CYP injections have on referred abdominal hypersensitivity in male mice, the procedure described above will be repeated. If similar results are attained, the extent of male baseline withdrawal thresholds will be explored to determine if larger filaments must be used.

FUTURE DIRECTIONS

We plan to fully characterize the time parameters of this model and identify the latest time point at which it has an effect in male and female mice. Additionally, using our understandings of the model's timeline, we plan to develop a model of chronic bladder pain in which the animals are exposed to early-life stress before they receive CYP injections, as many bladder pain patients report stressful childhoods⁸. It is hopeful that through these efforts we will develop a model of chronic bladder pain that accurately represents clinical symptomology, thus enabling researchers to explore the etiologies of bladder pain syndromes.

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