

Original Article

Swimming Exercise Attenuates Mechanical Hypersensitivity and Mitigates

Peripheral Nerve Degeneration in Rats with Painful Diabetic Neuropathy (PDN)

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Highlights

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- Swimming mitigated mechanical hypersensitivity in rats with PDN.

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- Rats performing swimming exercise retained higher IENFD.

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- Swimming exercise may mitigate peripheral nerve degeneration in rats with PDN.

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Abstract

Background: This study aimed to assess the effectiveness of swimming exercise in alleviating mechanical hypersensitivity and peripheral nerve degeneration associated with a pre-clinical model of painful diabetic neuropathy (PDN).

Methods: This study is a pre-clinical study conducted using the streptozocin (STZ)-induced PDN rat model. Rats were randomly allocated to three groups: a vehicle group of non-diabetic rats (Vehicle, n=9), a group of rats with PDN (PDN, n=8), and a group of rats with PDN that performed a swimming exercise program (PDN-SW, n=10). The swimming exercise program included daily 30-minute swimming exercise, 5 days per week for 4 weeks. Von Frey testing was used to monitor hindpaw mechanical sensitivity over 4 weeks. Assessment of cutaneous peripheral nerve fiber integrity was performed after the 4-week study period via immunohistochemistry for protein gene product 9.5-positive (PGP9.5+) intra-epidermal nerve fiber density (IENFD) in hind-paw skin biopsies by a blinded investigator.

Results: The results showed that swimming exercise mitigated but did not fully reverse mechanical hypersensitivity in rats with PDN. Immunohistochemical testing revealed that the rats in the PDN-SW group retained higher PGP9.5+ IENFD compared to the PDN group but did not reach normal levels of the Vehicle group.

74 **Conclusions:** The results of this study indicate that swimming exercise can mitigate
75 mechanical hypersensitivity and degeneration of peripheral nerve fibers in rats with
76 experimental PDN.

77 **Keywords (6)**

78 Diabetes, Diabetic neuropathy, swimming, physical exercise, intra-epidermal nerve fiber
79 density (IENFD)

80

Introduction

Painful diabetic neuropathy (PDN) is a common complication among patients with diabetes [9]. Unfortunately, current treatments for PDN are costly, can only partially relieve pain and often produce unwanted side effects [5, 13]. Therefore, a cost-effective management program is needed to improve the care of patients with PDN [23].

A key limiting factor of current medications for PDN is that they do not target the underlying mechanism that causes PDN, such as the progressive degeneration and concomitant regeneration of peripheral nerve fibers [11, 12]. Peripheral nerve degeneration is well documented in patients with diabetes. Patients often have slower nerve conduction velocity (NCV) and reduced intra-epidermal nerve fiber density (IENFD) in skin biopsies [11, 12]. Of note, degeneration of peripheral nerves often co-exists with peripheral nerve regeneration in patients with diabetic neuropathy [4, 22]. Current consensus is that the active degeneration of axons and the concomitant regeneration of unmyelinated fibers are major contributors to the pain associated with PDN [7]. Thus, a management strategy aimed at mitigating the progressive neural degeneration would be desirable for managing PDN [11].

Several studies have suggested that physical exercises may alleviate pain in patients [19] and pre-clinical models of PDN [2, 6, 15, 24, 26]. In addition, several pre-clinical

studies showed that physical exercise may mitigate the degeneration of peripheral nerve fibers in animals with PDN [8, 25], and one clinical study suggested that physical exercise may facilitate the regeneration of nerve fibers in patients with PDN [19].

However, these studies predominantly use land-based exercise, while the effect of swimming and aquatic exercise are not well studied. In the few studies that investigated the effect of swimming in pre-clinical models of PDN, it was shown that swimming alleviates mechanical and thermal hypersensitivity in rodent models of PDN [2, 24].

Some studies also showed that swimming is associated with improved nerve conduction velocity [21], or reduced severity of nerve degeneration [8, 25]. However, the effect of swimming on peripheral nerve degeneration associated with PDN has not been investigated using quantitative immunohistochemical methodology (e.g. IENFD). The aim of this study was therefore to evaluate the effect of swimming exercise on mechanical hypersensitivity and degeneration of peripheral nerve fibers associated with experimental PDN.

