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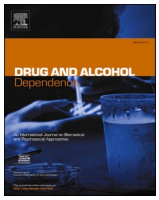

Short communication

Xylazine potentiates the lethal but not the rewarding effects of fentanyl in mice

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ABSTRACT

Background: Fentanyl is commonly laced with xylazine. People who use this combination report heightened effects, but it also increases death risk. Although no medication has been approved to counteract overdoses produced by fentanyl and xylazine, naloxone is frequently used. This paper studies the preclinical rewarding and lethal effects of fentanyl combined with xylazine and the efficacy of yohimbine or naloxone to prevent death.
Methods: Male Swiss Webster mice were treated with (in mg/kg, i.p.) xylazine (0.3, 1, 3, or 5.6), fentanyl (0.01, 0.3, or 0.1), or 1 xylazine plus 0.01 (non-effective) or 0.1 (effective) fentanyl doses during the conditioned-place preference (CPP) test. In addition, independent groups received (in mg/kg, i.p.): xylazine (31.6, 60, 74.2, or 100), fentanyl (3.1 or 10), or both substances at two doses: 31.6 xylazine + 3.1 fentanyl, or 60 xylazine + 10 fentanyl to analyze lethal effects. We determined whether yohimbine or naloxone (each medication tested at 10 or 30 mg/kg) could prevent the lethality produced by fentanyl/xylazine combinations. Female mice were also tested in key experiments.
Results: Xylazine neither induced CPP nor altered fentanyl's rewarding effects. In contrast, lethality was potentiated when fentanyl was combined with xylazine. Naloxone, but not yohimbine, effectively prevented the lethality of the fentanyl/xylazine combinations.
Conclusions: At the doses tested, xylazine does not increase the rewarding effect of fentanyl on the CPP in male mice but potentiates the risk of fatal overdose in male and female mice. A high naloxone dose prevents death induced by coadministration of fentanyl and xylazine in both sexes.

1. Introduction

Heroin and fentanyl samples have been recently adulterated with xylazine (Ruiz-Colón et al., 2014), a compound increasingly detected in people who died of opioid overdose (Alexander et al., 2022; Bowles et al., 2021; Johnson et al., 2021; Nunez et al., 2021). Using xylazine in humans is prohibited because of its life-threatening effects, such as hypotension, bradycardia, and respiratory depression (Alexander et al., 2022; Ball et al., 2022; Kariisa et al., 2023; Ruiz-Colón et al., 2014). In addition, the introduction of xylazine to the drug scene has evidenced that its repeated use causes necrotic skin lesions (Pergolizzi Jr et al., 2023).

Xylazine is an α_2-adrenoceptor agonist with non-opioid sedative and analgesic effects, approved exclusively for veterinary use by the Food and Drug Administration (Ruiz-Colón et al., 2014). α_2-adrenoceptors are mainly expressed in neuronal presynaptic terminals, and their activation decreases norepinephrine and dopamine release, resulting in generalized central nervous system (CNS) depression (Starke, 2001).

To date, there is no antidote approved for xylazine overdose; however, because it is frequently combined with opioids, the use of the opioid receptor antagonist naloxone is recommended (Kariisa et al., 2023). Some reports have shown that naloxone (2–10 mg) can counteract the overdose produced by clonidine (another α_2-adrenoceptor agonist) (Seger and Loden, 2018; Swift and Wilson, 2019). However, naloxone is not a proven treatment for xylazine intoxication (Ball et al., 2022; Duong et al., 2023; Norman and Nappe, 2023). According to a recent review, physicians have used 2 mg of naloxone to handle cases of xylazine intoxication with mixed results (Ball et al., 2022).

The association between the life-threatening effect of xylazine and fentanyl has only recently been studied at the preclinical level (Choi et al., 2023). However, to our knowledge, no studies have analyzed whether xylazine facilitates or potentiates fentanyl's rewarding and

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lethal effects. In this work, we aim to a) determine if xylazine promotes fentanyl-induced conditioned-place preference and increases fentanyl's lethality and b) test the effectiveness of naloxone, yohimbine, or their combination to prevent death produced by coadministration of fentanyl and xylazine. Since sex differences exist in some fentanyl effects (Little and Kosten, 2023), we also compared the lethal effects of both drugs in male and female mice.

