Effect of dibenz(b,f)-1,4-oxazepine aerosol on the breathing pattern and respiratory variables by continuous recording and analysis in unanaesthetised mice

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A R T I C L E   I N F O

A B S T R A C T

A riot control agent has to be a sensory irritant of a reversible type without pulmonary irritation as the later can cause lung injury. The aim of the present study is to continuously record and analyse breathing pattern and respiratory variables of dibenz (b,f)-1,4-oxazepine (CR) in unanaesthetised mice during and after exposure. The lowest concentration of 0.65 mg/m^3 did not produce any effect on the breathing pattern. As high as 500 fold increase (315.9 mg/m^3) in the concentration was used and no mortality was observed. CR produced a concentration dependent sensory irritation, without pulmonary irritation or airflow obstruction, showing that it may not cause any lung injury. The sensory irritation was initiated within 5 min of exposure due to the activation of TRPA1 receptors of the upper respiratory tract. Immediate recovery of normal breath without sensory irritation was observed in all the concentrations except the highest concentration of 315.9 mg/m^3. Corresponding to the sensory irritation there was concentration dependent respiratory depression. The 50 percent respiratory depression (RD_{50}) in this experiment was 152 mg/m^3 and the estimated threshold limit value for occupational exposure was 4.56 mg/m^3. The present study shows that CR causes sensory irritation only which is completely recoverable.

1. Introduction

Controlling civil unrest is necessary and important for avoiding property damage and human injury. This can be done either by physical methods which cause pain, immobilization and can be fatal, or by chemical methods which cause distraction, incapacitation and force the individuals to leave the place [1]. The chemicals used are peripheral chemosensory irritants and are known as riot control agents or tear gases. The chemicals are 1-chloroacetophenone (CN), 2-chlorobenzylidene malononitrile (CS), dibenz[b,f]-1,4-oxazepane (CR) and oleoresin of capsicum (OC) [2]. Eyes, nose, mouth, respiratory tract and skin, particularly the moist areas of the body are affected by the vapours and aerosols [3]. The symptoms are lacrimation, blepharospasm, cough, breathing difficulties and increase in blood pressure with bradycardia. The effects are transient and reversible, once the individuals leave the place.

Among the riot control agents, CN and OC are comparatively more toxic and at high concentrations can cause eye injury. Death following CN and OC exposure has been reported. CS and CR are most potent sensory irritants and have a large safety margin, and among them CR has the least systemic toxicity [3]. Extensive studies have been reported in animal models on the effectiveness of riot control agents and it has been proved that CR is the most potent sensory irritant with least mammalian toxicity [4–6]. However, the toxicity depends upon the exposure concentration and duration, and in confined areas the riot control agents can cause significant toxic effects [2]. Contamination with CR cannot be removed easily unlike the other riot control agents by the normal methods and the effect may persist for a longer time. CR is a highly stable compound and the sensory irritancy property is retained even after prolonged storage [7].

Sensory irritation caused by inhaled chemicals, reflexively induce respiratory depression in mice and the exposure concentrations are

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inversely proportional to the respiratory frequency. This phenomenon is used to predict occupational exposure limits for humans by the Alarie test [8]. This is further standardised as an ASTM method [9]. This knowledge has been utilised for the assessment of breathing pattern by an online computer program which can characterise respiration as normal, sensory irritation, pulmonary irritation and airflow obstruction in unanaesthetised mice [10]. This is a non-invasive mouse bioassay and using the program the respiratory frequency can be determined for each exposure concentration for the estimation of 50 percent respiratory depression (RD$_{50}$). The threshold limit value (TLV) for occupational exposure can be extrapolated by the reported formula (TLV = 0.03 x RD$_{50}$) [11,12]. Several studies have been carried out by many experts using variety of chemicals with sensory irritation potential for the estimation of TLV and reported to have very high correlation [13,14].

A riot control agent has to be a strong sensory irritant of a reversible type without pulmonary irritation as the later can cause lung injury. So far studies are carried out on analysis of the end results data. A continuous recording and real time analysis of data for various exposure concentrations are not reported. This is achieved by the online computer program. Hence, the aim of the present study is to utilise the computer program for continuous recording and analysis of breathing pattern and respiratory variables, and to quantify them during and after exposure to CR aerosols.