Methods and materials

Ethics Approval

This study was approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

117 *Animal model*

118 The streptozocin (STZ)-induced PDN rat model was used in this study. Adult, male
119 Sprague-Dawley rats that weighed between 280-300g were purchased from BioLASCO
120 (Yilan, Taiwan). Two to three rats were housed in plastic cages with shredded wood chips
121 and wooden toys. The housing facility maintained a constant temperature of $25\pm 2^{\circ}\text{C}$,
122 humidity of 50%, and a 12-hour day/night cycle. Unless otherwise specified, food and
123 water supplies were not limited.

124 The rats were randomly assigned using Microsoft Excel to receive STZ or normal
125 saline injection one day before the injection procedure. The rats were fasted overnight
126 before surgery, and anesthetized using intra-muscular injection of Zoletil (10mg/kg) and
127 Xylazine (5mg/kg), and STZ(65mg/kg) or normal saline was injected into the femoral
128 vein. On day three after injection, a blood glucose test was performed, and rats with a
129 fasting blood glucose level over 300mg/dl were considered to have developed diabetes.

130 *Study procedure*

131 Figure 1A summarizes the experimental procedure. Before STZ injection, rats
132 received two days each of familiarization in the test environment and swimming pool.
133 Thereafter, two baseline behavioral tests were performed at two and one days before
134 STZ/saline injection and their averages were used as baseline behavioral data. After the

injection of STZ/saline, five confirmatory behavioral tests were performed on the third to seventh day after injection. Rats with diabetes and reduced mechanical response threshold compared to baseline data at day 7 post-injection were considered to have developed PDN [6]. Rats that failed to develop PDN were withdrawn from the study, while rats with PDN were randomly allocated to PDN or PDN-Swimming (PDN-SW) group. The rats that received normal saline injection were designated as the Vehicle group.

Throughout the study, behavioral tests were performed to monitor the rats' sensory profiles. Rats in the Vehicle and PDN groups only underwent behavioral tests whilst the rats in PDN-SW group received a 4-week swimming exercise program (Figure 1A).

Swimming exercise program

The swimming exercise program included daily 30-minute swimming exercise, 5 days per week for 4 weeks. The swimming pool was a plastic pool 75x50x60 centimeters in dimension. The depth of the water was controlled at 20 ± 2 centimeters and the water temperature was maintained at $25\pm 2^{\circ}\text{C}$. After swimming exercise, the rats were dried with towels and placed in a cage with a heat lamp to recover.

Behavioral test

Von Frey test was used to measure the mechanical response threshold of both hind paws. The method was based on the simplified up-down method [3] and was described in

our previous studies [6, 31]. In brief, von Frey filaments weighing 0.6-26g were used to stimulate the center of walking pads in the hind paw, an area innervated by the tibial nerves [10]. The test was repeated twice for each foot. Results were averaged for each foot, and the mechanical response thresholds of left and right feet were averaged for each rat[6]. To accommodate for the non-linearity of the von Frey filaments, the averaged mechanical response threshold was converted into a Log₁₀ scale for analysis.

Skin biopsies and measurement of intra-epidermal nerve fiber density (IENFD)

At the end of the study, the rats were perfused transcardially with normal saline, followed by 30 minutes of 4% paraformaldehyde solution. Skin samples from the center of the hind paw walking pads were collected bilaterally and preserved in 4% paraformaldehyde solution for 24 hours, followed by cryopreservation in 30% sucrose solution for 72 hours before embedding in Optimal Cutting Temperature compound and storage at -80 degrees.

Skin samples were cut into 14 micrometer (μm) sections on a cryostat and mounted on gelatinized slides. The slides were dried at room temperature for an hour before adding a blocking solution and incubating at room temperature for 2 hours. Table 1 summarizes the antibodies and solutions used in this study. Primary protein gene product 9.5 (PGP9.5, a pan axonal marker) antibody was added onto the slides and incubated in a

4°C cold room overnight. On the next day, the sections were washed using a washing buffer before biotinylated antibody was added onto the sections and incubated for 2 hours at room temperature. The sections were washed again, and secondary streptavidin fluorescent antibody was incubated in the dark at 4°C for 2 hours. After another wash, mounting medium (Vectashield, Vector laboratories, Burlingame, CA, U.S.A.) was added and the slides were sealed for microscopy.