2. Materials and methods

2.1. Animals

We used 216 male and 70 female Swiss Webster mice weighing 25–32 g. Animals were housed in groups with water and food *ad libitum*, under a 12/12 h light/darkness cycle (lights on at 6:00 h) and controlled temperature (20–24 °C). All mice were handled and habituated to the experiment room for two days before testing. The experiments were carried out following our Institutional Internal Ethics Committee for the Care and Use of Laboratory Animals (CICUAL, protocol 0021–12) and the Guide for the Care and Use of Laboratory Animals by the United States National Research Council US Committee for the Update of the Guide for the Care, of of Laboratory Animals. (2011), which complied with the Mexican Official Norm (NOM-062-ZOO-1999) and the ARRIVE guidelines (Kilkenny et al., 2014).

2.2. Drugs

We purchased xylazine hydrochloride, naloxone hydrochloride, and yohimbine hydrochloride from Sigma-Aldrich (Toluca, Mexico). Laboratorios Psicofarma (Mexico City, Mexico) kindly donated fentanyl citrate. Drug doses were calculated as the free base, and all substances were dissolved in 0.9% saline and intraperitoneally injected in a maximum final volume of 0.2 ml. When using drug combinations, the individual substances were mixed in the syringe immediately before the injection. The doses of naloxone and yohimbine were selected based on their efficacy in antagonizing high opioid doses (Ruiz-Quiñonez et al., 2022; Rzasa Lynn and Galinkin, 2018) and xylazine (Komulainen and Olson, 1991).

2.3. Conditioned-place preference test (CPP)

We used a two-compartment apparatus (60×30×45 cm) with different contextual characteristics for the CPP test. One had dark walls, a steel bar floor, and indirect light; the other had black and white striped walls, a wire mesh floor, and dim light (Violante-Soria et al., 2023). The protocol includes three phases: habituation (pre-conditioning), conditioning (training), and testing (post-conditioning). During habituation, mice had free access to both compartments for 15 min. Three independent sessions (one per day) were performed on consecutive days. The last session was video-recorded to obtain a baseline preference from each compartment. We excluded six animals that spent less than 30% in one of the compartments during this pre-conditioning phase (McKendrick and Graziane, 2020). Conditioning was performed with a biased design in which drug administration was immediately received before being confined to the least-preferred compartment for 30 min (Mueller and de Wit, 2011). The next day, the vehicle was injected before confining the animals to the opposite compartment. A total of six conditioned sessions were conducted on consecutive days (Zhang et al., 2023). We tested the conditioned preference (post-conditioning test) 48 h after the last conditioning session by giving animals free access to both compartments in a drug-free state for 15 min and video-recorded the test for further analysis. CPP score was obtained by subtracting the time spent in the drug compartment during pre-conditioning from the time spent in the same compartment during the test.

2.4. Lethality test

We observed each mouse for 60 min after acute drug injection and checked them periodically during the next five and 24 hours after injection to determine the number of mice that died and the latency to death.

2.5. Statistical analysis

We used a nonpaired Student's t-test to compare two independent groups, a one-way ANOVA test for three or more independent groups, followed by the Dunnett test, and the Fisher exact probability test to analyze differences between groups in the proportion of animals that died. Data concerning latency to death are expressed as the mean ± the SEM. We considered a p-value <0.05 statistically significant and used the Prism 8.2.1 software (GraphPad, San Diego, USA) for statistical analyses.

2.6. Experimental design

2.6.1. Experiment 1. Effects of fentanyl and xylazine on the CPP

To determine if xylazine alone or combined with fentanyl was rewarding, independent groups of male mice (n=8 each) received xylazine (0.3, 1, 3, or 5.6 mg/kg), fentanyl (0.01, 0.03, or 0.1 mg/kg), or 1 mg/kg xylazine combined with either 0.01 or 0.1 mg/kg fentanyl on alternated days during the conditioning phase of the test. Control, non-conditioned (NC) animals received only vehicle in all conditioning sessions.