2. Materials and methods

2.1. Animals

Swiss albino male mice (25–30 g body weight), bred and maintained at DRDE (Defence Research and Development Establishment, Gwalior, India) were used for the experiments. The animals were housed in polypropylene cages in an environmentally controlled room (25 ± 1 °C; RH 40 - 60%) with sterilised paddy husk as the bedding material. Mice were provided, food (M/s Ashirwad India Ltd, Chandigarh) and water ad libitum. The study was approved by the Institutional Animal Ethics Committee of DRDE. The care and maintenance of the animals were as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, India).

2.2. Chemicals

CR was obtained from Synthetic Chemistry Division of DRDE and was found to be above 99 % pure by gas chromatographic analysis [15]. Analytical grade dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich (St Louis, Missouri, USA).

2.3. Inhalation chamber

A head-only inhalation exposure chamber made of glass (50 cm in length and 7 cm outer diameter) was used for exposing the mice. Glass nebuliser of 7 mL capacity fabricated at DRDE was used for nebulization. Body plethysmographs made of glass were used to accommodate the mice. A neck collar made of latex rubber and duct tape was fixed at the front end of the plethysmograph. The rear end was closed with a rubber stopper. An inlet at the middle of the plethysmograph and an outlet in the rubber stopper enabled passage of air for animal body ventilation. A differential volumetric pressure transducer (Grass PT-5, USA) was connected at the inlet. The mice were gently guided inside the plethysmographs. Once the head protrudes through the collar a piece of foam was place at the back side of the animal and the rubber stopper was placed. For generation of aerosol, solutions of CR in DMSO in different concentrations were loaded in the nebuliser. The outgoing air of the exposure chamber was passed through a series of wash bottles containing sodium hydroxide solution and a trap containing activated carbon for removal of chamber air. The whole assembly was housed in a fume hood [16,17].

2.4. Generation of CR aerosol

For generation of aerosol, CR solution in DMSO was nebulised at a pressure of 10 psi (pounds per square inch; 1 psi =0.075 kg/cm^2) at the inlet of the nebuliser. The exposure chamber air flow was adjusted to 20 LPM (litres per minute).

2.5. Exposure methodology

Four mice at one time, per concentration was exposed for each experiment. The mice were acclimatised in the body plethysmograph with the neck collar for 30 min. When the animals were quiet by observing the respiratory pattern in an oscilloscope (WindoGraf 930, Gould Instruments, USA), the baseline data (pre-exposure control) were recorded for 30 min. Different concentrations of CR were prepared in DMSO (w/w), 0.03, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 %. The animals were exposed to each concentration in a dynamic mode for 60 min followed by 30 min recovery period. Each CR concertation was repeated twice with 4 mice each (8 mice per concentration). DMSO alone was used for the exposure of vehicle control (4 mice). A total of 84 mice were used for the study, including the control exposure.

2.6. Particle size and CR exposure concentration

The CR solution loaded nebulisers were weighed, before and after exposure and the aerosol concentration was estimated from the rate of nebulization to the total airflow. The particle size was determined by LASER particle counter LASAIR HIF (USA).

2.7. Measurement of respiratory variables

The inspiration and expiration of mice caused small changes in air pressure inside the plethysmograph, which was sensed by the differential volumetric pressure transducer (Grass PT-5, USA) connected to the plethysmograph with a silicone tube. The transducer was connected to an amplifier (Universal Amplifier, Gould, USA) to amplify and condition the respiratory signals. The respiratory signals were recorded continuously on an oscilloscope (WindoGraf 930, Gould Instruments, USA) during the experiment to obtain typical respiratory pattern. The amplified signals were also fed to a computer through analog to digital converter card (Keithly Metrabyte, Model DAS-16, USA) for computing the respiratory parameters [16,17]. The respiratory signals were recorded at every 15 s interval and were analysed using a computer program capable of characterising the respiratory variables and the breathing pattern [10]. The respiratory variables, like respiratory frequency (f), tidal volume (VT), time of inspiration (TI), time of expiration (TE), time of brake (TB) after each inspiration, time of pause (TP) after each expiration and mid-expiratory flow (VD) were reordered and analysed, during and after exposure. The changes in breathing pattern viz., normal respiration (N), sensory irritation (S), pulmonary irritation (P), airflow obstruction (A), and their combinations (SP, SA, PA and SPA) were also measured during and after exposure. The measured variables were quantified with respect to changes from baseline data. Sensory irritation was characterized by a pause between inspiration and expiration, pulmonary irritation was characterized by a pause between two respirations, and airflow obstruction was characterized by slow expiration [10].