The sections were analyzed and photographed under a fluorescence microscope (Olympus BX51/DB80, Center Valley, PA). An investigator blinded to group allocation counted the intra-epidermal nerve fibers (IENFs) marked by PGP9.5 crossing the dermal-epidermal border down the microscope, adhering to published principles [20]. The length of epidermis was measured using CellSense software (Olympus, Center Valley, PA). The intra-epidermal nerve fiber density (IENFD, fibers/mm epidermis) was calculated by dividing the number of IENFs by the total length of epidermis. The IENFD from three sections were averaged for each paw, and the data from left and right sides were averaged for each rat.

Statistical analysis and sample size determination

We based animal numbers on our previous work with a similar experimental paradigm, which demonstrated that n=9-10 rats were sufficient to detect behavioral and

189 IENFD changes following neurodynamic intervention in animals with PDN [31].

190 Statistical analysis was performed using SPSS 17.0 (IBM, Armonk, NY, USA). The

191 behavioral data was analyzed using two-way (time, group) mixed model analyses of

192 variance (ANOVA). Tests that showed significant time*group interaction were followed

193 by post-hoc Fisher's test to compare the behavioral data 1) between different timepoints

194 within each group and 2) between groups at each timepoint. The IENFD data were

195 analyzed using one-way ANOVA and post-hoc Fisher's test to evaluate group differences.

196 The normality of the data was evaluated by inspecting histograms, and the assumption of

197 sphericity was evaluated by Mauchly's test. When the assumption of sphericity was not

198 met, the main effect was reported with Huynh-Feldt ($\epsilon > 0.75$) or Greenhouse-Geisser

199 correction ($\epsilon < 0.75$). Level of significance was set at $p < 0.05$.

200 **Results**

201 *Number of animals used in this study*

202 In total, 37 rats were used for the experiment, 10 rats were excluded (Figure 1B).

203 The remaining 27 rats were included in this study: 9, 8, and 10 rats in the Vehicle, PDN,

204 and PDN-SW groups respectively.

205 *Rats with PDN developed mechanical hypersensitivity*

206 The two-way mixed ANOVA test revealed a significant time*group interaction for

the mechanical response threshold. Within group post-hoc analysis showed that the mechanical response threshold of both PDN and PDN-SW groups were significantly decreased compared to the baseline data from the third day after injection, and this was maintained to the end of the study ($p<0.001$, Figure 2). For the Vehicle group, the mechanical response threshold remained unchanged throughout the study (Figure 2). Post-hoc analysis of the group difference at different timepoints confirmed that the mechanical response threshold of PDN and PDN-SW groups were comparable to the Vehicle group at baseline ($p=0.642$, $p=0.374$), but their data from the third day after injection to the end of the study were consistently lower compared to the Vehicle group ($p<0.001$, Figure 2)

These results indicate that the rats with PDN developed significant and continuing mechanical hypersensitivity, similar to the findings in other studies using the same model [14, 31].

Swimming exercise alleviated mechanical hypersensitivity in rats with PDN

The results of post-hoc Fisher's test showed that, except for a few timepoints (day 18, 21 and 24 after injection), the mechanical response thresholds of the PDN-SW group were significantly higher compared to the pre-treatment data (day 7 after STZ injection) after day 15 post-injection ($p<0.05$, Figure 2). In contrast, the mechanical response

thresholds of the PDN and Vehicle groups at post-treatment timepoints remained largely unchanged compared to the pre-treatment data ($p>0.05$). It is worth noting that the mechanical response thresholds of the PDN group were significantly lower compared to pre-treatment data at several post-treatment timepoints (days 15, 21 and 33), suggesting that the mechanical hypersensitivity in rats of the PDN group may further deteriorate over time ($p=0.038$, $p=0.031$ and $p=0.047$ for days 15, 21 and 33).

We also compared the mechanical response thresholds among the three groups at each timepoint using post-hoc Fisher's test. The mechanical response threshold of the PDN-SW group was significantly higher compared to the PDN group at days 15, 29 and 32 through 34 ($p=0.001$ for day 15; $p=0.006$ for day 29; $p=0.011$, 0.003 and 0.018 for days 32-34 respectively). However, it was still significantly lower compared to the Vehicle group at all timepoints after STZ injection ($p<0.05$).

These results indicate that the regular swimming exercise can gradually alleviate but not fully reverse mechanical hypersensitivity in rats with PDN.

