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Effects of Inhaled Aerosolized Carfentanil and Subsequent Naloxone Treatment on Real-Time Physiological Responses in Mice

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14. ABSTRACT
This study examined the real-time exposure-response effects of aerosolized carfentanil (CRF) on opioid-induced toxicity, respiratory dynamics, and cardiac function in mice. Clinical signs of opioid-induced toxicity were observed in a dose-dependent manner during exposure and at 24 hr post-exposure to CRF. Post-exposure administration of naloxone (NX, 0.05 mg/kg, i.m.) did not increase the minute volume of animals exposed to CRF to control levels within 24 hr, but decreased clinical signs of opioid-induced toxicity and the duration of respiratory depression. This is the first study to evaluate real-time respiratory dynamics and cardiac function during exposure and up to 24 hr post-exposure to CRF. The evaluation of toxicological signs and respiratory dynamics following exposure to CRF will be useful in the development of therapeutic strategies to counteract the ongoing threat of abuse and overuse of opioids and their synthetic variants.

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Abstract

This study examined the real-time exposure-response effects of aerosolized carfentanil (CRF) on opioid-induced toxicity, respiratory dynamics, and cardiac function in mice. Unrestrained, conscious male CD-1 mice (25-30 g) were exposed to 0.4 or 4.0 mg/m^3 of aerosolized CRF for 15 min (*Ct* = 6 or 60 mg×min/m^3) in a whole-body plethysmograph chamber, in which minute volume (MV) was recorded in real-time. Various clinical observations of classical opioid-induced toxicity were also collected during exposure and up to 24 hr post-exposure. Core body temperature (T_c), mean arterial blood pressure (MAP), and heart rate (HR) were evaluated in animals implanted with telemetric devices and exposed to CRF or sterile H_2O. Loss of consciousness and Straub tail were observed in under 1 min following initiation of exposure to 6 or 60 mg×min/m^3 of CRF. Clinical signs of opioid-induced toxicity were observed in a dose-dependent manner during exposure and at 24 hr post-exposure to CRF. Exposure to 6 or 60 mg×min/m^3 of CRF resulted in significant decreases in MV as compared to controls. MAP, HR, and T_c decreased over the 24 hr collection period in animals exposed to either 6 or 60 mg×min/m^3 of CRF as compared to controls. Post-exposure administration of naloxone (NX, 0.05 mg/kg, i.m.) did not increase the MV of animals exposed to CRF to control levels within 24 hr, but decreased clinical signs of opioid-induced toxicity and the duration of respiratory depression. This is the first study to evaluate real-time respiratory dynamics and cardiac function during exposure and up to 24 hr post-exposure to CRF. The evaluation of toxicological signs and respiratory dynamics following exposure to CRF will be useful in the development of therapeutic strategies to counteract the ongoing threat of abuse and overuse of opioids and their synthetic variants.

1. Introduction

Opioids are alkaloidal compounds that are intended for use as sedatives and analgesics. Side effects of opioid administration include nausea, dizziness, constipation, vomiting, physical dependence, and respiratory depression. Opioid toxicity results in depression of the central nervous system and inhibition and failure of the respiratory system. While the use of these chemicals is highly regulated and occurs under close supervision and scrutiny, death occurs from overdose and subsequent cardiorespiratory collapse. Widespread over-prescription of opioid analgesics and insufficient resources to support medical personnel trained in opioid medication management have led to recent increases in opioid-related deaths—the number in the United States alone more than quadrupling since 1999—rendering opioid misuse a major health concern and emphasizing the need for a more complete understanding of the potential toxicity of opioids (Cochran et al. 2016; Compton et al. 2015; Kolodny et al. 2015; Muhuri et al. 2013; Tefera et al. 2016).

Opioids and their derivatives act through their interaction with multiple endogenous peptide ligands such as endorphins, enkephalins, and dynorphins, each having affinities for the mu (μ), delta (δ), and kappa (κ) receptors, respectively (Feng et al. 2012). These G-protein-coupled receptors are widely distributed throughout the body and are present not only in the nervous system, but also peripherally in the lung, heart, liver, and gastrointestinal tract (Wittert et al. 1996). Regardless of the route of administration, opioids and their analogues are highly lipid soluble and can reach the brain quickly, providing rapid onset of effects. Opioid receptors have a role in various physiological and pathophysiological processes, including the regulation of membrane ionic homeostasis, cell proliferation, emotional response, epileptic seizures, immune function, feeding, obesity, and respiratory and cardiovascular control, as well as some neurodegenerative disorders (Feng et al. 2012). Pharmacologically, general systemic side effects of opioid toxicity include, but are not limited to, euphoria, life-threatening bradycardia, respiratory depression, hypoxia, miosis, hypothermia, decreased gastric motility, and seizures (Feng et al. 2012). The availability and potency of this class of medications increase the likelihood of intentional and accidental exposure resulting in health hazards, morbidity, and potential lethality.

Carfentanil (CRF) is a narcotic analogue of the synthetic opioid fentanyl and is not intended for use in humans. It is classified by the United States Comprehensive Drug Abuse Prevention and Control Act of 1970 as a Schedule II drug, meaning that it has a high potential for abuse and a currently accepted medical use in treatment in the United States or a currently accepted medical use with severe restrictions, and that its abuse may lead to severe psychological or physical dependence. It is widely used as a commercially available tranquilizing agent (Wildnil, Wildlife Laboratories) for large livestock and wildlife in zoological and wildlife facilities under very strict control and with assistants always standing by with antidote. It is 10,000 times more potent than morphine and 100 times more potent than fentanyl (George et al. 2010). CRF has been used in studies with goats, Rocky Mountain elk, bison, bear, and rats as well as in pharmacokinetic studies in humans (Halverson and Teare 1989; Heard et al. 1996; Moresco et al. 2001; Mutlow et al. 2004). These studies investigated challenges on the scale of μg/kg on a range of physiological, cardiopulmonary, and pharmacological parameters. CRF is considered to be safe, with a therapeutic safety margin of 8,460 and a potency ratio of 16,600 (Haigh and Gates 1995; Haigh et al. 1983). In medical laboratories and clinics, CRF is used as a radio-ligand for positron emission tomography (PET) imaging of brain opioid receptors (Ly et al. 2013; Titeler et al. 1989). Data on toxicity and dose assessments of CRF in humans are unknown and require additional investigation.