2.8. Calculation of RD$_{50}$ and TLV

The total exposure time of 60 min was divided into three different time periods; 0–10 min, 10–30 min and 30–60 min for computing respiratory depression. The concentration of CR that induced 50 % depression in the respiratory frequency of the exposed animals (RD$_{50}$) was calculated individually for all the three time periods. The graph for determining RD$_{50}$ was drawn by plotting percent respiratory depression

corresponding to nominal concentration of CR. The RD_{50} was determined by obtaining linear curve fitting equation. The TLV was calculated by multiplying the RD_{50} values by the factor 0.03 as per the Alarie test [14].

2.9. Statistical analysis

User defined transformations were made in SigmaPlot worksheet for the quantification of the acquired variables of 8 animals from each exposure concentration. The data were expressed as mean + SE and were analysed by one-way ANOVA with Dunnett's multiple comparisons test using 0.65 $mg.m^3$ concentration as reference. A probability of 0.05 and less was taken as statistically significant. Statistical significance is indicated for time periods of 10, 30, 60 (exposure period) and 90 min (recovery period). All the statistical analysis and graph plotting was done using SigmaPlot 13 (Systat, USA).

3. Results

Ten concentrations (logarithmic) of CR in DMSO solution was used for the nebulization viz., 0.03, 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0 and 16.0 % The corresponding aerosol exposure concentrations were 0.65, 1.5, 2.9, 5.8, 11.3, 23.3, 45.5, 88.7, 158.2 and 315.9 mg/m^3 of CR respectively (0.08–39.56 ppm). The particle size of the CR aerosol was found to be in the respirable range (size range of 90–95 % particles was 2 μm and less).

After the acclimatization period the mice breath normally at the rate of 250 ± 25 breaths/min (Table 1). The mice were exposed to different concentrations of CR for 60 min. The highest concentration of CR used was 315.9 mg/m^3 while the minimum concentration was 0.65 mg/m^3. No mortality was observed in any of the concentrations, during or after exposure. The exposed animals were observed for 14 days after exposure and their body weights were recorded for all 14 days post exposure. None of the animals showed delayed toxicity in terms of decrease in body weight, and their body weights were maintained 14 days post exposure. At CR concentration of 0.65 mg/m^3 the normal breath was 100 % till the end of exposure and also during the 30 min recovery period (Fig. 1). Dose dependant decrease in normal breath of aerosol exposed groups (at concentrations higher than 0.65 mg/m^3) was observed as compared to the baseline values (Fig. 2). At concentrations ranging from 1.5 to 315.9 mg/m^3 the normal breath reduced from 80 % to as low as 10 % within 5 min of exposure. Similarly, sensory irritation increased in a dose dependant manner at concentrations ranging from 1.5 to 315.9 mg/m^3 from normal levels to 70–90 % within 5 min of start of exposure. The normal breath and sensory irritation followed an inverse relationship with CR aerosol exposure. There was no change in pulmonary irritation and airflow obstruction in any of the exposed concentrations (Fig. 2).

The respiratory frequency (f) showed a dose dependent decrease during CR exposure. Respiratory frequency decreased to a minimum of 20 % (i.e., respiratory depression 80 %) at the highest CR concentration of 315.9 mg/m^3 just after 5 min of start of exposure. The respiratory depression was more than 60 % even after 30 min of recovery period. The other concentrations showed recovery of varying percentages (Fig. 3). Corresponding to the decrease in respiratory frequency, the tidal volume showed an increase. But, the increase in tidal volume did not show a dose dependent change. A two fold increase in tidal volume
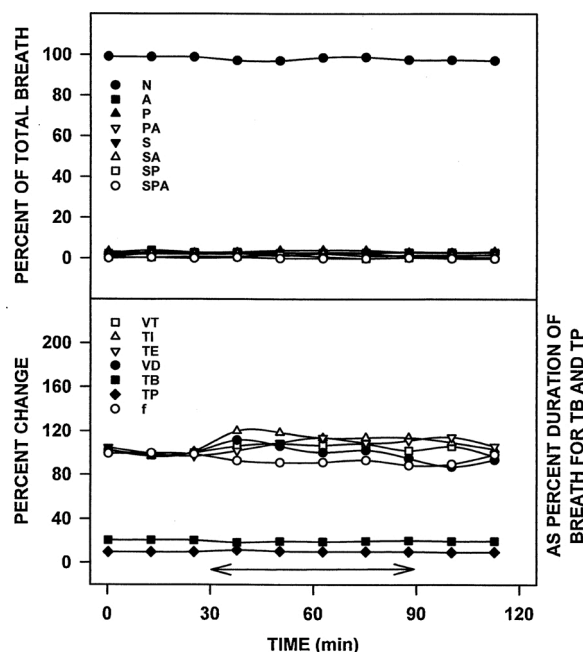
Table 1
Normal respiratory variables of Swiss mice.