Swimming exercise retained higher PGP9.5+ IENFD in rats with PDN

One-way ANOVA revealed a significant difference among the three groups for the IENFD data ($p<0.001$). Post-hoc Fisher's test showed that the IENFD of the PDN and PDN-SW groups were significantly lower compared to the Vehicle group

($p < 0.001$ / $p = 0.001$, Figure 3). This indicated that the rats in both PDN groups had extensive degeneration of intra-epidermal nerve fibers as previously reported [7].

However, the IENFD of the PDN-SW group was significantly higher compared to the PDN group ($p < 0.001$, Figure 3). This suggests that swimming exercise mitigates the progression of peripheral nerve degeneration in rats with PDN.

Discussion

The results of this study demonstrate that swimming exercise ameliorates mechanical hypersensitivity, and mitigated the degeneration of cutaneous peripheral nerve fibers in a preclinical model of PDN.

Our findings that swimming exercises ameliorated mechanical hypersensitivity associated with PDN is similar to previous studies that utilized land-based exercises in rodent models of PDN [6, 15, 26]. In previous studies investigating swimming in PDN models, it was shown that 8-10 weeks of swimming intervention alleviated thermal hypersensitivity [2, 24]. However, these studies did not measure the effect of swimming on mechanical hypersensitivity. Our findings of alleviation of mechanical hypersensitivity complement previous work.

Although our findings confirm that 4-week swimming exercise can ameliorate mechanical hypersensitivity in rats with PDN, it remains unclear whether this effect can

be maintained. Two previous studies suggest that although aerobic exercise can mitigate mechanical hypersensitivity in rats with early STZ-induced PDN, the hypoalgesic effects were not significant at 6-10 weeks after STZ injection [6, 26]. We did not include timepoints beyond the fifth week after STZ injection, since the STZ model is often associated with serious long-term complications such as cataracts or foot wounds that may interfere with behavioral tests [30]. herefore, the long-term effects of swimming exercise and their effects in longstanding PDN require further study.

The results from our histological analysis showed that the rats in the PDN-SW group had retained higher IENFD compared to the rats in the PDN group. Previous studies have suggested that swimming may have a pro-regenerative effect on neurons in the context of diabetic neuropathy [8, 21, 25]. These studies showed that swimming exercise can improve nerve conduction velocity [21] and the amplitude of compound motor action potential [25], as well as maintain the integrity of myelinated nerve fibers [8, 25].

Here we show that swimming mitigates the degeneration of small cutaneous fibers, which is a hallmark of diabetic neuropathy [29]. Similar findings were also shown in pre-clinical and clinical studies that utilized other aerobic exercises such as treadmill and walking [16, 19, 28], suggesting that physical exercise has a pro-regenerative effect on peripheral nerve fibers in patients or pre-clinical models of PDN.

The findings in our study indicated that swimming had reduced, but did not fully prevent the degeneration of peripheral nerve fibers in animals with PDN. Given the short timespan between the injection of STZ and the collection of skin samples, as well as the fact that regeneration of nerve fibers was associated with hypersensitivity in PDN [7, 22], we believe that the higher IENFD observed in the rats of PDN-SW group was not the result of an isolated regenerative effect. Rather it is suggestive of a protective effect that prevents the degeneration of cutaneous nerve fibers [11]. This is in line with our previous findings of mitigation of nerve degeneration using neural mobilization in rats with PDN [31]. Our findings, along with findings in previous studies suggested that physical exercises, including swimming, can mitigate the progress of peripheral nerve degeneration related to PDN. Further studies that investigate the optimal intensity, duration, and length of exercise interventions are needed to translate the findings into exercise prescriptions for clinical use.

Limitations

We did not include female animals to minimize the potential influence of the estrous cycle on the behavioral tests [18]. This decision limits the generalizability of our study as sex differences exist in preclinical models and patients with PDN [1, 17]. Due to logistical issues, we could not blind the behavioral analyses, which therefore reduces

confidence in these findings. Nevertheless, the hypoalgesic effect of swimming identified here is in line with previous findings from blinded analyses [27]. Importantly, the IENFD analyses were thoroughly blinded. Although our study provided valuable evidence for the beneficial effects of exercise in a preclinical model of PDN, studies are needed to examine their effect in patients with PDN.

Conclusion

Our study revealed that swimming exercise is effective in reducing mechanical hypersensitivity in rats with experimental PDN. In addition, swimming exercise mitigates the degeneration of cutaneous nerve fibers.

