Sex Differences in Organophosphate Model of Benzodiazepine-Refractory Status Epilepticus and Neuronal Damage

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DFP, diisopropylfluorophosphate; EEG, electroencephalogram; FJB, fluoro-jade B; MDZ, midazolam;
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Abstract

Sex differences are common in human epilepsy. Although men are more susceptible to seizure than women, the mechanisms underlying sex-specific vulnerabilities to seizure are unclear. The organophosphate (OP), diisopropylfluorophosphate (DFP), is known to cause neurotoxicity and status epilepticus (SE), a serious neurologic condition that causes prolonged seizures and brain damage. Current therapies for OP poisoning and SE do not consider neuronal variations between male and female brains. Therefore, we investigated sex-dependent differences in electrographic seizure activity and neuronal injury using the DFP model of refractory SE in rats. EEG recordings were used to monitor DFP-induced SE, and the extent of brain injury was determined using fluoro-jade-B staining to detect cellular necrosis. After DFP exposure, we observed striking sex-dependent differences in SE and seizure activity patterns as well as protective responses to midazolam treatment. Following acute DFP exposure, male animals displayed more severe SE with intense epileptiform spiking and greater mortality than females. In contrast, we observed significantly more injured cells and cellular necrosis in the hippocampus and other brain regions in females than in males. We also observed extensive neuronal injury in the somatosensory cortex of males. The anticonvulsant effect of midazolam against SE was limited in this model and found to be similar in males and females. However, unlike males, females exhibited substantially more protection against neuronal damage after midazolam treatment. Overall, these results demonstrate significant sex-dependent differences in DFP-induced refractory SE and neuronal damage patterns, suggesting that it may be possible to develop sex-specific neuroprotective strategies for OP intoxication and refractory SE.

Significance Statement

Sex-dependent differences in neurotoxicity and SE are key biological variables after OP exposure. Here, we investigated sex-dependent differences in SE and brain injury after acute DFP exposure. Male rats had more severe SE and less survival than females, while females had more neuronal damage. Females had more neuroprotection to midazolam than males, while both sexes had similar but partial anticonvulsant effects. These findings suggest that a sex-specific therapeutic approach may prevent neurological complications of OP-induced SE.
Introduction

Status epilepticus (SE) is a serious neurologic condition in which seizures lasting more than five minutes, or multiple seizures, occur without recovering consciousness in between. In the United States, approximately 200,000 SE cases occur annually, resulting in approximately 55,000 deaths (DeLorenzo et al., 2009). The incidence of SE increases with age, with the highest rates in people over 60 years of age. The underlying conditions associated with SE include stroke, traumatic brain injury, and exposure to organophosphates (OP). Early and effective treatment of SE is essential for optimal outcomes and to prevent long-term neurological complications. Refractory SE can occur with chemical exposures that cause prolonged and persistent seizures (Reddy and Colman, 2017). New onset SE and refractory SE, caused by OP chemicals and nerve agents, are associated with devastating morbidity and permanent neurological dysfunction. Accidental exposure to, or intentional poisoning by, OP chemicals (e.g., diisopropylfluorophosphate, DFP) and nerve agents (e.g., sarin) are highly neurotoxic (Chen et al., 2012; Abou-Donia et al., 2016). These agents have been used in combat as chemical warfare weapons or as bioterror agents against civilians, as in the 2013 Syrian sarin attack (Dolgin et al., 2013) and Tokyo subway sarin gas attack (Okumura, et al., 1996). OP pesticides and nerve agents use similar mechanisms to irreversibly inhibit acetylcholinesterase, triggering a massive cholinergic crisis (Reddy et al., 2019). Acute OP intoxication causes prolonged seizures and refractory SE, eventually leading to excitotoxicity and death (McDonough et al., 2010; Wu et al., 2018; Reddy et al., 2021b). The current treatment regimen after OP intoxication includes the muscarinic receptor blocker, atropine, 2-pralidoxime (2-PAM), acetylcholinesterase reactivator, and midazolam (MDZ), an anticonvulsant and allosteric modulator of γ-aminobutyric acid (GABA) type A receptors (Reddy et al., 2019). Despite their effectiveness when administered early after OP exposure, these therapeutic regimens do not protect survivors adequately against long-term neurological deficits, such as structural brain damage and persistent neurological deficits. Therefore, novel medical countermeasures are needed to effectively control SE and manage long-term consequences in victims of OP exposures.