Variables	Abbreviation	Mean	SD
Respiratory frequency (breaths/min)	f	250	25
Tidal volume (mL)	VT	0.11	0.01
Time of inspiration (s)	TI	0.11	0.01
Time of expiration (s)	TE	0.15	0.01

Fig. 1. Time response analysis for breath classification and respiratory variables for a group of four mice, before, during and after inhalation exposure to CR aerosols at 0.65 $mg.m^3$. The arrow indicates the duration of exposure.
N normal, S sensory irritation, A airway obstruction, P pulmonary irritation, and SA, SP, PA and SPA are combinations of breath classification.
VT tidal volume, TI time of inspiration, TE time of expiration, VD mid expiratory flow, TB time of brake, TP time of pause and f respiratory frequency.

was observed at the concentration of 315.9 $mg.m^3$ within 5 min of exposure. Though, there was an increase, a progressive decrease in the tidal volume was observed with the exposure time. At the recovery period the tidal volume was close to the normal values in most of the exposure concentrations, except 315.9 $mg.m^3$ concentration (Fig. 3).

Fig. 4, represents the variation in time of brake (TB), time of pause (TP) and mid expiratory air flow (VD). These were computed online and were utilised for the quantification of sensory irritation, pulmonary irritation and airflow obstruction respectively. TB increased 2–5 fold from the baseline value. No change in TP was observed in any of the concentrations of CR showing that CR did not cause pulmonary irritation. VD increased with increase in CR concentration but was not dose dependant. This parameter would increase with increase in TB.

With each exposure concentration of CR aerosol, the respiratory frequency decreased immediately and slight recovery was observed after 30 min. Hence, to calculate the mean respiratory depression, three separate time periods of 0–10 min, 10–30 min and 30–60 min were taken (Fig. 5). For each concentration of CR solution, percent respiratory depression was plotted against nominal exposure concentrations of CR (0.65–315,9 $mg.m^3$ corresponding to 0.031–16 %). The RD_{50} values at different time intervals were 152 mg/m^3 (0–10 min), 158 mg/m^3 (10–30 min) and 184 mg/m^3 (30–60 min). The lowest value of 152 mg/m^3 was used for the estimation of TLV. The TLV was calculated as (0.03 x RD_{50}) = (0.03 × 152 mg/m^3) = 4.56 mg/m^3.

4. Discussion

Olfaction, taste and somatosensation are the major sensory systems that detect chemicals in the environment. The trigeminal somatosensory neurons in oral and nasal mucosa detect environmental stimuli particularly pressure, temperature and irritant chemicals, and transmit the sensory information [18]. The peripheral nerves innervating the nose, mouth, and throat protect the organism against chemical irritants. When they are stimulated stinging and burning sensation occurs and various
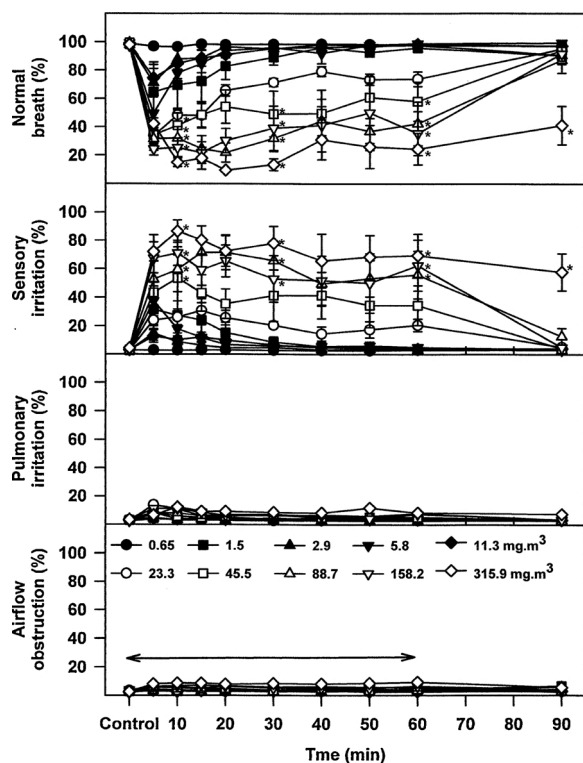
Fig. 2. Percent change in normal breath, sensory irritation, pulmonary irritation and airflow obstruction, during and after inhalation exposure to CR aerosols at different concentrations. The arrow indicates the duration of exposure. The values are mean ± SE of 8 mice.