Although opioids are analgesics, their role as lethal agents has also been considered, thus requiring further investigation into their toxicity. Studies have shown that the FDA-approved opioid antagonist naloxone (NX) is capable of rapidly counteracting the respiratory depression resulting from exposure to morphine and fentanyl (Miller et al. 1996; Olofsen et al. 2010). Inhalation exposure of mice to volatilized heroin, morphine, codeine, fentanyl, and meperidine resulted in antinociception, as measured by the tail-flick test, which was decreased or reversed following administration of NX (Lichtman et al. 1996). NX is typically administered to individuals suffering from opioid-induced toxicity until symptoms are reversed. However, the therapeutic potential of antagonists such as NX has not been extensively studied for the ability to counteract opioid-induced toxicity of extremely potent opioids and their variants that have diverse binding affinities and selectivities for receptor complexes. The rapid elimination of NX, biophase equilibration half-lives, and receptor kinetics complicate reversal of high-affinity opioids, such as CRF (Dahan et al. 2010). Following its metabolism, CRF can bind to systemic tissues, recirculate, and trigger "renarcotization." Additional studies evaluating various antagonist therapies against opioids with varying membrane binding affinities would provide valuable information in the development of medical treatment strategies.

This study was designed to evaluate the toxic effects of aerosolized CRF using a high-throughput mouse inhalation model with integrated real-time respiratory monitoring capabilities, since respiratory depression is the primary cause of opioid-induced lethality. Various clinical observations and physiological parameters, including loss of consciousness, Straub tail, minute volume (MV), mean arterial blood pressure (MAP), heart rate (HR), and core body temperature (T_c) were used to evaluate the toxicity of CRF in mice before, during, and up to 24 hr post-exposure. In addition, we tested the ability of the standard treatment, NX, to ameliorate the systemic sequelae of inhaled aerosolized CRF. Collectively, the robust exposure model and studies described herein were used to establish and assess opioid-induced toxicity in order to test potential therapeutic countermeasures.

2. Materials and Methods

2.1 Animals

Adult male albino CD-1 mice (25-30 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Mice were housed in groups of 10 until the agent exposure and then individually housed afterward. Housing consisted of polycarbonate micro-isolator cages in temperature-controlled rooms. Temperature was maintained between 20°C and 26°C, with humidity maintained between 30% and 70%. Rodents had free access to food and water and were subjected to a 12-hour light/dark cycle with no twilight. The study protocol was approved by the Institutional Animal Care and Use Committee, USAMRICD, Aberdeen Proving Ground, Maryland, and this research complied with the Animal Welfare Act and implementing Animal Welfare Regulations, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and adhered to the principles noted in The Guide for the Care and Use of Laboratory Animals (NRC, 2011).

2.2 Chemicals

Carfentanil (CRF), methyl 4-[(1-oxopropyl) phenylaminol-1-(2-phenylethyl)-4-piperdine carboxylate, >95% pure ($C_{24}H_{30}N_2O_3$; MW 394.51), was obtained in crystalline form as a citrate salt from the chemical synthesis laboratory at the Edgewood Chemical Biological Center, APG, MD. From this, stock solutions of 2.5 and 25 mg/mL were made in sterile water and stored at -80° C until needed. Xylazine and ketamine were purchased from Webster Veterinary Supplies

(Devens, MA). Naloxone (NX), 4,5-epoxy-3,14-dihydroxy-17-(2-propenyl) morphine-6-one HCl, was obtained from Sigma Chemical Co. (St. Louis, MO).

2.3 Inhalation Exposures

Exposures to CRF and controls were conducted within a custom inhalation system as shown in Figure 1. CRF- and control-exposed animals were contained within whole-body plethysmograph (WBP) chambers (Data Sciences International, St. Paul, MN). Aerosols were generated by placing the entire contents of the exposure aliquot into a Collison nebulizer (BGI Incorporated, Waltham, MA) and operating the nebulizer according to the manufacturer's instructions. A CRF concentration-time product (*Ct*) of 6 or 60 mg×min/m^3 (0.4 or 4.0 mg/m^3 for 15 min) was used throughout the study. Mass median aerodynamic diameter (MMAD) and particle concentration were determined to be 3-4 µm and 130 mg/m^3, respectively, using an Aerodynamic Particle System (TSI Model 3321, Shoreview, MN). Nebulizers were pressurized using a medical air compressor (Jun-Air, Benton Harbor, MI) to 25-30 psi to generate CRF aerosol, which was then fed into a custom-designed air manifold to which four WBP chambers were connected. Exposure chambers were modified WBP units (FinePointe Series Whole Body Plethysmography Rat Chamber; Data Sciences International; St. Paul, MN, USA) capable of collecting respiratory parameters before, during, and after exposure. Bias flow generators (Bias Flow Fresh Air Pump; Data Sciences International; St. Paul, MN, USA) connected to the WBPs generated a slight vacuum to pull gas and aerosolized CRF from the manifold into the chamber, ensuring agent flow to each exposure unit at physiologically compatible and sufficient rates. Exit flows from the manifolds and bias flow units passed through a custom-built activated charcoal decontamination unit.

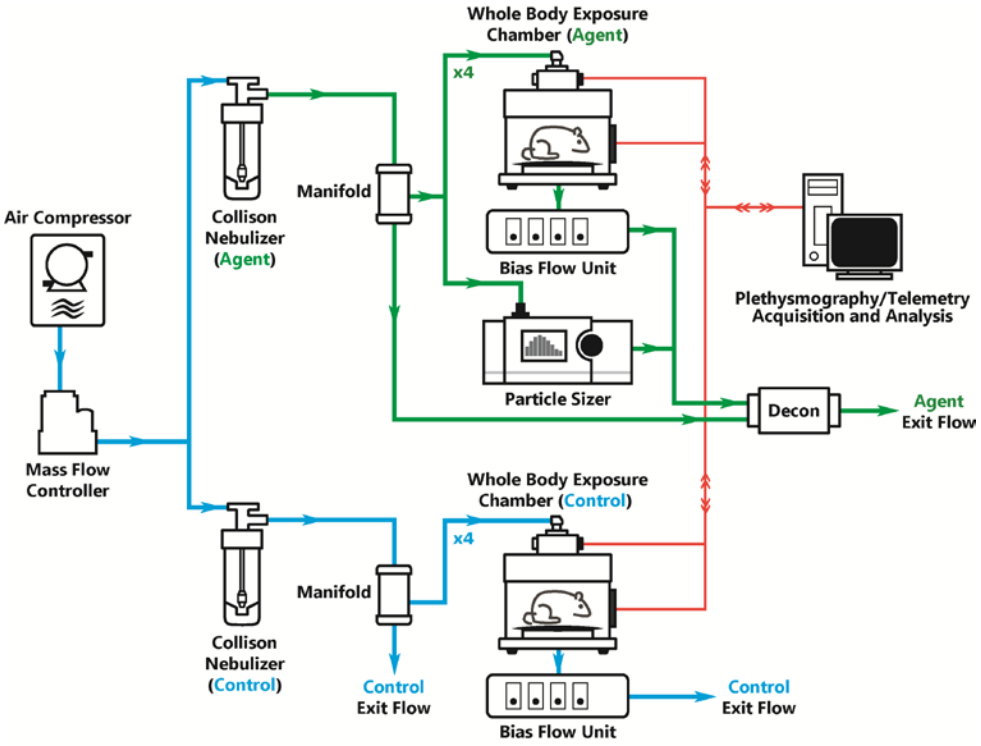

Figure 1. Configuration of CRF inhalation exposure system.