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Tables

Table 1. Summary of antibodies and solutions used for immunohistochemistry analysis in this study.

Antibody	Specification	Dilution ratio	Manufacturer
Bio-rad 7863-1004 Protein Gene Product 9.5 (PGP9.5) antibody 31A3 clone	Mouse anti-human	1:200	Bio-rad (CA, U.S.A.)
Vector lab BA2001 Biotinlyated antibody	Horse anti-mouse	1:100	Vector laboratories (CA, U.S.A.)
Invitrogen S11223 Alexa 488 Streptavidin fluorescent antibody	Streptavidin	1:500	Invitrogen (CA, U.S.A.)
Solution	Content		
Blocking Solution	5% normal donkey serum, 1% bovine serum albumin, 1% dimethyl sulfoxide, 0.5% milk powder, 0.3% Triton X-100, 0.1% sodium azide in PBS		
Washing Buffer	0.05% Triton X-100 in PBS		

Figure legends

Figure 1. Study procedure (A) and animal usage (B).]

Figure 2. Behavioral von Frey data. The x-axis represents time (days after STZ/Saline injection) and the y-axis represents the mechanical response threshold expressed in log(10) scale. Data are shown as mean (SD) with 9, 8 and 10 rats in Vehicle, PDN and PDN-SW group. # $p < 0.05$ compared to pre-treatment data (day 7 post-injection) of the same group, * $p < 0.05$ compared to PDN group at the same time point.

Figure 3. Representative protein gene product 9.5 (PGP9.5) immunohistochemical staining of a rat plantar hind paw skin of the Vehicle group (A), PDN group (B) and PDN-SW group (C). PGP9.5-positive nerve fibers crossing into the epidermis are marked with arrows. Figure (D) presents the quantified data of PGP9.5+ IENFD of the three groups. Data are shown in mean (SD) with 9, 8 and 10 rats in Vehicle, PDN and PDN-SW group. * $p < 0.05$ compared to the PDN group, # $p < 0.05$ compared to Vehicle group.