Statistical significance was tested compared to 0.65 mg.m³ concentration at 10, 30, 60 and 90 min.

*Indicates statistically significant at respective time periods.

Fig. 3. Percent change in respiratory frequency and tidal volume, during and after inhalation exposure to CR aerosols at different concentrations. The arrow indicates the duration of exposure. The values are mean ± SE of 8 mice.

Statistical significance was tested compared to 0.65 mg.m3 concentration at 10, 30, 60 and 90 min.

*Indicates statistically significant at respective time periods.

reflexes of sneezing and cough are triggered. Occupational and environmental exposure to irritating chemicals and inflammatory mediators can produce cough [19]. Accidental exposure to high concentrations or chronic environmental irritant exposure may sensitise the trigemino-vascular system and cause headache and migraine [20]. Low concentrations of irritant chemicals produce decrease in respiratory frequency. Mice, exposed to formic, acetic, propionic and butyric acid caused a rapid decrease in the respiratory frequency [21]. The potency of a chemical to cause sensory irritation can be used by a mouse bioassay for assessing the concentration-dependent decrease in the respiratory rate (50 % respiratory depression; RD_{50}) [13,21]. A riot control agent should by a peripheral chemosensory irritant with reversible effect, produce temporary incapacitation and should be effective as vapour or aerosol [1]. The chemical irritant should be non-corrosive and should cause reversible change at the contact site [22].

The role of Ca^{2+} permeable, cation transient receptor potential (TRP) channels in airways of normal and disease conditions are gaining importance [23–25]. The TRP channels function as an important endogenous protective mechanism of the respiratory system by detecting noxious stimuli of various oxidative, chemical irritant and temperature stimuli, and ensure protection by physiological response. But, the altered expression, activation and regulation may also contribute to pathogenesis and respiratory diseases [26]. The important channels are the irritant receptor – transient receptor potential ankyrin 1 (TRPA1), the heat sensitive ion channel – transient receptor potential vanilloid 1 (TRPV1), the pressure sensitive ion channel – transient receptor potential vanilloid 4 (TRPV4), and the cold-sensitive ion channels – transient receptor potential melastatin 2 and 8 (TRPM2 and TRPM8) [18]. These ion channels are stimulated and perform a variety of overlapping effect in airway hyperreactivity, inflammation, asthma, chronic obstructive
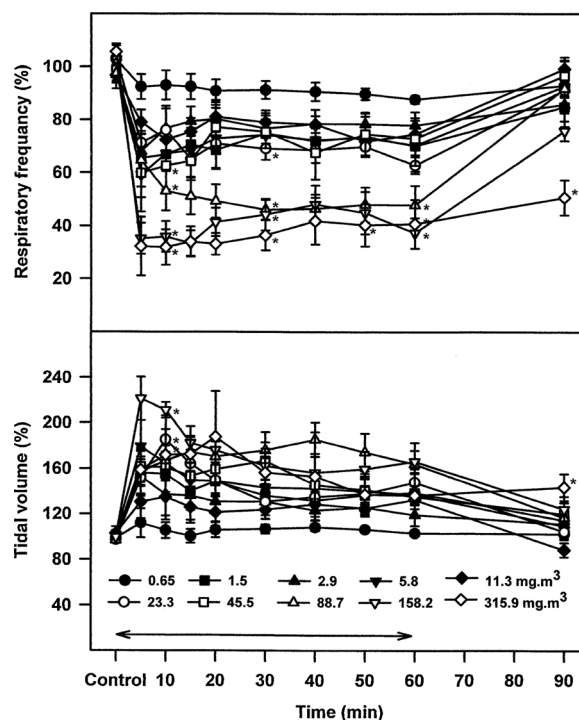
pulmonary disease and other respiratory disorders through the expression of C-fibres and Aδ-fibres. In addition, inhibitory two-pore potassium channels (TASK1 and TASK3) are also implicated in trigeminal chemo-sensation [27]