Immediately before exposure, all mice were allowed to acclimate to the exposure chamber for 10 min, and baseline recordings were collected for 30 min. A 15 min aerosolized CRF exposure then followed baseline respiratory data collection. Concurrently, control mice were exposed to aerosolized sterile H_2O for the same duration using a separate but identical exposure setup. Exposures were controlled by custom computer programs (LabView®, National Instruments Corporation, Austin, TX). The primary parameter of respiratory dynamics measured was minute volume (MV), the product of tidal volume and respiratory rate. Data collection occurred at 15-second intervals, and the acquisition software (FinePointe, DSI) utilized a rejection index to exclude statistical inaccuracies and external noise. Mice remained in the WBP with continuous recording of respiratory dynamics for 24 hr. Animals were provided with a high protein rodent liquid diet (Bio-Serv, Frenchtown, NJ). All exposures were conducted within a custom-designed certified glovebox (Baker Co., Sanford, Maine).

2.4 Clinical Observations

Clinical observations were collected for all CRF- (6 and 60 mg×min/m^3) and control-exposed animals during exposure and at 24 hr post-exposure. Events recorded during the 15 min exposure period included the onset of loss of consciousness (defined as the inability of a mouse to right itself within 15 sec), and the Straub reaction (wherein the tail becomes rigid and erect across the back of the animal in an S-shaped curve) (Bilbey et al. 1960; Straub 1911). Additional clinical observations included general (dyspnea, lack of grooming, piloerection and ocular protrusion), gastrointestinal (abdominal bloating, urinary incontinence, and rectal prolapse), movement (ataxia and restlessness), and behavior (stressor-provoked response and natural behavior) effects. The observed clinical signs were defined as no effect, minimal, mild, moderate and severe.

2.5 Telemetry

Animals were surgically implanted with telemetry probes (HD-X11 PhysioTel Hybrid Digital, Data Sciences International, Inc), which were placed in the ventral abdomen by Data Sciences International Inc. (DSI) surgical staff and shipped to USAMRICD after one week of recovery. The transmitter sends out the digitized data in the radio frequency range to a receiver located beneath the WBP. The biopotentials collected from the telemetry units for all experimental animals were verified post-implantation by both DSI and USAMRICD staff. Heart rate (HR), core body temperature (T_c) and mean arterial blood pressure (MAP) were collected during the baseline (30 min), exposure (15 min), offgas (15 min) and post-exposure (the first 15 minutes of every hour, up to the 24 hr endpoint) periods.

2.6 Treatment Protocol

Animals in this section were divided into several groups based on their exposure to (1) aerosolized sterile H_2O followed by treatment with 0.05 mg/kg NX, (2) aerosolized CRF (60 mg×min/m^3), and (3) aerosolized CRF (60 mg×min/m^3) followed by treatment with 0.05 mg/kg NX. At 15 min post-exposure, mice in the treatment groups were given a single i.m. administration of NX at 0.05 mg/kg and immediately returned to the WBP chamber for collection of MV.

Clinical observations were collected up to 24 hr post-exposure from animals following post-exposure administration of NX and consisted of assessment of general (dyspnea, lack of grooming, piloerection and ocular protrusion), gastrointestinal (abdominal bloating, urinary incontinence, and rectal prolapse), movement (restlessness), and behavior (stressor-provoked response and natural behavior) effects.

2.7 Data Analysis

Statistical analysis of differences between the controls, CRF-exposed NX-treated, and CRF-exposed animal groups was performed using GraphPad Prism (GraphPad Prism v5.04, Graph Pad Software Inc. San Diego, CA). MV, HR, T_c, and MAP were evaluated by two-factor repeated measures (time point as the repeated factor and exposure dose as the other) analysis of variance (ANOVA) followed by Bonferroni post-tests to assess differences between all groups within a factor. Calculated probability values (*p*) less than or equal to 0.05 were considered statistically significant.

3. Results

3.1 Effects of inhaled aerosolized CRF on clinical observations

The evaluation of clinical observations in CRF- and control-exposed animals during exposure and at 24 hr post-exposure is shown in Table 1. The onset of loss of consciousness was observed at an average of 50 and 30 sec into the 15 min exposure period in animals exposed to 6 or 60 mg×min/m^3 of CRF, respectively. Straub tail was recorded at an average of 32 and 20 sec following exposure to 6 or 60 mg×min/m^3 CRF, respectively. Additionally, dyspnea, lack of grooming, piloerection, ocular protrusion, abdominal bloating, urinary incontinence, rectal prolapse, ataxia, restlessness, and alterations in stressor-provoked response and natural behavior were observed in a dose-dependent manner in animals exposed to 6 or 60 mg×min/m^3 of CRF. All controls appeared normal and did not exhibit any of the observed clinical signs of CRF exposure.

Table 1. Clinical observations in mice following inhalation exposure to CRF.

