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Induction and Evaluation of Acute Inflammatory Model of Sulfur Mustard Analogue (CEES) in C57BL/6 Mice

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ABSTRACT

The acute threat model with mustard analogs has been produced and synthesized due to the dangers that sulfur mustard can pose to users in experimental work conditions and the creation of sulfur and numerous analogs to prevent its dangers. To evaluate the confirm of 2-Chloroethyl Ethyl Sulfide (CEES) as an analog of SM, we set up a new model of CEES systemic injection & exposure to be as close as possible role of this toxin analog effects on innate immune inflammation. Female C57BL/6 mice, 8-10- week old at the onset of the study, were exposed to CEES (10 mg / kg). The administration route was Intraperitoneal (IP) injection. At the end of the study, the mice's lung fluid, and peritoneal lavage, spleen lymphocyte and lung tissue were extracted for future histopathological assessments.

INTRODUCTION

The acute threat model with mustard analogs has been produced and synthesized due to the dangers that sulfur mustard can pose to users in experimental work conditions and the creation of sulfur and numerous analogs to prevent its dangers. The vesicular damage of this lethal inhalation toxin occurs through the mechanism of alkylation of macromolecules in cells, genetic content, DNA and proteins. Both sulfur mustard (SM) and CEES (which is the analogue 2- chloroethyl ethyl sulfide of the sulfur mustard) are similar in pathophysiology and used for better understanding of chemical effects (1). Is CEES a suitable analog for sulfur mustard? Many studies have investigated this safe alternative and they used it as a suitable model in the investigations of acute and chronic models, including in cases of acute and systemic inflammation, especially the chronic type, which is very similar to the human events of exposure to poisonous gas (2). Obtaining the results of the induction of such models is especially important in organs such as lungs, brain, skin, and vision. In these organs, the occurrence of oxidative stress and the reduction in levels of glutathione are among the above disorders (3). It is difficult to study the effects of sulfur mustard in experiments because it is so dangerous to handle. The synthesis and production of analogues such as 2-Chloroethyl Ethyl Sulfide have been able to partially control these risks with minimal environmental damage. In such a way as to be effective in dealing with rodents. This analog in direct contact with the skin and in case of inhalation, causes lesions very similar to those caused by contact with sulfur mustard in real conditions (4). Erythema and resulting necrosis occur in contact with these analogs, quite similar to sulfur mustard. Many experiences such as models, conducted in vitro or in vivo, have been successful in the field of similarity and working of this analog.

Sulfur mustard analog 2-chloroethyl ethyl sulfide (CEES), as an alternative mono - functional *SM analog* with systemic diffusion, now are using as SM analogs in the lab for less toxicity. Similar character is- tics to SM (being oily, hydrophobic, and volatile) (1). However, this chemical compounds needs to be careful for lab worker and special attention to avoid contamination in researcher and co- workers. Its personnel injury from environmental pollution may be a high risk issues. Numerous investigations have experienced the systemic body effects associated with

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an excess frequency of inflammation of respiratory tract in animal models but could not be explained by sever peritoneal inflammation. Although there are many examples of inducing the model with the help of this analog, there are few experiences in the field of experimental exposure to the systemic form and the effect on acute symptoms in the innate defense system through systemic diffusion. In the current study protocol, acute inflammatory effects caused by systemic exposure to analogs in target organs, including: spleen, lung, peritoneal cavity blood circulation as innate inflammatory prone organ, have been reviewed and considered.

Pulmonary and Intraperitoneal Inflammation Induced by Sulfur Mustard analog (CEES) by systemic exposure

PROTOCOL

Objectives:

This experimental study protocol delineates the methodologies employed and seeks to offer guidance to fellow researchers for improving their comprehension of the toxic and inflammatory impacts of CEES in laboratory investigations and animal programs. All animal care and experimental protocols received approval from the Iran Ministry of Health and adhered rigorously to the guidelines specified in the Declaration of Helsinki.

METHODS

To assess the confirm of CEES, we established a novel model involving systemic injection and exposure to CEES. This approach aimed to closely mimic the impact of this toxic analog on innate immune inflammation. To achieve this objective, it was imperative to utilize an animal model that would facilitate clinical observations over a span of 14 days. The selected model was C57BL/6 mice, a strain extensively employed in immunological research and notably in studies related to SM.