2.6.2. Experiment 2. Effects of fentanyl and xylazine on lethality in male mice

Based on preliminary lethality data from our laboratory, we injected two independent groups of mice (n=10 each) with 3.1 mg/kg (non-lethal dose) or 10 mg/kg fentanyl (lethal dose at 10%, LD_{10}). To choose the dose of xylazine to combine with fentanyl, we did a dose-response curve for the lethal effects of xylazine (31.6, 60, 74.2, or 100 mg/kg; n=10 each) and selected two high non-lethal doses to be combined with fentanyl. Thus, independent groups of animals received either 31.6 mg/kg xylazine + 3.1 mg/kg fentanyl or 60 mg/kg xylazine + 10 mg/kg fentanyl.

2.6.3. Experiment 3. Efficacy of yohimbine and naloxone to prevent death in male mice

Next, we tested if yohimbine (a competitive α_2-adrenoceptor antagonist), naloxone (a non-selective opioid receptor antagonist), or the combination of both substances were effective in preventing the death caused by the highest fentanyl/xylazine combination used in the previous experiment. Each receptor antagonist (10 and 30 mg/kg yohimbine or 10 and 30 mg/kg naloxone), alone or combined (n=10 each), was injected 20 min before the administration of fentanyl + xylazine.

2.6.4. Experiment 4. Effects of fentanyl, xylazine, and naloxone on lethality in female mice

Based on the results obtained in males, we analyzed the lethality of 31.6 or 60 mg/kg xylazine, 3.1 or 10 mg/kg fentanyl, or two xylazine/fentanyl combinations (31.6/3.1 mg/kg or 60/10 mg/kg) in independent groups of mice (n=10 each). An additional group received 30 mg/kg naloxone before (−20 min) the 60/10 mg/kg xylazine/fentanyl combination.

3. Results

3.1. Xylazine, at the doses tested, lacks rewarding effects on the CPP assay in male mice

Xylazine was not rewarding for mice at the doses used, as none

produced CPP [$F_{(4, 35)} = 0.43, p = 0.785$]. We could not test doses higher than 5.6 mg/kg because the animals were too sedated to explore the chambers. At 0.1 mg/kg, fentanyl produced CPP, but 0.01 and 0.03 mg/kg did not [$F_{(3, 28)} = 8, p<0.001$]. The CPP scores corresponding to the coadministration of 0.01 mg/kg fentanyl and 1 mg/kg xylazine were not different from those observed with each drug alone [$F_{(2, 21)} = 0.017, p = 0.983$] (Fig. 1). Combining 1 mg/kg xylazine with 0.1 mg/kg fentanyl produced an effect statistically similar to that produced by fentanyl alone (t = 1.5; $p = 0.156$).

3.2. Xylazine potentiates the lethal effect of a high fentanyl dose, and naloxone prevents it in male mice

The dose-response curve for xylazine's lethal effect in males was steep, with 60 mg/kg being ineffective and 100 mg/kg causing 90% lethality. At 31.6 and 60 mg/kg, xylazine impaired motor coordination for approximately 4–5 min. This effect was followed by long-term sedation (1–2 h), urination, and excessive sweating. With the 100 mg/kg xylazine dose, death occurred within the first 5 min after injection.

On the other hand, 3.1 mg/kg fentanyl was not lethal. Instead, it produced hyperactivity and the characteristic opioid-induced Straub tail in 80% of mice. The rest of the group showed a sedative effect that lasted approximately 20 min, after which animals regained mobility. A higher fentanyl dose (10 mg/kg) was lethal for 1 out of 10 mice.

Coadministration of 31.6 xylazine and 3.1 fentanyl caused death in 40% of male mice (latency: 21 ± 7.2 min, [range 6–37 min]). The surviving animals were hyperactive and had Straub tail during the first 3–5 min after injection. Then, they became sedated for approximately 30 min, were hyperactive again, and presented myoclonus and miosis for approximately 1.5–2 h. Proptosis was evident in all mice treated with this drug combination. This sign was conspicuous and appeared approximately 20–30 min after injection.

Combining 60 mg/kg xylazine with 10 mg/kg fentanyl resulted in 90% mortality within 15 min instead of the 10% produced by fentanyl alone (Fig. 2 A). Animals treated with this combination showed increased locomotion for 5–10 s immediately after injection, but afterwards they stayed motionless and with breathing difficulties.