DFP is widely used within the CounterACT research field as a chemical threat agent and as a nerve agent surrogate to test medical countermeasures (Deshpande et al., 2010; Pouliot et al., 2016; Siso et al., 2017; Wu et al., 2018; Reddy et al., 2020a). While benzodiazepines, such as diazepam or MDZ, are first-line anticonvulsants used to treat SE and nerve agent seizures, they have significant limitations (Reddy et al., 2019). Importantly, they must be administered within 30-min of exposure, after which protection against seizures is reduced and progressive neuronal damage occurs (Apland et al., 2014; Reddy and Reddy, 2015; Reddy, 2019). In most cases, this timeline is not practical. For example, it is
critical to control seizures early after OP exposure to achieve neuroprotection, survival and to prevent brain dysfunction (Shih et al., 1999; Reddy et al., 2020b). Recently, we and other teams have extensively studied the DFP-induced, benzodiazepine-resistant model of SE in male rats (Deshpande et al., 2010; Kuruba et al., 2018; Wu et al., 2018). Because the acute neurotoxic effects of OP have primarily been studied in male animals (Wu et al., 2018; Kuruba et al., 2018; Guignet et al., 2020; González et al., 2021), there is limited information about the potential effects of sex on DFP-induced, refractory SE and neuronal injury. Research on the effects of sex on OP neurotoxicity and SE is needed to simulate a civil mass casualty caused by OPs that would affect diverse populations of various ages and genders, especially women. Currently, there is a concerning gap in understanding the influence of sex on acute and chronic neurotoxic effects following OP exposure and response to therapeutic interventions (Rauh et al., 2012, Comfort and Re, 2017).

In this study, we investigated the effect of sex on seizure activity and neuronal injury patterns using the DFP model of refractory SE in male and female rats. To simulate a practical therapeutic window in case of chemical emergencies, we also investigated the efficacy of anticonvulsant MDZ when given 40 min post-exposure. Our results demonstrate striking differences in seizure activity and neurodegeneration patterns in male and female animals, as well as differences in MDZ sensitivity.
**Materials and Methods**

**Animals.** Adult Sprague-Dawley male and female rats (3 months old) (Taconic Farms, Rockville, MD) were used in this study. Rats were housed in standard plastic cages in an environmentally controlled vivarium (22 ± 2 °C, 40–50% humidity, 12-hour light/dark cycle). Food and water were provided ad libitum. All experimental procedures were conducted under a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee as per the NIH guidelines.

**EEG Electrode Implantation.** Rats were anesthetized using ketamine (100 mg/kg, intraperitoneal (i.p.) injection) and xylazine (10 mg/kg, i.p.). After positioning rats in the stereotactic apparatus (David Kopf Instruments, CA, USA), a stainless-steel EEG surface electrode (Protech International Inc., Boerne, TX) was implanted over the right frontoparietal cortex. An intracranial depth electrode was placed into the right dentate gyrus (DG) using the coordinates: AP= -4.0 mm, ML= 2.3 mm, and DV= 3.4 mm (Wu et al., 2018). A reference electrode was implanted over the left cerebellum. Rats were allowed to acclimate and recuperate for 1-2 weeks following surgery. Power and sample sizes were determined using the proposed statistical tests, e.g., one-way repeated measures ANOVA for neuropathological results. Behavioral seizures severity was assessed using a five-point modified Racine’s scale (Racine, 1972), where stage 0 represented normal behavioral activity, stage 1 corresponded to rapid cholinergic signs (salivation, lacrimation, urination, defecation), stage 2 was linked to myoclonic jerks, stage 3 involved fast tremors, and muscle fasciculations, stage 4 involved fast tremors with forelimb clonus, straub tail, and extended body posture, and stage 5, indicated symptoms such as tremors, forelimb clonus with hindlimb extension, as well as episodes of wild jumping with tonic-clonic seizures.

**DPF Model of Status Epilepticus.** To induce persistent SE, DFP (3.2 mg/kg, s.c.) was administered in both male and female animals (Wu et al., 2018; Kuruba et al., 2018; Reddy et al., 2021b). DFP (Sigma-Aldrich, St. Louis, MO) dissolved in ice-cold phosphate-buffered saline solution was prepared freshly before each experiment. Female rats were randomized for DFP model but not stratified into sub-groups based on the estrous cycle stage before administering DFP (Gage et al., 2022; Rao et al., 2022). Prior to DFP injection, rats were treated with pyridostigmine bromide (0.026 mg/kg, i.m.) for 30 min. One minute following DFP injection, SE was induced by injecting rats with a standard treatment regimen consistent with those used by NIH CounterACT Program investigators (Deshpande et al., 2010; Pouliot et al., 2016; Siso et al., 2017), including pralidoxime chloride (2-PAM, 25 mg/kg, i.m.) and atropine methyl nitrate (AMN, 2 mg/kg, i.m.), which do not significantly cross the blood-brain barrier and thus do not affect seizure severity (Kenley et al., 1982; Shih et al., 2010). Seizure activity progression was
assessed by continuously monitoring behavior and EEG activity for 24 h after DFP. Mortality was observed for 24 h following DFP injection (see DFP exposure experimental protocol, Fig. 1A).

**Video-EEG Recording and Analysis.** Behavior and EEG activity were monitored for 24 h post-DFP treatment to observe seizure initiation and progression. Before each experiment, a digital EEG system (Grass-Astromed, Warwick, RI) was used to record a 1-hour baseline EEG in rats that were awake and moving freely in cages fitted with a swivel. DFP-induced EEG seizure activity, high-amplitude EEG spikes (at least double the baseline wave amplitude for period-spike analysis), and repetitive discharges (>0.5 Hz) were monitored. Power spectrograms were generated as previously described (Golub et al., 2023). Absolute power in each frequency band was expressed as mV^2/Hz and estimated using Welch’s periodogram (Reddy et al., 2023) and a composite Simpson’s rule. The estimated frequency band powers include Delta (1-4 Hz), Theta (4-7 Hz), Alpha (7-14 Hz), Beta (15-30 Hz), and Gamma (31-70 Hz).