Animals:

C57Bl/6 mice 8 weeks old at entry to the study were used in the Laboratory pathophysiological experiments. After purchasing them, the mice were allowed a minimum acclimation period of 7 days before initiating any injections. An additional two weeks were provided for the mice to adapt to the environment before administering the intraperitoneal injection for the acute inflammatory model in female C57BL/6 mice with eight to ten- week- old. Throughout the study, all animals were provided with unlimited access to tap water and pelleted standard maintenance diet. This aligned with the directives set by the Ethical Committee of Shahid Beheshti University of Medical Sciences. Animal Care Committee approved all experimental protocols (IR.SBMU.MSP.REC 1399. 339).

Institutional permissions

- All studies with animals must be conducted in accordance with the Guide of Laboratory.
- 8-10 week old C57BL/6 female mice, housed in the animal facility under pathogen - free conditions, constant temperature and humidity with 12 h light / dark cycle, and were given free access to water and standard pellet food.
- The mice should be acclimated for at least 7 days before beginning the experiment.
- The number of mice should be selected according to study endpoints Intraperitoneal injection of CEES

CEES - induced complication model

Chloroethyl Ethyl Sulfide preparation

- To handle 2- Chloroethyl Ethyl Sulfide safely, it is essential to work under a chemical hood.
- Take the appropriate amount of 2- Chloroethyl Ethyl Sulfide (Sigma Company, St. Louis, USA, Cat number: 242640), considering its density (1.07 g/mL), and dissolve it in different solvents. Intraperitoneally inject 100 µL of the solution, while the control group should receive the befit solvent.
- Make sure to appropriately mark the tube with the resuspension date, store it at 4°C, and make use of the contents within a 24- hour period.
- Allow the CEES solution to reach room temperature before administering the instillation.
- Keep in mind that each mouse strain may have varying sensitivity to CEES.
- When performing injections, closely follow the personal and environmental safety guidelines outlined by the manufacturer of CEES. This encompasses the utilization of a chemical mask, gloves, gowns, and a laminar flow hood featuring a charcoal filter (2).

Intraperitoneal injection of CEES

- Weigh the animal, record the weights, and calculate the required volume of the solution according to the animal's weight
- Disinfect the top of the container with alcohol and allow it dry.
- Gently remove animal from the cage and restrain the mouse in an appropriate manner. Restrain the mouse with your non-dominant hand such that the animal's head is slightly below the abdomen.
- Disinfect the abdomen of the mouse with a swab dipped in 70% alcohol and perform CEES injection as an IP.
- Fill a syringe that is sterile with a needle (25 gauge, 8.5 inches) with 100 µL of the CEES solution.
- Place the mice back into their cage and supervise them until complete recuperation.

- Conduct daily weighing of the animals and keep a watch for signs of discomfort or distress. Subsequently, from that point until the designated sacrifice time, mice should be weighed weekly and observed for indications of discomfort and the emergence of skin wounds. Weight loss stands out as the predominant indicator of deteriorating health in investigations.

Cardiac puncture/Transcardial depletion/serum samples

Necessary items encompass animal, anesthesia substance, cloth, cotton; needle sized 19 to 25G along with a syringe of 1 to 5 ml capacity, surgical blade, single-use plastic bag, and tubes for collecting blood samples.

- Generally, for the final phase of the study, the recommended approach for acquiring a substantial volume of high - quality blood sample from experimental animals is through cardiac puncture. This procedure takes place while the animal is in a state of terminal anesthesia.
- While collecting blood, the animal will remain in a state of terminal anesthesia to ensure proper manipulation and minimize any discomfort.
- For obtaining blood samples, the suitable needle will be employed, whether with or without thoracotomy. The preferred site for blood extraction will be the heart, specifically the ventricle, in a slow and careful manner to prevent heart collapse (5).