Parameter	**Control**	**CRF (6 mg×min/m^3)**	**CRF (60 mg×min/m^3)**
Timed events to onset:			
Onset of loss of consciousness (sec)*	-	50 sec	30 sec
Straub Tail (sec)*	-	32 sec	20 sec
General:			
Dyspnea/labored breathing***	-	++++	++++
Lack of grooming**	-	++	+++
Piloerection**	-	+++	+++
Ocular protrusion***	-	++	++
Gastrointestinal:			
Abdominal bloating**	-	++	++++
Urinary incontinence (leakage)**	-	++	+++
Rectal prolapse***	-	++	+++
Movement:			
Ataxia (loss of balance)*	-	++	++
Restlessness (Spinning/flipping/circling)***	-	+++	++++
Behavior:			
Stressor-Provoked response***	-	Weak	Very weak
Natural behavior***	-	Less alert	Less alert

Time of Observation: During exposure*, 24 hr post-exposure**, and during and 24 hr post-exposure ***
Grading Scale: No effect/Normal -, Minimal +, Mild ++, Moderate +++, Severe ++++

3.2 Effects of inhaled aerosolized CRF on MV

Trends of MV over 24 hr in animals exposed to sterile H_2O and CRF at 6 and 60 mg×min/m^3 are shown in Figure 2. During exposure, animals exposed to CRF at either *Ct* showed a decrease in MV as compared to controls. Statistically significant reductions in MV were observed at multiple time points following exposure to 6 or 60 mg×min/m^3, as compared to controls. The most prominent MV reduction in CRF-exposed animals was observed from 5 to 19 hr post-exposure. Additionally, at 5 hr post-exposure, the MV in animals exposed to 6 and 60 mg×min/m^3 was decreased by 120% and 130% in comparison to controls, respectively.

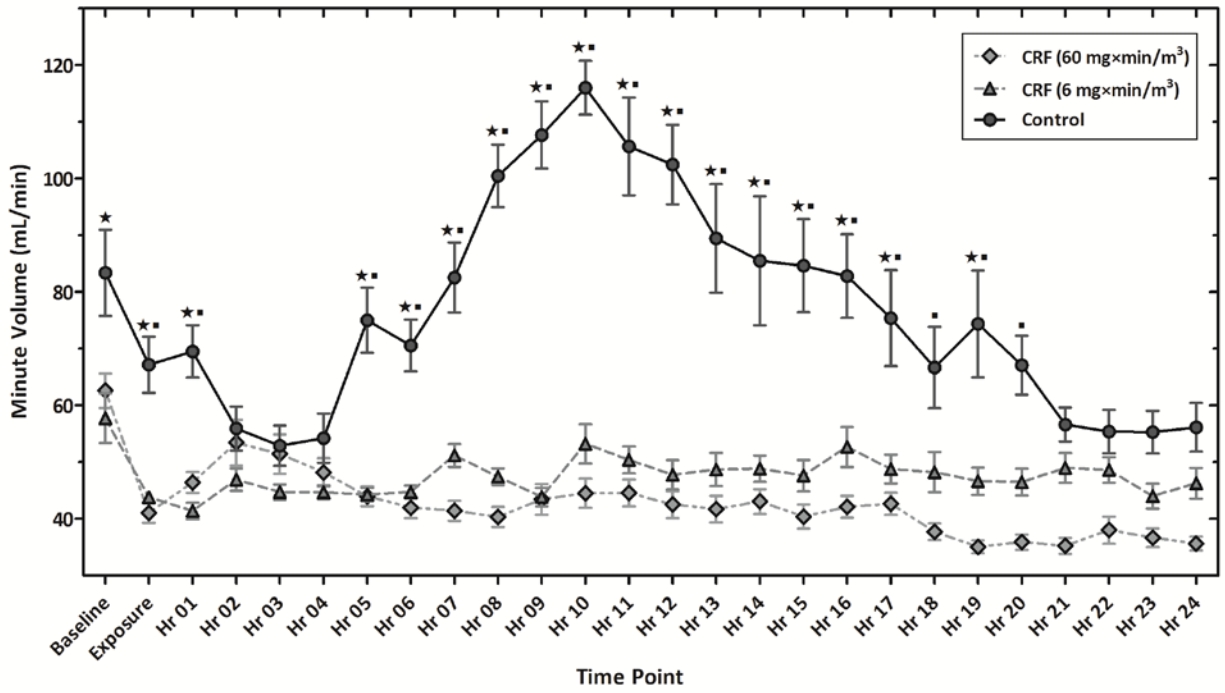

Figure 2. MV in mice following a 15 min exposure to CRF. MV shows minimal temporal dose-response between 6 and 60 mg×min/m^3 CRF-exposed animals. This effect is more pronounced from 16-24 hr post-exposure. *n*=16 for each group. The increased MV peak around Hr10 is expected due to the diurnal cycle of mice and correlates to expected time of highest wakeful activity. ▪ indicates statistical significance (p<0.05) between control and 60 mg×min/m^3 CRF-exposed groups. ★ indicates statistical significance (p<0.05) between control and 6 mg×min/m^3 CRF-exposed groups.

3.3 Effects of inhaled aerosolized CRF on cardiac function

Trends of MAP, HR, and T_c over 24 hr in control animals and those exposed to 6 or 60 mg×min/m^3 of CRF are shown in Figure 3. MAP decreased in all CRF-exposed animals as compared to controls during exposure, but was not observed to be statistically significant. Statistically significant decreases in MAP were observed in animals exposed to CRF as compared to controls from 9 to 14 hr post-exposure. During the exposure period and at 1 and 5 hr post-exposure to 60 mg×min/m^3 of CRF, statistically significant decreases in heart rate (Figure 4) were observed. Exposure to 6 mg×min/m^3 of CRF resulted in a statistically significant decrease in heart rate at 2 hr post-exposure. Addtionally, the heart rate of animals exposed to 6 or 60 mg×min/m^3 of CRF was decreased in a statistically significant manner during the 5 to 10

hr period following exposure. T_c following exposure to 6 and 60 mg×min/m^3 CRF and controls is shown in Figure 5. Statistically significant decreases in T_c in comparison to controls were observed in animals exposed to 60 mg×min/m^3 CRF from 5 to 15 hr post-expsore. Exposure to 6 mg×min/m^3 CRF resulted in statistically significant decreases in T_c in comparison to controls from 3 to 14 hr post-exposure, but was statistically insignificant for all other data collection periods.