**Midazolam Treatment.** To study the effect of MDZ on seizure semiology and acute neurotoxicology, male and female rat groups each received a single injection of MDZ (2 mg/kg, n = 8) 40 min after DFP exposure. The effect of MDZ on behavior, electrographic seizure, and mortality was determined by continuously monitoring behavior and EEG activity for 24 h after DFP.

**Brain Histology and Immunohistochemistry.** Neuronal damage associated with DFP-induced SE has been assessed by FJB(+) staining (Wu et al., 2018; Reddy and Abeygunaratne, 2022). Briefly, 3 days after DFP rats were anesthetized with a mixture of ketamine (100 mg/kg)-xylazine (10 mg/kg) and transcardially perfused with a 4% paraformaldehyde solution in sodium phosphate buffer (pH 7.4). After removal, brains were fixed in 4% paraformaldehyde for 24h and then sequentially treated with 10%, 20%, and 30% sucrose solution for 48h each prior to staining. Rat forebrains containing the cortex, hippocampus, and amygdala were cut coronally from the bregma into 30 μm thick serial sections. Sections taken every 600-μm were placed into 24-well plates filled with sodium phosphate buffer and processed for FJB(+) staining. Minimal variation in slice thickness was observed.

**Stereology Quantification of Neurodegeneration.** Cell density, number of dying neurons, and tissue volume in FJB(+) stained hippocampal sections were quantified using a stereology protocol as previously described (Golub et al., 2015). The stereology system includes an Olympus BX53 microscope (Olympus, Tokyo, Japan) and newCAST software (version VIS 4.0; Visiopharm, Hørsholm, Denmark). Dying neurons were sampled at 5% of the total FJB (+) cell region area with a 60X objective.
in the CA1, CA3, and DG subfields and sampled at 10% of the total region area in the CA2 and dentate hilus (DH) subfields. Subfield volume was estimated using the criteria of at least 200 counting points with a 10X objective (Boyce et al., 2010; Dorph-Petersen and Lewis, 2011; Golub et al., 2015). To evaluate FJB(+) cells in extra-hippocampal regions, including the thalamus, hypothalamus, amygdala, and somatosensory cortex, the neuropathology score rating system (Wu et al., 2018) was used. Briefly, 27 brain regions were evaluated per extra-hippocampal region per rat. Each region of interest received a score describing the severity of tissue damage based on the estimated percentage of tissue involved: 0 = no neuropathology; 1 = minimal with 1-10%; neuropathology 2 = mild with 11–25% neuropathology; 3 = moderate with 26–45% neuropathology; 4 = severe with >45% neuropathology. The scores were averaged to create a neuropathological score for each brain region.

**Data Analysis.** All parametric results were expressed as mean ± standard error mean (SEM) unless specified for non-parametric datasets. Analysis of variance was used for statistical comparisons of seizure activity and neurodegeneration outcomes, and post hoc analyses by Tukey’s honestly significant difference were used for multiple group comparisons. The non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U test, was used to analyze the seizure stage and other non-parametric outcomes. All statistical tests, including multiple comparisons, were performed using OriginPro 2021 software (OriginLab Corporation, Northampton, MA). All statistically significant test differences were set at p < 0.05.
Results

Impact of Sex on DFP-induced Mortality. DFP exposure produced rapid neurotoxic effects, including early signs of cholinergic crisis such as salivation, lacrimation, urination, and defecation in both male and female animals. However, following DFP-induced SE, we observed a significantly higher mortality rate in male animals than in female animals. Furthermore, MDZ treatment produced better overall survival rates in DFP-exposed male animals, which also differed significantly from both DFP control male and female groups (Fig. 1B).

Impact of Sex on DFP-induced Behavioral and Electrographic SE. DFP exposure triggered intense SE, characterized by two to four clonic seizure episodes (stage 3), with secondary generalization leading to generalized tonic-clonic seizures (GTCSs) (stage 4), with rearing and falling (stage 5). We monitored EEG seizures and behavioral seizure scores for 24 h after DFP exposure (Fig. 2A and B). We recorded the highest behavioral seizure scores in rats within 10-s epochs at 10-min intervals. Seizure monitoring started immediately after DFP injection, and animals were considered to be out of SE when they became immobile irrespective of EEG. A seizure score of 3 or more corresponds with a convulsive phase of SE in adult DFP rats (Reddy et al., 2021b). In our results, with both male and female rats, seizure stages 3-5 represented the majority proportions and contributed to ~50% of seizure-associated behaviors in the initial 12 h following DFP. However, the mean seizure score was notably higher in DFP male rats than in female rats (p < 0.05) (Fig. 2B). Male rats had seizure scores ≥ 4 for almost 4 h, whereas females had seizure scores ≥ 4 for almost 3 h during the 24 h monitoring period (Fig. 2B). We also observed higher seizure severity in DFP male rats during the waning phase of SE, which eventually subsided. There were no differences in latency to SE initiation and termination between male and female rats.