Figure 1

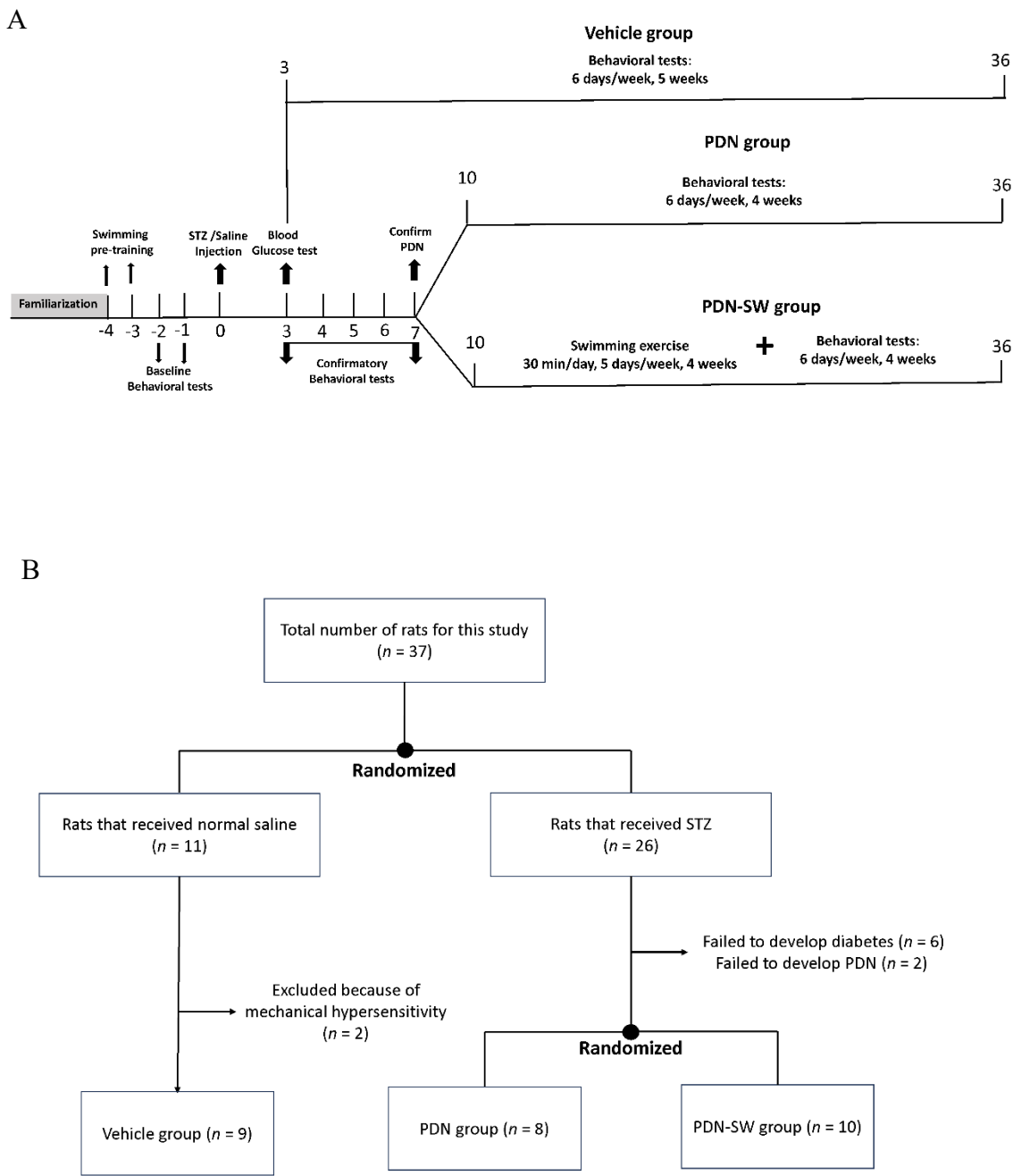


Figure 2

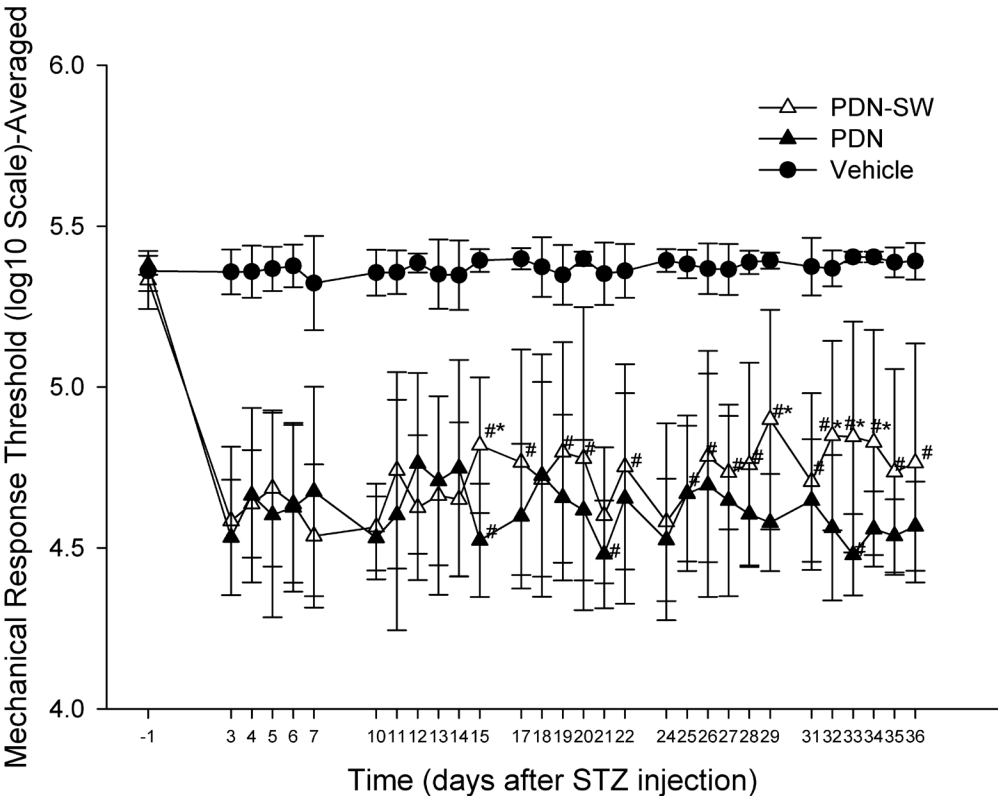


Figure 3