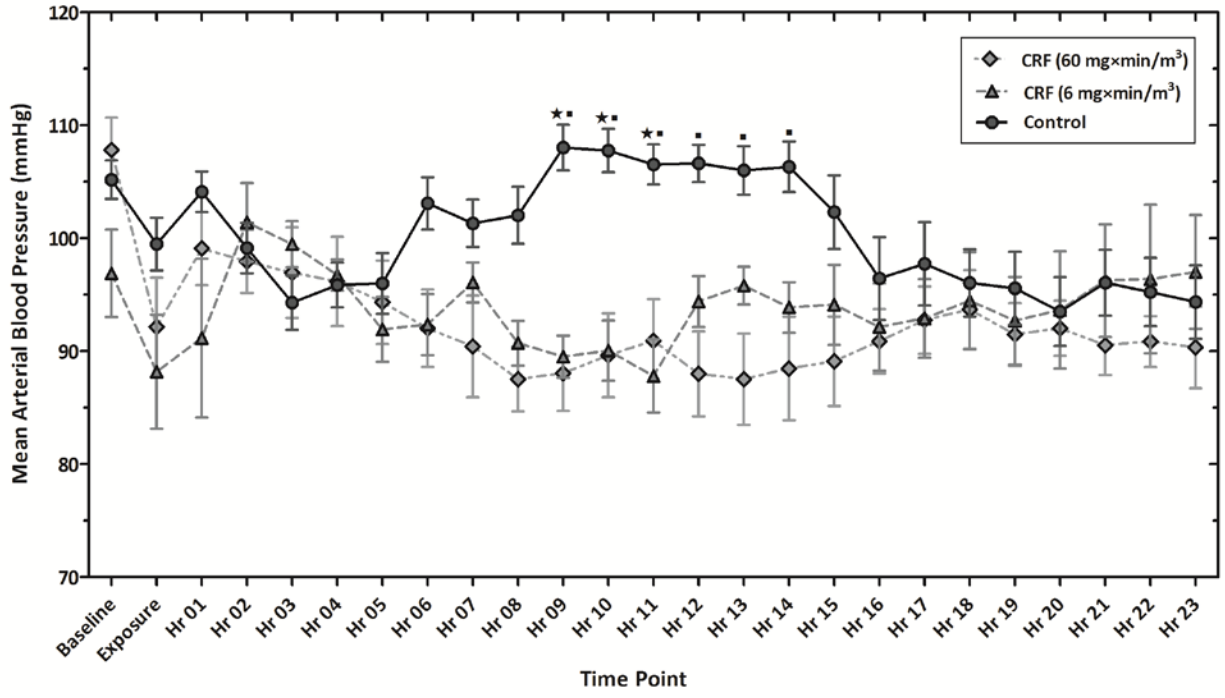

Figure 3. MAP in mice following a 15 min exposure to CRF. Exposure to 6 and 60 mg×min/m^3 of CRF resulted in statistically significant decreases in MAP, as compared to controls, in the 9-14 hr post-exposure window. *n*=8 for each group. ▪ indicates statistical significance (p<0.05) between control and 60 mg×min/m^3 CRF-exposed groups. ★ indicates statistical significance (p<0.05) between control and 6 mg×min/m^3 CRF-exposed groups.

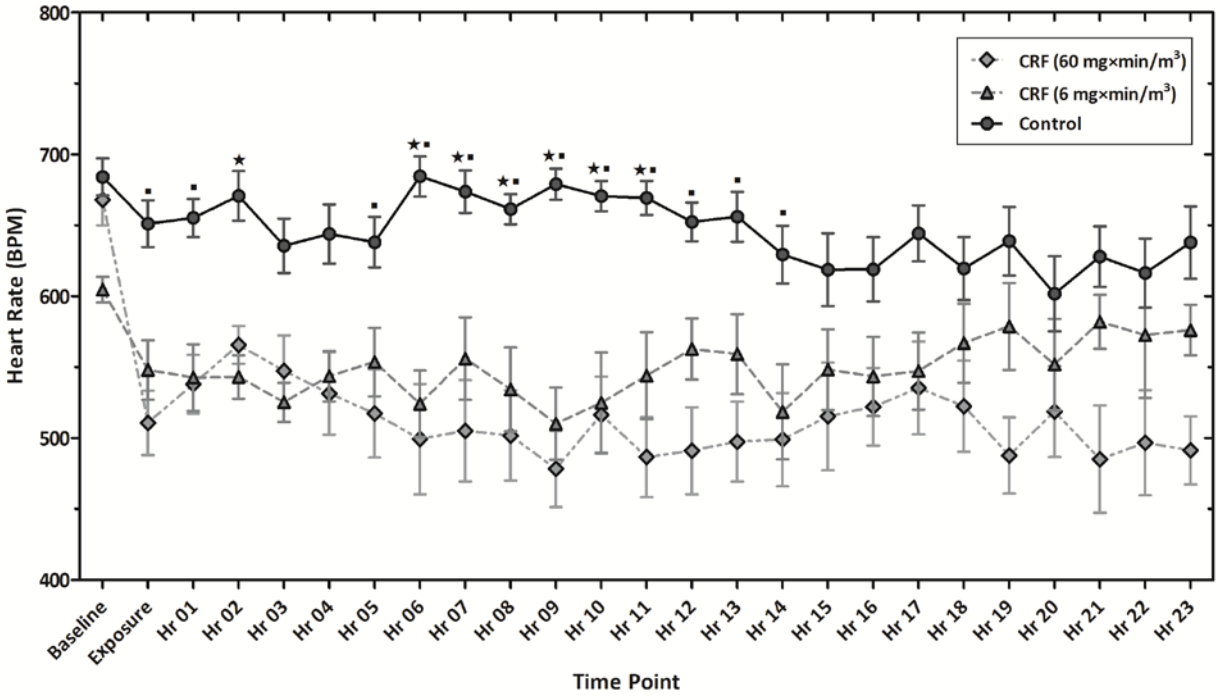

Figure 4. HR in mice following a 15 min exposure to CRF. Exposure to 6 and 60 mg×min/m^3 of CRF resulted in statistically significant decreases in HR, as compared to controls, especially in the interval from 5-14 hr post-exposure. *n*=8 for each group. ▪ indicates statistical significance (p<0.05) between control and 60 mg×min/m^3 CRF-exposed groups. ★ indicates statistical significance (p<0.05) between control and 6 mg×min/m^3 CRF-exposed groups.