Hippocampal EEG recordings showed progression of DFP-induced focal intermittent clonic seizures, depicted as high-frequency, low-/high-amplitude spike-wave discharges (SWDs) primarily in theta, alpha, and beta frequencies. SE onset was characterized by a shift from blue to green and red colors in the EEG power spectrum analysis. However, single spikes were observed between these early seizures. After 2-4 episodes of early intermittent focal seizures, they eventually continued into the autonomous/continuous SE phase. The autonomous phase is characterized by higher SWD frequencies in delta, theta, and alpha ranges. Interestingly, in male DFP rats, we also observed intermittent, faster discharges (high-/low-amplitude faster discharges [HAFDs/LAFDs]) within continuous high-frequency rhythmic SWDs (Figs. 2D and 3). Overall, power spectrum analysis revealed continuous high-power activity in delta, theta, and alpha ranges with sustained lower power activity in beta and lower gamma
frequencies, which typically continued for ~4 h following DFP. Subsequently, amplitude and frequency began to decrease, with the appearance of periodic low-amplitude SWDs. Surprisingly, during the waning phase, we did not observe periodic discharges that are typically seen in other models of chemical-induced SE. The power spectrum showed bouts of high power in the delta and theta frequencies and lesser power in alpha and beta frequencies. This phase continued for up to 24 h, and activity never returned to baseline.

Similar to male animals, DFP female animals displayed continuous high-frequency, low-/high-amplitude SWDs, but did not observe HAFDs/LAFDs. The power spectrogram showed continuous high power only in the delta and theta ranges interspersed with lower power activity in alpha and beta frequencies (depicted as yellow streaks interspersed in continuous red color). Intense high power persisted for ~3 h after DFP (Fig. 3A). Thereafter, continuous seizure activity transitioned to generalized periodic discharges interspersed with bursts of low amplitude polyspike discharges. The power spectrum showed lower power in delta and theta frequencies, which continued for ~16 h. Over time, periodic discharges decreased in amplitude and frequency until there was no high amplitude or SWDs, but activity never returned to baseline. Consistent with power spectrum analysis, 18% less spike activity was seen in the first 4 h after DFP challenge in the female groups than in the male groups, and the two groups did not differ significantly in spike activities in the 24 h after DFP challenge. Therefore, considering behavior, survival, and EEG power outcomes, male animals were more responsive to DFP challenges than females.

Impact of Sex on the Anticonvulsant Effect of Midazolam in DFP-induced SE. MDZ treatment, at 40 min post-DFP, reduced behavioral seizures in male and female rats (Fig. 2B). The majority of observed behavioral seizures were mild, including behaviors such as head bobbing and facial automatisms (stages 1 and 2) and contributed to ~80% of seizure-associated behaviors. MDZ treatment significantly decreased SE termination time and improved survival in male (n=15/20) and female (n=21/24) animals at 24 h following DFP (Fig. 1B). Notably, improved survival rates were more pronounced in male animals. We also compared compressed snippets of typical EEGs at different intervals and power spectra to better understand the duration of SE and the seizure intensity in MDZ-treated male and female animals (Fig. 2D and 3). SWDs in the first 4 h were 30% and 37% less intense in MDZ-treated male and female groups than in DFP animals, respectively. MDZ-treated male and female animals also had recurrent seizures after initial suppression of spike activity (Fig. 2D and 3), indicating the development of pharmacoresistant SE. These results confirm previously described
limitations of using benzodiazepines as anticonvulsant antidotes following OP intoxication (McDonough et al., 2010; Apland et al., 2014; Wu et al., 2018). However, behavioral seizure scores and seizure termination times did not significantly differ in MDZ-treated male and female rats. Electrographically, in MDZ-treated animals, continuous seizure activity evolved into generalized periodic discharges with interspersed bursts of polyspike discharges at 4-6-h post-DFP. Although frequency and amplitude decreased over the next few hours, periodic discharges persisted much longer in MDZ-treated males (24 h) than in females (12 h).

MDZ also decreased total power in all frequencies at 4-6 h; however, continuous high-power activity decreased in male rats at ~ 6 h, followed by high power bouts in the delta and theta frequencies together with significant power at alpha and beta frequencies (Fig. 3A and B). Although the frequency of these high-power delta and theta bouts decreased at 24 h for males, baseline power activity did not recover. However, in females, a decrease in continuous power was observed at 4 h after DFP injection. MDZ treatment decreased activity in all powers, but bouts of high-power delta and theta frequencies interspersed with low-power alpha and beta, and low-power gamma were observed for 24 h. Therefore, considering behavior, survival, and EEG power, female animals were more responsive to MDZ treatment than males.