TRPA1 is present in trigeminal neurons of nasal cavity and vagal neurons of trachea and lung, and are activated by cold, mechanical stimulation, and irritant and spicy chemicals. They cause respiratory depression, bradypnea, cough, glandular secretions and other protective responses [28,29]. TRPA1 is distributed throughout the human body and are involved in different physiological and pathological conditions [30]. TRPA1 is a nonselective cation channel and activated by a number of natural, environmental and pungent chemicals, and inflammatory mediators such as isocyanates, isothiocyanates, thiosulphinates, chlorine, acrolein, acetophenone, 2-ethylhexanol, trimethylcyclohexanol, vehicle exhaust, cigarette smoke, cyclopentenone prostaglandins and riot control agents [19,28,31,32]. The activating compounds contain reactive, electrophilic ligands that covalently react with cysteine residues in the active site of the TRPA1 channel, and are generally reversible [33].

The riot control agents. CN, CS, OC and CR are potent and selective activators of the human TRPA1 receptor [23]. They are electrophilic in nature and the lacrimation and blepharospasm are due to TRPA1 receptor activation similar to garlic [34]. The stimulation of TRPA1 in the upper respiratory tract results in respiratory depression and restricts absorption of the chemical, and there by systemic toxicity. High concentration exposure would stimulate the lower respiratory tract also resulting in the activation of TRPA1 of vagal sensory afferents and produce central reflexes, dyspnoea, changes in breathing pattern of pulmonary irritation [19]. Cigarette smoke and particularly 9,10-phenanthrenequinone from cigarette smoke is an activator of TRPA1. Other than TRPA1, cigarette smoke activates, TRPV1 and nicotinic acetylcholine receptors (nAChR). The sensory effect of cigarette smoke is more due to nAChR and TRPV1, and the activation may cause cough
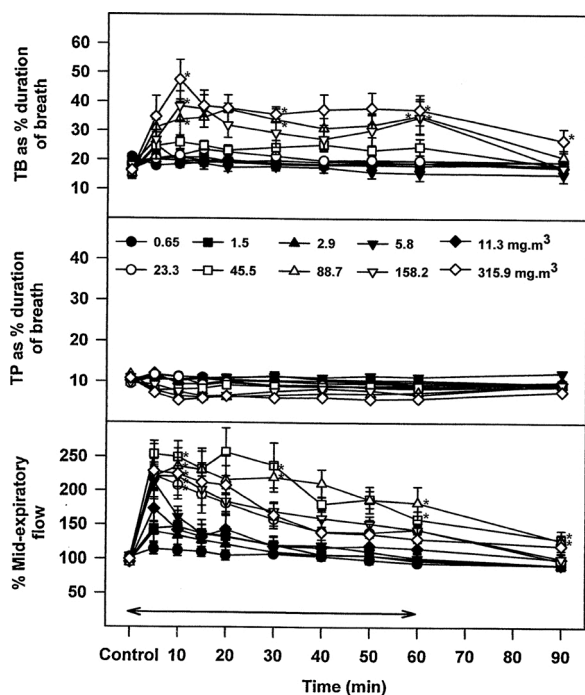
Fig. 4. Percent change in time of brake (TB) between inspiration and expiration, time of pause (TP) between two respirations and mid expiratory airflow, during and after inhalation exposure to CR aerosols at different concentrations. The arrow indicates the duration of exposure. The values are mean ± SE of 8 mice.

Statistical significance was tested compared to 0.65 mg.m^3 concentration at 10, 30, 60 and 90 min.