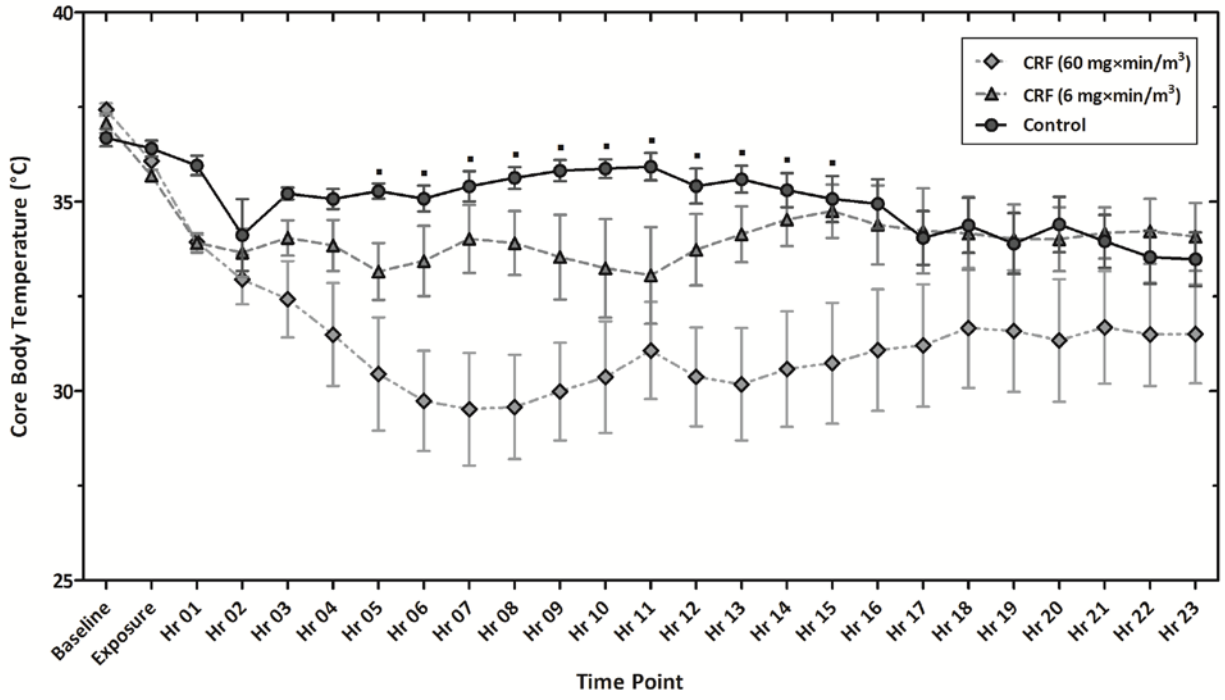

Figure 5. T_c of mice following a 15 min exposure to CRF. Exposure to 6 and 60 mg×min/m^3 CRF resulted in statistically significant decreases of approximately 15-20% starting at 5 hr post-exposure at the higher dose. *n*=8 for each group. ▪ indicates statistical significance (p<0.05) between control and 60 mg×min/m^3 CRF-exposed groups. ★ indicates statistical significance (p<0.05) between control and 6 mg×min/m^3 CRF-exposed groups.

3.4 Effects of a single administration of NX following aerosolized CRF exposure on clinical observations and physiological recovery

The clinical observations of animals exposed to sterile H_2O, 60 mg×min/m^3 CRF alone, and 60 mg×min/m^3 CRF followed by NX at 0.05 mg/kg 15 min post-exposure are shown in Table 2. All assessments in animals administered NX were conducted up to 24 hr post-exposure. Data and observations for animals exposed to sterile H_2O followed by no treatment or treatment with an equivalent volume of saline could not be reasonably distinguished from those exposed to sterile H_2O and treated with NX and is not shown. Animals exposed to CRF and administered NX at 0.05 mg/kg appeared to exhibit fewer, if any, signs of CRF intoxication as compared to untreated, CRF-exposed animals. A lack of grooming, piloerection, abdominal bloating, restlessness, decreased stressor-provoked response, and abnormal natural behavior were present in all animals exposed to 60 mg×min/m^3 CRF but were absent in NX-treated animals. Labored breathing, ocular protrusion, urinary incontinence, and rectal prolapse were still observed in the NX-treated animals, but were decreased in comparison to CRF-exposed, untreated animals.

Table 2. Assessment of 24 hr post-exposure clinical observations in NX-treated controls, CRF-exposed animals, and CRF-exposed animals treated with NX.

Parameter	Control + NX (0.05 mg/kg)	CRF (60 mg×min/m^3)	CRF (60 mg×min/m^3) + NX (0.05 mg/kg)
General:			
Dyspnea/labored breathing	-	++++	++
Lack of grooming	-	+++	-
Piloerection (fur poofing)	-	+++	-
Ocular protrusion	-	++	+
Gastrointestinal:			
Abdominal bloating	-	++++	-
Urinary incontinence (leakage)	-	+++	++
Rectal prolapse	-	+++	++
Movement:			
Restlessness (Spinning/flipping/circling)	-	++++	-
Behavior:			
Stressor-provoked response	-	Very weak	-
Natural behavior	-	Less alert	-

Grading Scale: No effect/Normal -, Minimal +, Mild ++, Moderate +++, Severe ++++

The MV of animals exposed to sterile H_2O, 60 mg×min/m^3 CRF, and 60 mg×min/m^3 CRF followed by NX at 0.05 mg/kg 15 min post-exposure is shown in Figure 6. Statistically significant inhibition of MV in comparison to controls was still observed between 9 to 14 hr post-exposure to CRF despite administration of NX at 0.05 mg/kg 15 min post-exposure. However, substantial recovery of observed behaviors (Table 2) typically occurs within 1-2 minutes of NX administration. Over the course of 24 hours, the behavior of animals treated with NX more closely resembles that of controls than that of CRF-exposed, untreated animals, despite sustained reductions in MV.