**Impact of Sex on DFP-induced Neuronal Damage in Hippocampal Subfields.** To evaluate the neuroprotective effects of MDZ 72 h after DFP exposure, brain sections were stained with FJB (+), and injured neurons or cell death in the hippocampal subfields of CA1, CA2, CA3, and DG were quantified by stereology. Because FJB (+) primarily stains cell bodies or necrotic cells, damaged neurons are shown at low and high magnifications (Fig. 4A and B), along with stereological quantification of damaged cells. Injured neurons in DFP groups were identified by bright green fluorescence in FJB-stained sections (Fig. 4A). We observed more FJB(+) cells in the DG of female rats than in male rats (Fig. 4). DFP exposure produced a massive and significant increase (p < 0.0001. Fig. 5) in the stereology-based count of FJB(+) cells in the hippocampus and its subfields (CA1, CA2, CA3, DG, and DH) in male and female rats (Fig. 4 and 5). However, FJB (+) cells were significantly higher in DFP-intoxicated female rats (P < 0.01 vs. DFP male) than in DFP-intoxicated male rats, particularly in the DG and DH hippocampal subfields (Fig. 4 and 5). We also determined normalized neuronal protection by comparing the treated and untreated DFP-exposed female groups (normalized as 0% protection), while control rats without FJB(+) staining were normalized as 100% protection. Both male and female DFP-exposed animals showed significantly less survival than non-DFP-exposed, naïve female groups.
We also observed higher survival and less neurodegeneration of DG and DH neurons in male DFP-exposed animals than in female DFP-exposed animals. Further, stereological counts showed significantly higher FJB (+) cell density in hippocampal regions of DFP-exposed, than non-DFP-exposed male and female animals. Volumes of the hippocampus (74.66 ± 0.3 mm³ in control) and its subfields CA1, CA2, CA3, DG, and DH (n = 10) did not differ significantly (p = 0.1) among all examined groups, indicating that tissue volumes do not change after acute DFP exposure (Fig. 5D). Taken together, these histopathological results suggest that DFP induces significant neuronal damage in the hippocampus.

**Impact of Sex on DFP-induced Neuronal Damage in Extra-hippocampal Regions.** To assess neuronal injury in extra-hippocampal regions, we used neuropathology-based relative quantification as described previously (Wu et al., 2018). Male and female DFP-intoxicated animals had significant neuronal damage and necrosis in the thalamus, hypothalamus, amygdala, and somatosensory cortex (Fig. 6). Notably, the hypothalamus had the fewest FJB(+) cells among extra-hippocampal brain regions. However, DFP male rats had more cortical neurodegeneration, as indicated by a higher neuropathology score and a normalized neuronal survival rate.

**Impact of Sex on the Neuroprotective Effects of Midazolam on DFP-induced Neuronal Damage in Hippocampal and Extra-hippocampal Regions.** To evaluate the neuroprotective effects of midazolam, we evaluated normalized neuronal protection as previously described. MDZ treatment significantly attenuated DFP-induced neuronal degeneration (60%-90% protection) across all hippocampal subfields (Fig. 4 - 6), including CA1 (p < 0.001), CA2 (p < 0.001), CA3 (p < 0.001), DG (p < 0.001), and DH (p < 0.05) regions. However, DFP-exposed female animals showed remarkably less neurodegeneration after MDZ treatment, especially in the DG and DH hippocampal subfields. MDZ treatment also reduced the neuronal injury in several extra-hippocampal regions, including the amygdala, somatosensory cortex, and entorhinal cortex (p< 0.05) (Fig. 6). In summary, our findings suggest that MDZ treatment significantly protects against DFP-induced neuronal injury and necrosis in both hippocampal and extra-hippocampal areas, particularly in hippocampal areas of DFP-exposed female animals.
Discussion

In this study, we identified remarkable variations between sexes in their responses to DFP-induced SE. These variations included differences in EEG patterns, occurrence of behavioral seizures, mortality rates, as well as patterns and intensity of neurodegeneration. We also identified sex-specific differences in the therapeutic efficacy of MDZ. The salient findings of this study are: (i) DFP-exposed male animals had more severe behavioral seizures with associated electrographic patterns and reduced survival following SE; (ii) DFP-exposed female animals had less severe behavioral seizures and electrographic changes but greater neurodegeneration; (iii) MDZ treatment reduced the severity of electrographic and behavioral seizures, improved survival, and protected against DFP-induced neuronal injury and necrosis in both male and female animals; (iv) MDZ treatment provided better protection against neuronal injury and hippocampal necrosis in DFP-exposed female animals; and (v) MDZ treatment significantly prevented post-SE mortality in male animals. This study also supports previous work reporting that DFP-induced SE is refractory to delayed treatment with benzodiazepines (Kuruba et al., 2018; Wu et al., 2018). The implications of these findings are discussed below.