Neither 10 mg/kg nor 30 mg/kg yohimbine altered the proportion of male mice that died with 10 mg/kg fentanyl + 60 mg/kg xylazine [10 mg/kg yohimbine vs. fentanyl/xylazine: $p = 0.99$; 30 mg/kg yohimbine vs. fentanyl/xylazine: $p = 0.99$; Fisher test]. In contrast, 10 and 30 mg/kg naloxone dose-dependently reduced the lethality to 40% and 0%, respectively. Coadministration of the low yohimbine and naloxone doses significantly reduced the percentage of animals that died, but only to 30% (Fig. 2B). The latency to death was prolonged only with the yohimbine/naloxone combination (Fig. 2 C).

3.3. Potentiation of fentanyl's lethal effects in female mice

To determine whether sex differences existed, we tested two doses of xylazine and fentanyl alone or combined in female mice. The 31.6 mg/kg xylazine dose was not lethal. Although one animal died with 3.1 fentanyl (latency: 73 min), none did it with the 31.6 mg/kg xylazine + 3.1 mg/kg fentanyl treatment. This drug combination produced proptosis at 10–15 min post-injection, sedation, and immobility for approximately 30 min, and hyperactivity, miosis, and Straub tail for another 1.5–2 hours.

At 60 mg/kg, xylazine caused one animal to die; 10 mg/kg fentanyl was lethal for three mice, whereas coadministration of xylazine and fentanyl at the same doses caused death in all ten animals. Pretreatment with 30 mg/kg naloxone reduced lethality from 100% to 10% (latency: 5 min) (Fig. 3). These effects were similar to those seen in males. Table 1 shows the latencies to death and number of fatalities produced by high doses of xylazine, fentanyl, and their combination in both sexes.

4. Discussion

4.1. Effects of xylazine/fentanyl combination in the CPP test

Present results show that xylazine was not rewarding at the doses tested, nor did it alter fentanyl effects in male mice in the CPP. Further studies are needed to determine if this also occurs in females. To our knowledge, this is the first study to test xylazine's effects on the CPP assay. We used subanesthetic, non-sedative doses of this drug to avoid mnemonic processes or locomotor impairment that could interfere with the contextual associations in this test. Although the literature about the putative hedonic effects of xylazine is scarce, people who use it report that it increases and prolongs fentanyl's euphoria (Friedman et al., 2022). Higher doses may be rewarding, but they would be challenging to test in any behavioral assay due to xylazine's CNS depressant effects.

As to fentanyl, we found that low doses (0.01 and 0.03 mg/kg) were not rewarding, but 0.1 mg/kg effectively induced CPP. These results are similar to those reported by Zhang and coworkers (2023) using C57BL/6 mice. The reason for testing these three doses was to choose an effective and a subeffective one, under our experimental conditions, to be used in xylazine/fentanyl combinations. Other researchers have found that 0.01 and 0.03 mg/kg fentanyl induced CPP; however, those results were observed after a longer conditioning phase than ours (eight or ten conditioning sessions instead of six) and in C57BL/6 and A/J mice (Du et al., 2021; Harp et al., 2022). Differences could be due to conditioning time, strains, and contextual cues (Cunningham et al., 2006).