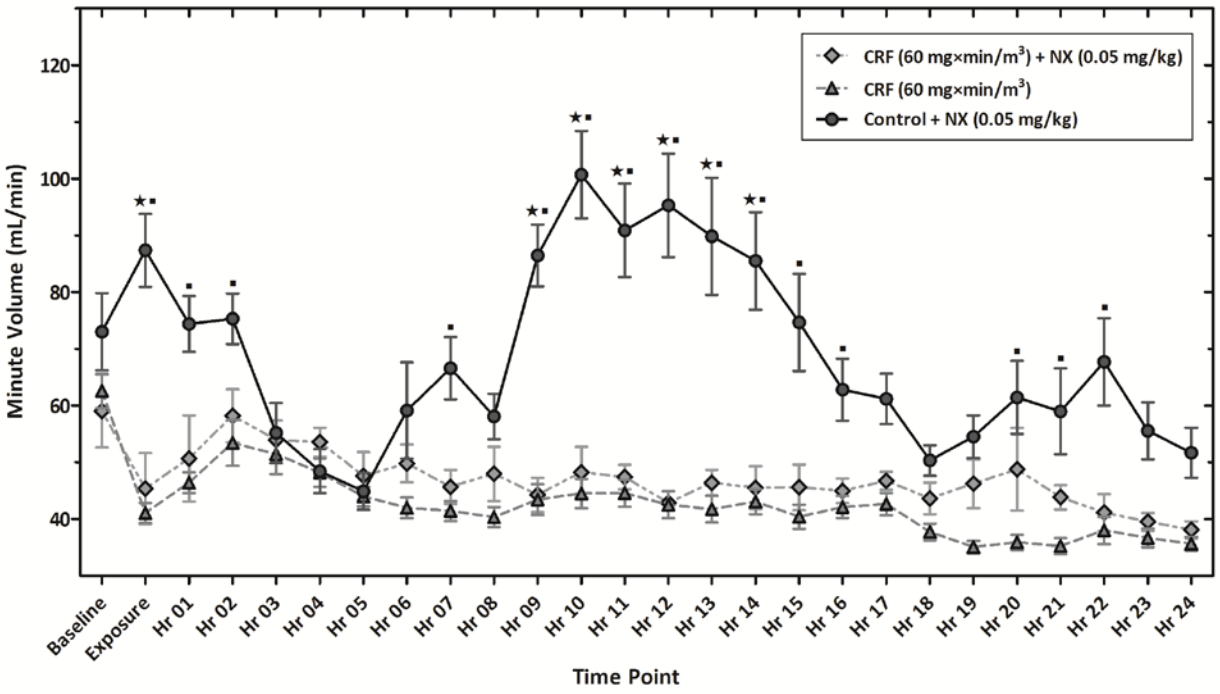

Figure 6. MV of NX-treated controls, CRF-exposed animals (60 mg×min/m^3 CRF) with and without NX treatment at 0.05 mg/kg. A single i.m. administration of NX at 0.05 mg/kg failed to restore MV to levels comparable to controls. n=16 for each group. ▪ indicates statistical significance (p<0.05) between controls and CRF-exposed (60 mg×min/m^3) groups. ★ indicates statistical significance (p<0.05) between controls and CRF-exposed (60 mg×min/m^3) groups treated with NX (0.05 mg/kg).

4. Discussion

Respiratory depression and opioid-induced toxicological symptoms following exposure to synthetic opioids and their variants are an increasing concern. Respiratory depression is thought to be a result of opioid receptor-mediated depression of neuronal transmission in the respiratory center of the brain (Feng et al. 2012; Hurle et al. 1985). Limited information exists on the chemical properties and binding efficiency of various opioids, including CRF. Increase in opioid use has driven the need for additional evaluation of therapeutic strategies to aggressively monitor pain management, decrease sedation, and manage respiratory depression that can lead to serious opioid-related adverse events (Jarzyna et al. 2011; Jungquist et al. 2011). In this study, we tested the hypothesis that the potency of aerosolized CRF would produce acute effects resulting in increased mortality, especially with regard to its use as a large animal sedative. In contrast to the expected increase in mortality, we have shown that mice exposed to 6 or 60 mg×min/m^3 CRF only showed statistically significantly compromised physiological function, as measured by respiratory dynamics and systemic hemodynamics. Further research will be needed to determine the precise reason for the lack of mortality in this study, but species-dependent differences in metabolism, pharmacokinetics/pharmacodynamics, physiology, and anatomy should be considered central to answering this question. In combination with other studies, the collective body of literature indicates that respiratory depression can be a severe complication following exposure to CRF. However, this is the first study to evaluate the toxicity of aerosolized CRF in real-time using respiratory parameters

before, during, and after exposure for up to 24 hr. Additional studies are necessary to determine the toxicity and assess respiratory depression following exposure to CRF and other inhaled opioids and their variants to determine potential therapies to reverse opioid-induced toxicity.

Obvious and immediate dose-dependent signs of opioid-induced intoxication were observed following exposure to 6 or 60 mg×min/m^3 CRF. Exposure resulted in loss of consciousness almost immediately following exposure at both *Ct*s, verifying the ability of this aerosolized inhalation model to produce rapid, opioid-induced sedation. Consistent with historical work with morphine in mice (Bilbey et al. 1960; Heinekamp 1922; Leimdorfer 1948; Straub 1911), Straub tail was also observed almost immediately following exposure to CRF, indicating that the aerosolized CRF generated within this exposure system was able to rapidly produce signs of direct spinal cord stimulation and centrally mediated toxicity, even though definitive proof of CRF in the brain or CNS was not established. The observed gastrointestinal effects, such as abdominal bloating, urinary incontinence, and rectal prolapse, are consistent medical complications resulting from opioid overuse and toxicity. Clinically, respiratory toxicity and constipation have been considered two of the most prominent symptoms following exposure to opioids (Jarzyna et al. 2011; Jungquist et al. 2016). Persistent alterations in behavior, observed from time of exposure until the experimental endpoint, implied that the cognitive ability of the animals may have been compromised up to 24 hr post-exposure. Behavioral studies utilizing this model have been initiated and will provide useful information regarding the cognitive and learning abilities of mice following inhalation exposure to CRF.

Respiratory dynamics following exposure to 6 or 60 mg×min/m^3 CRF were altered during the entire 24 hr recording cycle, with dose-dependent inhibition of MV observed. Despite the 10-fold difference in exposure dose, statistically significant differences in MV were absent between the dose cohorts. Some controversy surrounding the nature of opioid-induced respiratory depression exists, with arguments being whether tidal volume (TV) or respiratory frequency (*f*) is the primary driver. One theory is that opioids produce respiratory depression primarily through reductions in tidal volume (TV), a view experimentally supported by several animal studies (Bouillon et al. 2003; Heard et al. 1990; Heard et al. 1996; Manral et al. 2009; Yeadon and Kitchen 1990). However, these studies also show that depression in respiratory frequency does occur, but appears to recover faster than tidal volume depression. The precise anatomical and physiological origins of the observed effects of opioids on *f* and TV have recently been a subject of vigorous scientific discourse (Lalley 2008; Lalley et al. 2014a, b; Montandon and Horner 2014a, b), with no consensus to date. However, as the product of TV and *f*, MV can be considered a quantifiable net physiological average that is a scientifically relevant and informative compromise. Significant inhibition of MV, concentration-dependent hypoventilation, and increased irregularity of breathing was observed in patients breathing spontaneously via a continuous positive airway pressure mask following intravenous infusion of the opioid alfentanil (Bouillon et al. 2003). Monitoring of alterations in respiratory dynamics continues to be vital to medical management of opioids as analgesics and sedatives. In evaluating medical claims in which postoperative opioid-induced respiratory depression was observed, 88% of the events occurred within 24 hr of surgery, 77% of claims resulted in severe brain damage or death and 97% were judged as preventable with better monitoring and response (Lee et al. 2015). This further highlights the need for additional evaluation of opioid-induced respiratory toxicity to better manage toxic effects.