DFP exposure produces electrographic and behavioral seizures accompanied by cholinergic crisis (Deshpande et al., 2010; Poulion et al., 2016; Guignet et al., 2020). In male and female rats, DFP injection led to the development of early intermittent focal seizures that rapidly progressed to SE characterized by continuous behavioral seizures lasting for at least 4-6 h. Hippocampal EEG recordings showed high-frequency discharges predominantly in the delta, theta, alpha, and beta frequencies, followed by rhythmic SWDs in the theta frequency, consistent with previous reports of EEG recordings in DFP-intoxicated rats (Reddy et al., 2021b). There were no significant differences in the latency and termination of SE between male and female rats. This finding suggests that the factors contributing to the onset and resolution of seizures in rats were not dependent on sex-based variations. Although gender may not be a critical determinant in seizure generation, there are many other ictal measures that are sex dependent. Adult male rats had more severe behavioral and electrographic seizures, consistent with previous observations (Gage et al., 2020; Supasai et al., 2020; González et al., 2021). These sex-specific differences in seizure severity may be due to sexually dimorphic expression of excitatory and inhibitory receptors in the brain. Glutamatergic, cholinergic, and GABAergic receptors, essential for initiation and maintenance of seizures, are differentially expressed in brains of male and female animals (Akman et al., 2014, Scharfman and MacLusky, 2014a; 2014b). Glutamate receptors, including NR1, NR2A, and GluR1, are critical to maintaining seizures following OP intoxication (Barker-Haliski and White, 2015,
Hanada, 2020) and are more highly expressed in males than in females (Hsu et al., 2000, Bian et al., 2012, Damborsky and Winzer-Serhan, 2012). Interestingly, cholinergic receptors are also more highly expressed in male than in female rats (Potier et al., 2005).

Other mechanisms are also known to contribute to sex-specific differences in epilepsy, seizure susceptibility, and antiseizure medication treatments, including increased levels of endogenous neurosteroids and steroid hormones in females (Christian et al., 2020). Neurosteroid fluctuation during the ovarian cycle could affect seizure susceptibility (Reddy, 2017) due to their potent modulation of both synaptic and extrasynaptic GABA-A receptors, with a higher affinity for extrasynaptic receptors (Reddy, 2010; Rogawski et al., 2013; Reddy, 2015; Reddy and Estes, 2016). The antiseizure potency of neurosteroids is higher in females than in males, likely due to the increased abundance of extrasynaptic δ-GABA-A receptors in females that mediate neurosteroid-sensitive tonic currents and protect against seizures (Reddy et al., 2019). Neurosteroids also increase the frequency and duration of chloride channel opening mediated by GABA-A receptors and inhibit excitability by enhancing tonic inhibition (Reddy, 2018; Chuang et al., 2018).

The decreased intensity of seizure activity seen in female animals could also be attributed to circulating hormones such as estrogen and progesterone, which play important roles during seizure development. Progesterone binds to its receptors to negatively impact excitatory transmission and is metabolized into the inhibitory neurosteroid allopregnanolone, further simulating the anticonvulsant effect (Brinton et al., 2008; Motta et al., 2013). Conversely, while estrogen is known to promote epilepsy in animals and humans (Scharfman et al., 2006), recent studies reported that it also has anticonvulsant properties mediated through the neuroprotective effects of β-estradiol (Velišková et al., 2000; Velišková and DeSantis, 2000). Taken together, differences in seizure activity that we observed in male and female rats are likely due to increased expression of excitatory receptors and greater effects of endogenous neurosteroids in females.

DFP-induced SE was associated with higher mortality rates in male animals, potentially due to severe seizures stemming from the topographic architecture of neurons and male sex hormones. GTCSs can cause significant autonomic activation, leading to tachycardia, hyperthermia, elevated plasma glucose, lactic acidosis, and hypertension. During SE, elevated intracranial pressure and hypertension associated with GTCSs reduce cerebral perfusion pressure and enhance neurological and systemic dysfunction (Boggs, 2004). The high mortality rates in male animals clearly indicate that GTCSs can be life-threatening, as previously reported in a mouse model of SE that is based on electrical stimulation.
Also, in clinical settings, the survival of epilepsy patients depends on factors including GTCSs, age, history, underlying etiology, and type of epileptiform discharges. GTCSs also increase the risk of sudden unexpected death in epilepsy (SUDEP), which contributes significantly to mortality in epilepsy (Lhatoo et al., 2012). In 25-80% of SUDEP cases, death is preceded by GTCSs (Langan et al., 1985; Walczak et al., 2001). Furthermore, in recent studies using continuous hippocampal stimulation and kainic acid-induced SE, central apnea was associated with a high frequency of SWDs (Villiere et al., 2017; Lewczuk et al., 2018). Therefore, frequent occurrence of GTCSs and high-amplitude fast discharges most likely contribute to high mortality in male animals following DFP-induced SE.