4.2. Xylazine potentiates fentanyl's adverse effects

Animals treated with 31.6/3.1 mg/kg xylazine/fentanyl showed

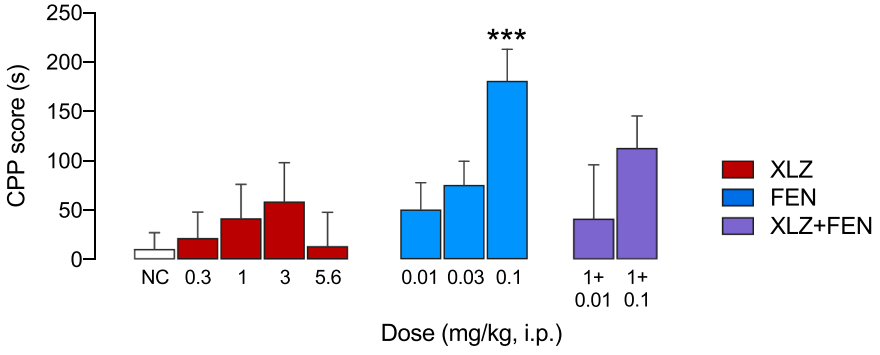

Fig. 1. Dose-response curves for CPP scores corresponding to xylazine (XLZ, 0.3–5.6 mg/kg, red), fentanyl (FEN, 0.01–0.1 mg/kg, blue), and a non-conditioned (NC) group in male mice. Purple bars show CPP scores corresponding to the combination (XLZ+FEN) of a subeffective dose of xylazine (1 mg/kg) with a subeffective or effective fentanyl dose (0.01 and 0.1 mg/kg, respectively). Data represent the mean + SEM. ***$p < 0.001$; one-way ANOVA, followed by Dunnett test, n=8 each.

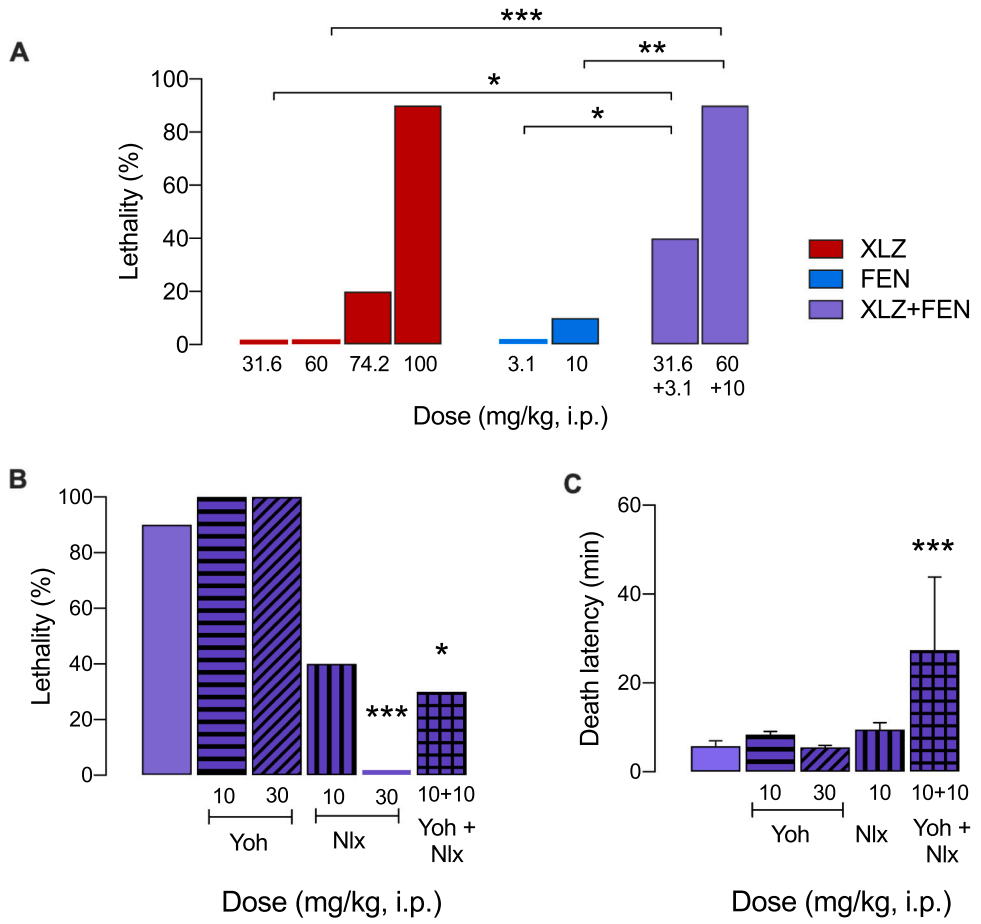

Fig. 2. (A) Dose-response curve for the lethal effects of xylazine (XLZ, red) in male mice, and lethality produced two fentanyl (FEN) doses (blue), or the combined administration of two doses of xylazine and fentanyl (XLZ+FEN, purple). (B) Lethality produced by the 60 mg/kg xylazine/10 mg/kg fentanyl combination in animals pre-treated (−20 min) with 10 or 30 mg/kg yohimbine (YOH), 10 or 30 mg/kg naloxone (NLX), or 10 mg/kg YOH plus 10 mg/kg NLX. (C) Death latency for the groups shown in B. Data are expressed as percentages or as the mean + SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Fisher test or one-way ANOVA followed by Dunnett (n=10 each).