The results herein indicate that inhalation exposure to CRF produces dose-dependent changes in MAP, HR, and T_c. CRF appears to suppress normal circadian rhythm, and we speculate that the mice are in a state of partial sedation, characterized by decreased cardiac

output. At this time, we cannot determine whether this is a centrally or locally driven response to CRF inhalation; however, we can infer that a central component is linked to core body temperature since the hypothalamus serves as the temperature servomechanism in the brain (Hardy 1973; Rodbard 1948). Heat loss is controlled by the neurons in the heat conservation center of the posterior hypothalamus, and damage or neuronal paralysis may eliminate heat production, thereby causing heat loss under certain environmental conditions. Additional studies evaluating the various opioid receptors in multiple tissues following inhalation exposure to CRF will provide useful information on the activity, selectivity, and binding affinity of different opioids and assist in determining targets and location of toxicological action.

The preliminary evaluation of a single administration of NX at 0.05 mg/kg to sterile H_2O-exposed animals indicated that NX did not alter respiratory function in a statistically significant manner as measured by MV. Conversely, inhalation exposure to 60 mg×min/m^3 CRF induced statistically significant reductions in MV from 5 to 20 hr post-exposure in untreated animals (Figure 1), and administration of NX to 60 mg×min/m^3 CRF-exposed animals resulted in statistically significant decreases in MV in comparison to controls from 9 to 16 hr post-exposure. Although no statistical significance was found in the effects of NX treatment on MV following exposure to CRF, NX did decrease the amount of time the animals were in a state of respiratory depression, defined as an MV equal to or less than 60% of the control average value at the same time point (Table 3).

Table 3. CRF-Induced Minute Volume (mL/min) Depression and Effects of NX on Recovery

Group	Time Point												
	BL	EXP	Hr 01	Hr 02	Hr 03	Hr 04	Hr 05	Hr 06	Hr 07	Hr 08	Hr 09	Hr 10	Hr 11
Control + NX (0.05 mg/kg)	73.0	87.4	74.4	75.3	55.2	48.5	44.9	59.2	66.6	58.1	86.5	100.7	90.9
60% of Control Threshold	*43.8*	*52.4*	*44.6*	*45.2*	*33.1*	*29.1*	*26.9*	*35.5*	*40.0*	*34.9*	*51.9*	*60.4*	*54.5*
CRF (60 mg×min/m^3)	62.6	**41.1**	46.4	53.4	51.4	48.2	44.0	42.0	41.4	40.3	**43.4**	**44.5**	**44.6**
CRF (60 mg×min/m^3) + NX (0.05 mg/kg)	59.1	**45.4**	50.7	58.2	54.0	53.6	47.6	49.8	45.7	48.0	**44.3**	**48.3**	**47.4**

Group	Time Point												
	Hr 12	Hr 13	Hr 14	Hr 15	Hr 16	Hr 17	Hr 18	Hr 19	Hr 20	Hr 21	Hr 22	Hr 23	Hr 24
Control + NX (0.05 mg/kg)	95.3	89.8	85.5	74.7	62.8	61.2	50.3	54.5	61.5	59.0	67.7	55.6	51.7
60% of Control Threshold	*57.2*	*53.9*	*51.3*	*44.8*	*37.7*	*36.7*	*30.2*	*32.7*	*36.9*	*35.4*	*40.6*	*33.3*	*31.0*
CRF (60 mg×min/m^3)	**42.5**	**41.7**	**43.0**	**40.4**	42.1	42.6	37.7	35.1	**35.9**	**35.2**	**38.0**	36.7	35.6
CRF (60 mg×min/m^3) + NX (0.05 mg/kg)	**42.9**	**46.4**	**45.5**	45.6	45.0	46.7	43.6	46.2	48.8	43.8	41.1	39.5	38.1

Although NX showed the capacity to reduce the respiratory toxicity resulting from CRF exposure and is recommended to be administered until symptoms subside, a single administration was unable to entirely reverse the respiratory depression over the following 24 hours. In contrast, clinical signs of CRF intoxication were rapidly reversed after NX administration. Animals that were exposed to CRF and subsequently treated with NX typically required several minutes to go from an incapacitated, unresponsive state to a fully aware and responsive state. Over the next 24 hours, the clinical signs and observed behaviors of exposed and treated animals were otherwise indistinguishable from controls. The difference in timelines for behavioral and physiological recovery may be due to differences in central versus peripheral activity of both CRF and NX or due to the difference in route of exposure versus route of treatment; however, any conclusion cannot be definitive in the absence of data on species-specific opioid receptor distribution. However, the human equivalent dose of 0.05 mg/kg in a murine model is approximately 4 μg/kg, and further studies using this inhalation exposure system will evaluate multiple administrations and/or dosages of NX to better assess its efficacy against CRF. Other options, such as peripherally selective opioid-mediated therapies, have been investigated because of concerns with NX and potential withdrawal symptoms (Lewanowitsch and Irvine 2002), and studies have suggested that antagonists focused on the delta receptor of the brain may provide additional protection against respiratory depression by a potent analgesic like fentanyl (Freye et al. 1991). Further evaluation of other opioid antagonists, such as naltrexone or nalmefene, may provide insight into an optimal treatment against potent opioids.

4.1 Conclusions

Our results clearly indicate that inhalation aerosol exposure to CRF causes dose-dependent changes in physiological parameters, including MV, MAP, HR and T_c, in a murine model. Animals experienced signs of opioid-induced toxicity, such as labored breathing and alterations in gastrointestinal function, movement, and general behaviors. CRF may depress respiratory function by its direct effect on the lung itself or via the inhibition of respiratory neuron activity in the brain. However, reactions to opioids depend on the agonist activity, which can in turn be dependent on the conformational states of the receptor or receptors involved. It is possible that agonist/receptor complex formation may be altered to the extent that affinity binding is dependent upon the formation of the altered receptor complex, resulting in enhanced responses in either the lung and/or at the CNS level (Kenakin 2014). Continued investigation into complications and side effects resulting from opioid-induced toxicity—most notably respiratory depression following exposure to potent opioid agonists such as CRF—is essential considering the increased availability and use of opioids.

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Abbreviations:

CRF – carfentanil
Ct – concentration-time product
HR – heart rate, beats/min, bpm
i.m. – intramuscular
LCt_{50} – lethal concentration-time product for 50% of the population
MAP – mean arterial blood pressure, mmHg
MV – minute volume, mL/min
NX – naloxone
ppm – parts per million
psi – pounds per square inch
T_c – core body temperature, °C
WBP – whole-body plethysmograph