We observed region-specific differences in neurodegeneration patterns induced by DFP, with the hippocampus, amygdala, somatosensory cortex, and thalamus being the most severely affected in both male and female rats. Specifically, female animals exhibited higher hippocampal neurodegeneration, specifically in the DG and DH subfields, which is consistent with recent evidence that acute DFP intoxication can lead to neurodegeneration in adult rats, even in the absence of seizures (González et al., 2020). OPs have also been shown to cause significant brain damage at sub-convulsive doses in patients (Chen, 2012). Our results also confirmed that seizure intensity is not related to neurodegeneration. Instead, DFP-exposed male rats showed more neurodegeneration in the somatosensory cortex, consistent with a recent study that reported a positive correlation between seizure severity and increased involvement of cortical regions (Dabrowska et al., 2019; Singh et al., 2020; 2022; Adotevi and Kapur, 2022). Clinical studies also suggested that cortical structures involved in focal hippocampal seizures contribute to the manifestation of more severe convulsions in patients with epilepsy (Kwan and Brodie, 2000; Bragin et al., 2005).

Because benzodiazepines are the primary treatment for SE, we examined sex-specific antiseizure and neuroprotective effects of the MDZ following DFP-induced SE. While previous research demonstrated the antiseizure, anti-inflammatory, and neuroprotective effects of MDZ, its potential sex-specific effects have not been studied extensively (Wu et al., 2018; Reddy et al., 2021b; Rao et al., 2022). We confirmed that MDZ treatment protects against seizure and neurodegeneration in male and female animals but also showed that female rats exhibited more neuroprotection in hippocampal brain regions than seen in male rats, possibly due to the effect of sex hormones such as estrogen and progesterone on GABA receptors (Wu et al., 2013; Reddy 2018; Reddy et al., 2019; Reddy et al., 2021a). Interestingly, while MDZ treatment did not significantly improve survival in female rats, it did
significantly improve survival in male rats. GTCSs, which are associated with higher mortality and SUDEP, were effectively reduced by MDZ treatment in males, leading to a lower incidence of SUDEP and better survival.

In conclusion, the results of this study demonstrate that acute DFP intoxication has sex-specific effects that induce refractory SE, overall survival, and neurodegeneration patterns in adult male and female rats. The temporal relationship between developing prolonged seizures and neurodegeneration, as well as differential responses to MDZ, provide key insights into the extended neurological effects of acute OP intoxication in male and female rats. This study also confirms the limited efficacy of MDZ in treating refractory SE caused by acute OP intoxication when administered at 40 min, regardless of sex. Taken together, these findings further highlight sex as a biological variable that will require us to design or optimize sex-specific medical countermeasures for OP intoxication and refractory SE. Sex-specific treatments involve tailoring therapeutic interventions based on the unique physiological and biological characteristics of males and females. This approach may include optimizing drug dosages, adjusting dose frequencies, and using protective compounds that work more effectively in one gender over the other.
Acknowledgments

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Conflict of Interest

The authors declare no competing financial interests.

Author contributions

Participated in research design: Reddy.
Conducted experiments: Wu, Singh, Reddy.
Performed data analysis: Wu, Singh, Ramakrishnan, Reddy.
Wrote or contributed to the writing of the manuscript: Singh, Ramakrishnan, Wu, Reddy.
References


Figure Legends

**Fig. 1.** Diisopropylfluorophosphate (DFP) model of status epilepticus (SE) in rats. (A) Experimental paradigm of DFP-induced SE and neurotoxicity in rats. Rats were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min before DFP (3.2 mg/kg, s.c.) exposure. One minute following DFP injection, rats were injected with pralidoxime (2-PAM, 25 mg/kg, i.m.) and atropine methyl nitrate (2 mg/kg, i.m.) to increase survival rates. Midazolam (MDZ, 2 mg/kg, i.m.) was given at 40 min post-DFP exposure. All cohorts were monitored continuously up to 24 h post-DFP by recording behavioral and EEG seizure activity. Brains were collected for histology at 72 h post-DFP exposure. (B) Comparative survival outcomes in various subgroups. In the DFP-alone male group, survival rate was less than 50% in this experimental cohort (n = 29; 14 of 29 rats survived). Bar graph show percent survival in each group. Survival outcome was calculated 24 h after exposure to DFP by the non-parametric Wilcoxon signed-rank test. M: male; F: female. Each bar represents the mean value. *p<0.05 vs female DFP; #p<0.05 vs DFP group within same sex group (n = 20-29 rats/group; exact group sizes are listed in B).

**Fig. 2.** Temporal profile of status epilepticus (SE) progression and effects of midazolam (2 mg/kg, i.m.) on behavioral seizures and EEG seizure activity in adult male (M) and female (F) rats after acute DFP exposure. (A) Time-course of EEG seizure suppression by midazolam treatment 40 min after DFP exposure in male and female rats. (B) Temporal pattern of behavioral seizures recorded from male and female rats during the first 24 h post-DFP exposure. Data represent mean ± SEM (C) Effect of midazolam on seizure termination latency after DFP. Each bar represents the mean ± SEM. *p<0.05 vs. control (no DFP. within sex group). (D) Time-course changes in EEG seizure activity in male and female rats in response to MDZ treatment. Traces represent 1-min EEG epochs in the hippocampus. Seizures began ~8–10 min after DFP exposure with intermittent bursts of spikes that progressed into persistent high-amplitude spikes.