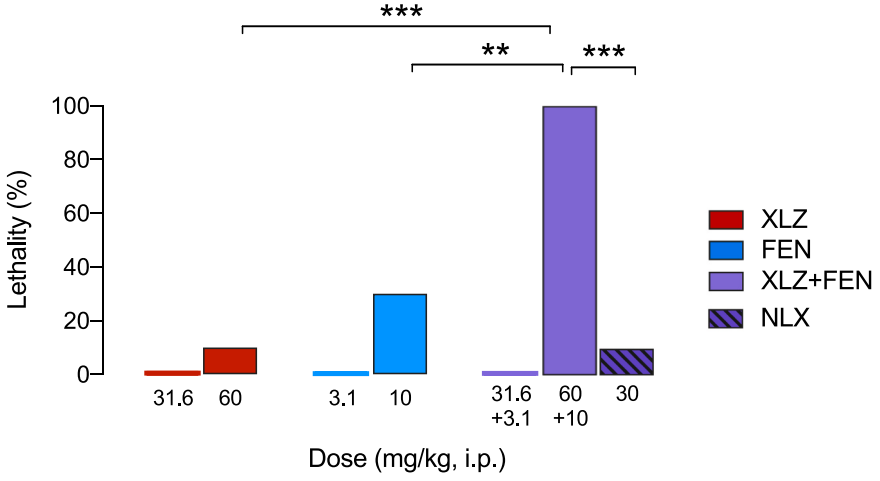

Fig. 3. Lethal effects of two high xylazine (XLZ, red) or fentanyl doses (FEN, blue) alone or in combination (XLZ+FEN, purple) in female mice. The dashed bar corresponds to animals treated with the high dose combination pretreated (−20 min) with 30 mg/kg naloxone (NLX). **$p < 0.01$; ***$p < 0.001$, Fisher test (n=10 per group).

proptosis, urination, and sweating that were not evident with the same doses of the individual drugs. Extreme miosis also occurred with this combination, possibly due to an additive effect of the well-described opioid effect and the miosis produced by high xylazine doses in humans and animals (Burke and Potter, 1986; Capraro et al., 2001). To our knowledge, no reports exist of xylazine-induced proptosis. More studies addressing the mechanism involved in this response are warranted.

Table 1
Number of male and female mice that died and latency to death (mean ± SEM) with different drug treatments.

	Males		Females	
Drug Treatment (mg/kg, i.p.)	Latency [Range] (min)	(n)	Latency [Range] (min)	(n)
60 Xylazine (Xlz)	NA	(0)	5	(1)
10 Fentanyl (Fen)	12	(1)	36 ± 12 [18–59]	(3)
60 Xlz/10 Fen	5.8 ± 1.2 [1–15]	(9)	6.4 ± 0.7 [3–12]	(10)
60 Xlz/10 Fen + 10 naloxone	NA	(0)	5	(1)

NA: not applicable.

The main effect observed here was that a non-lethal xylazine dose highly increased the number of male and female animals that died with an otherwise LD_{10} or LD_{30} fentanyl dose. Several forensic reports show that fatalities associated with xylazine and opioids have increased recently (Alexander et al., 2022; Bowles et al., 2021; Johnson et al., 2021; Nunez et al., 2021). While we cannot extrapolate our findings to humans, present results suggest that subjects exposed to fentanyl mixed with xylazine are at higher risk of experiencing severe side effects, including a fatal overdose, even after a single use.