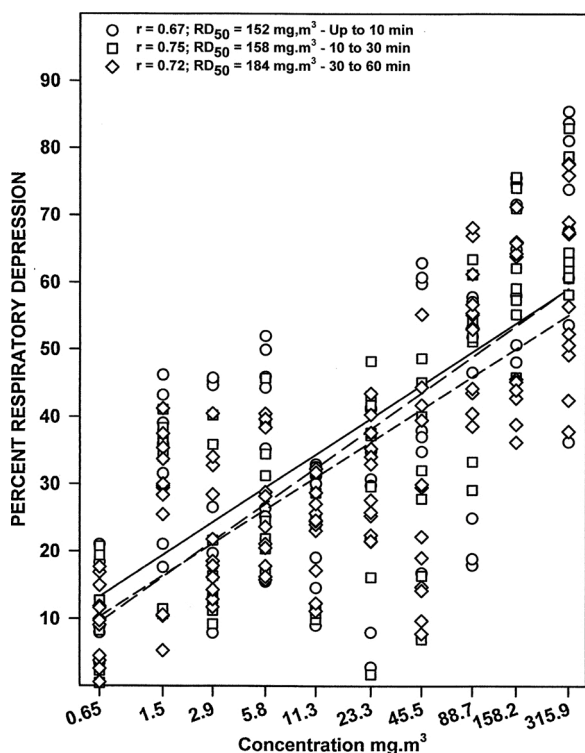*Indicates statistically significant at respective time periods.

Fig. 5. Percent respiratory depression at various concentrations of CR aerosol exposure for three time periods – upto 10 min, 10–30 min and 30–60 min. Eight mice for each exposure concentration for the estimation of percent respiratory depression (RD$_{50}$).

[31,35]. In an experiment using mouse model inhaling through a tracheal cannula, by passing the trigeminal nerves, pulmonary irritation caused less decrease in respiratory rate. Also, at low concentration, pulmonary irritation was less than the sensory irritation, showing that sensory irritation is a protective reflex than the pulmonary irritation [21]. TRPA1 and TRPV1 would be activated in the lower respiratory tract by reactive chemicals causing lung inflammation, lung injury and cough [22,36].

There is increasing importance of TRPA1 channel as a pharmacological target for agonists and antagonists. The mouse bioassay for estimating RD$_{50}$ showed positive correlation on the TRPA1 activation, indicating the later as a replacement for the bioassay [37]. But another study did not show relationship of nerve-compound interaction [32]. The sensory irritation or pulmonary irritation depends upon the type of compound, lipophilicity, electrophilic nature, electrostatic or covalent bonding, involvement of TRPA1 or TRPV1, the region of receptor activation, the concentration and the duration of exposure [38].

CR has been shown to be a safe riot control agent both by oral and inhalation routes [5,17]. The present study has shown that CR produced a concentration dependent sensory irritation only, without pulmonary irritation or airflow obstruction, as the later may permanently affect the respiratory system [39]. Even at the highest concentration of exposure, pulmonary irritation or airflow obstruction was not observed showing the high safety of CR. The sensory irritation was initiated within 5 min of exposure due to the activation of TRPA1 receptors of the upper respiratory tract [23]. Immediate recovery of normal breath without sensory irritation was observed in all the concentrations except the highest concentration of 315.9 mg/m^3. Corresponding to the sensory irritation there was concentration dependent respiratory depression which also was completely recovered, restricting the systemic absorption of CR. Though, there was an initial increase in the tidal volume it was stabilised after a period. The RD$_{50}$ in this experiment was 152 mg/m^3 (19 ppm) with the estimated TLV of 4.56 mg/m^3 (0.57 ppm) for CR aerosols. It is to be mentioned that CR causes instant pain in the eyes with profuse tears at very low concentrations compared to irritation of nose, mouth and the respiratory tract [3],

5. Conclusion

In the present study, unanaesthetised mice were exposed to CR aerosols, dynamically and the breathing pattern and respiratory variables were continuously recorded and analysed, real time by online computer program, for 60 min duration of exposure with 30 min recovery period. The lowest concentration of 0.65 mg/m^3 did not produce any significant effect on the breathing pattern and the respiratory variables and hence, can be considered as no observed effect level (NOEL) for acute exposures. As high as 500 fold increase (315.9 mg/m^3) in the concentration was used for the inhalation exposure and no mortality was observed during or after the exposure showing the safety of CR. The present study shows that CR causes sensory irritation only which is completely recoverable. The 50 percent respiratory depression (RD$_{50}$) in this experiment was 152 mg/m^3 and the estimated threshold limit value for occupational exposure was 4.56 mg/m^3.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.08.022.

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