**Fig. 3.** Time-course of SE progression and midazolam (MDZ, 2 mg/kg, i.m.) response on EEG spectral power using the DFP model in male and female rats. (A) Time-frequency and EEG power spectrum analysis showing continuous high-power activity within 4 h of DFP challenge in male and female rats. (B) Absolute EEG spectral power features in each frequency band in male and female rats. Each bar represents the mean ± SEM. *p<0.05 vs DFP female group; #p<0.05 vs DFP+MDZ female group.

**Fig. 4.** Neuronal injury revealed by Fluoro-Jade B–positive (FJB (+)) neurons in hippocampal subfields at 72 h post-DFP exposure in male and female rats. (A) Representative sections comparing neurodegeneration in hippocampal subfields (CA1, CA3, DG) in control, DFP, and midazolam (MDZ, 2 mg/kg) treated groups post-DFP for both female (control-F, DFP-F, and DFP+MDZ-F) and male (control-M, DFP-M and DFP+MDZ-M) rats. Note the significant neurodegeneration in DFP treated animals compared to few degenerating neurons in midazolam-treated male and female rats. (B) High-resolution magnification images of neurodegenerative cells showing FJB positivity in male and female DFP groups in the presence and absence of MDZ, using a 60x objective. (C) Confocal 3D volume
images of the DG subfield in male and female DFP groups present a comprehensive, high-resolution view of internal structures, including neurodegenerative cells distributed throughout the tissue layers. Each group consists of 5-10 rats.

**Fig. 5.** Quantitative neuronal damage by stereology analysis of Fluoro-Jade B–positive (FJB (+)) neurons in hippocampal (HPC) subfields at 72 h post-DFP exposure in male and female rats. (A) Stereology data showed significantly more FJB(+) cells in DFP treated rats in CA1, CA2, CA3, dentate gyrus (DG), and dentate hilus (DH), and neuroprotective effect of midazolam depicted as few degenerating neurons in both male and female rats (n=5-10). (B) Normalized percent neuroprotection by midazolam relative to FJB (+) cells in the untreated DFP group (as 0% protection). Normalized neuroprotection was calculated using the control female group as 100% protected due to the absence of FJB (+) cells in any region. (C) FJB(+) cell density by stereology in hippocampal subfields. Comparative stereology data showed significantly fewer FJB(+) cells post-midazolam treatment in male and female rats. (D) Brain slice thickness/volume in male and female rats. We did not observe any significant difference in tissue volume for each slice counted in all the groups. Each bar represents the mean ± SEM (n=5-10 per group). *p<0.05 vs. control within sex group; #p<0.05 vs. DFP group within sex group (except B, vs. DFP female group). &p<0.05 vs. same treatment in the female group.

**Fig. 6.** Analysis of neuronal damage by Fluoro-Jade B–positive (FJB (+)) neurons in extra-hippocampal areas at 72 h post-DFP exposure in male and female rats. (A) Representative sections comparing neurodegeneration in extra-hippocampal areas of control, DFP, and midazolam (MDZ, 2 mg/kg) treated groups post-DFP for female and male rats. DFP-treated animals had significant neurodegeneration in the thalamus (Thal), hypothalamus (Hypo), amygdala (Amy), and somatosensory cortex (CX). Hypothalamus showed the lowest loss of neurons in DFP-exposed male and female animals. Note extensive neuronal injury in the somatosensory cortex of male animals. Dramatic neurodegeneration is seen in many regions within the DFP-alone group, which was strikingly reduced in the midazolam-treated group. (B) Rat brain atlas showing regions selected for analysis of FJB (+) staining. (C) Neuropathology scoring in extra-hippocampal areas of female and male rats. DFP exposure was associated with severe damage in these extra-hippocampal regions with high neuropathology scores. Values in the bar graph represent the mean ± SEM (Kruskal-Wallis test followed by the Mann-Whitney U test). *p<0.05 vs control within sex group; #p<0.05 vs DFP group within sex group; &p<0.05 vs same treatment in the female group.
Figure 1

A) EEG Seizure Monitoring (24 h)

TIMELINE

-30 min

Pyridostigmine Atropine+2-PAM

DFP exposure

0 1 10 40 120 24 72

Minutes Hours

MIDAZOLAM

Acute Histology

B) Percent Survival

<table>
<thead>
<tr>
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<th>F</th>
<th>M</th>
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<tr>
<td>DFP</td>
<td>17/21</td>
<td>14/29</td>
</tr>
<tr>
<td>DFP+MDZ</td>
<td>21/24</td>
<td>15/29</td>
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</tbody>
</table>
Figure-5

A

Control, female
DFP, female
DFP + MDZ, female
Control, male
DFP, male
DFP + MDZ, male

HPC | CA1 | CA2 | CA3 | DG | DH

B

FJB+ Cell Numbers (×1000)

HPC | CA1 | CA2 | CA3 | DG | DH

C

FJB+ Cell Density (×1000/mm²)

HPC | CA1 | CA2 | CA3 | DG | DH

D

FJB+ Neuroprotection (% DFP in Female)

HPC | CA1 | CA2 | CA3 | DG | DH

Tissue Volume (mm²)

HPC | CA1 | CA2 | CA3 | DG | DH