Of interest, we found that a high naloxone dose effectively prevented the lethality produced by the higher fentanyl/xylazine combination tested here. This result, together with the finding that 10 mg/kg xylazine has mild antinociceptive effects in the tail-flick test, which can be antagonized with 5 mg/kg naloxone (Romero et al., 2013), suggests that at least some of xylazine's effects are mediated by opioid receptors. This point deserves further study because naloxone doses (1.2 – 2 mg i.v.) that antagonize heroin-induced overdoses have not been effective in xylazine-related overdoses in humans (Ayub et al., 2023; Ball et al., 2022; Capraro et al., 2001). Fentanyl differs from other opioids in its high potency, lipid solubility, and rapid onset of action. In addition, it induces muscle rigidity, has an affinity for α_1 adrenoceptors, and requires more naloxone to block its effects (Kelly et al., 2023; Torralva and Janowsky, 2019). It is worth mentioning that high naloxone doses (up to 10 mg/kg) can be effective in counteracting the intoxication produced by high doses of the α_2-adrenoceptor agonist clonidine (Seger and Loden, 2018), which could have contributed to the efficacy of this antagonist to prevent death.

We administered high naloxone doses before the xylazine/fentanyl combinations to ensure equilibrium conditions for testing the hypothesis that opioid receptors played a role in xylazine's effects. Undoubtedly, further studies are needed to determine if naloxone is also effective as an antidote when administered after this drug combination.

Several studies have shown that the effects of opioids and α_2-adrenoceptor agonists can synergize (Chabot-Doré et al., 2015; Vilardaga et al., 2008). As an α_2-adrenoceptor agonist, xylazine activates $G\alpha_{i/o}$ protein-coupled receptors (Bylund, 2007; Comer and Cahill, 2019). As such, it inhibits adenylyl cyclase, increases K^+ conductance, and decreases Ca^{2+} entry, causing cell hyperpolarization (Bylund, 2007; Comer and Cahill, 2019). Since the intracellular signaling of μ-opioid receptors is the same as α_2-adrenergic receptors, fentanyl and xylazine coadministration may produce an intracellular signaling convergence, causing excessive CNS depression and death.

There is also evidence that adrenergic receptor agonists (e.g., xylazine) can interact with opioid receptors and vice versa via allosteric modulation (Vilardaga et al., 2008). In particular, opioid receptors have an allosteric binding site for adrenergic agonists, and adrenoceptors have an allosteric binding site for opioids. On the other hand, it has been proven that clonidine binding to the κ-opioid receptor prevents its internalization and enhances the GPCR-mediated signaling induced by opioids (Root-Bernstein, 2022). In addition, μ-opioid receptors and adrenergic receptors are colocalized in several brain regions and can form heterodimers (Chabot-Doré et al., 2015; Vilardaga et al., 2008). The combination of possible allosteric interactions, heterodimer formation, and similar intracellular and systemic effects (i.e., breathing difficulties, hypotension, and sedation) makes adulteration of fentanyl with xylazine a significant threat for people exposed to these substances (even after a single use) and a challenge for service providers.

4.3. Lack of sex differences in xylazine and fentanyl's lethal effects

The lethality produced by high doses of xylazine, fentanyl, or xylazine plus fentanyl in the present study was not statistically different in male and female mice. A high naloxone dose to prevent death was equally effective in animals of either sex. To our knowledge, xylazine is similarly effective as a muscle relaxant in male and female animals, suggesting that sex differences are not evident for this compound. In addition, some studies have not found significant sex differences for CPP among animals conditioned with opioids (Barattini et al., 2023; Bardo et al., 1995), whereas others suggest that CPP can be induced by lower fentanyl doses in males compared to females (Gaulden et al., 2021). In addition, males show higher analgesia, more pronounced muscle rigidity, and decreased oxygen saturation than females in response to fentanyl (Little and Kosten, 2023). The similar efficacy found in the present work could be due to the use of high doses of each substance, which could mask possible sex differences.

5. Conclusions

Our results contribute to the knowledge of the preclinical effects of combining xylazine with fentanyl. Although xylazine, at non-sedative doses, did not modify fentanyl's rewarding effects, it dramatically increased the lethality induced by fentanyl. A high naloxone dose effectively prevented death produced by fentanyl/ xylazine. There were no differences between males and females in the lethal effects of xylazine/fentanyl coadministration.

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CRediT authorship contribution statement

Palmira Acosta-Mares: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Valeria Violante-Soria:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Thom Browne Jr.**: Conceptualization, Validation, Funding acquisition. **Silvia L. Cruz:** Conceptualization, Validation, Funding acquisition, Writing, Data analysis, final review, and editing. All authors read and accepted the final version of this manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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