Peritoneal lavage / Immunophenotyping

- The experiment commenced by euthanizing the animals using pentobarbital sodium (purchased from Sigma Aldrich, Louis, USA) at a dosage of 120 mg / kg. The animals were considered fully unconscious when they no longer responded to toe - pinch.
- Utilizing scissors and forceps make an incision in the outer skin of the peritoneum and gently retract it to expose the inner lining of the peritoneal cavity.
- Afterward, the peritoneal membrane was carefully separated from beneath the abdominal musculature, subsequently; administer 5-7 mL of frigid PBS into the peritoneal cavity through a 27g needle (6).
- The peritoneum was gently massaged to ensure even coverage with cold RPMI 1640 medium, and then the medium was carefully removed from the peritoneal cavity using aspiration (7).
- To initiate the process, murine peritoneal cells were retrieved from the peritoneal cavity using a rinse of 5 mL RPMI 1640 medium (procured from Invitrogen, Darmstadt, Germany). Following this, the cells were subjected to centrifugation at 200×g for 10 minutes and subsequently washed using PBS.
- If visible blood contamination is observed in step 6 or 7, discard the contaminated sample.
- Following the washing steps, the cells were cultivated in a complete culture medium of RPMI 1640 and maintained at 37°C for a period of 4 hours (8).
- Peritoneal macrophage as adherent cells used for ongoing cultivation to conduct MTT assays (6).

Flow cytometry analysis of peritoneal macrophages in acute model

- To assess peritoneal macrophages, antibodies against CD11b-PE / Cy7 (clone M1/70), F4/80- PE (clone BM8) purchased from Bio Legend in San Diego, CA, USA were used to evaluate surface markers (Table 1). After counting the cells, 1x106 cells were stained with Near- IR Dead Cell viability dye (Thermo Fisher Scientific, US) following the manufacturer's instructions.
- Following blocking of nonspecific binding by incubation with FcR blocker, Immunophenotyping antibody was added and the cells were stained at 4°C for 15 minutes. The cells were washed and analyzed using Flowjo version 10. Compensation was performed at the start of the experiment using the Compensation Bead (Invitrogen, Carlsbad, CA) (9).

Table 1. Antibodies

Name	Company	Cat #	Size
PE anti - mouse F4/80 Antibody	Biolegend	123110	100 µg
PE Rat IgG2a, κ Isotype Ctrl	Biolegend	400507	25 µg
PE / Cy7 anti-mouse/human CD11b Antibody	Biolegend	101216	100 µg

Nitric Oxide Test

Peritoneal macrophages were cultured to a 4- hour culture, and subsequently, the culture supernatant and floating cells were gathered. The complete culture medium was then replenished. The cultivation process was continued for an additional 4 hours, and the supernatant was collected once more and preserved for nitric oxide measurement (sib Zist Novin, Iran, Cat number: 3201-200).

Broncho alveolar lavage/ Protein measurement

Introducing the catheter into the trachea

- Administer a lethal dose of barbiturate anesthetic (120 mg / kg) to euthanize the mouse through intraperitoneal injection via a 26 G needle. Verify appropriate

anesthetization by pinching the mouse's rear paw by forceps to check if the foot reflex is absent.

- Position the mouse in a supine orientation on a surgical platform and stabilize it by restricting the movement of its limbs.
- Disinfect the neck by spraying 70% ethanol. Make a cut in the skin (incision) of the neck close to the trachea by using a scalpel.
- Expose the salivary glands by opening the skin. Utilize pincers / forceps to isolate the salivary glands and reveal the sternohyoid muscle. Cut around the trachea with pincers / forceps to reveal it.
- Gently place a cotton thread beneath the trachea by using forceps / pincers.
- Gently insert a 26 G needle into the central area of the revealed trachea, located between two cartilage rings, being cautious to avoid inflicting additional harm to the tracheal structure.
- Introduce the catheter into the trachea, ensuring a depth of approximately 0.5 cm. be cautious not to insert it too deeply, as this could potentially harm the structure of the lungs.
- Fasten the catheter securely by binding the trachea around it with the cotton thread positioned during step 5. Inadequate tying of the catheter could result in the infused balanced salt solution flowing toward the upper respiratory tract instead of reaching the lungs.

Collect the lavage fluid

- Fill a syringe with 1 mL of sterile cold medium.
- Attach the syringe to the catheter and delicately infuse the solution into the mouse's lung.
- Massage the mouse's thorax while gently aspirating the solution back into the syringe. If the solution is not apparent within the syringe, carefully adjust the position of the catheter slightly up or down the trachea.
- Detach the syringe from the catheter and move the retrieved lavage fluid into a 15 mL tube that has been positioned on ice. Typically, 700 to 900 microliters of BAL can be recovered from 1 mL of injected solution.
- Repeat steps 2 and 3 two more times. NOTE: If the intention is to examine the non-cellular components, it is advisable to concentrate the combined samples if concerns regarding sensitivity arise.

Collecting the Cellular and Noncellular Components of the BAL Fluid

- The lavage fluid is Centrifuge for 7 min at 400 x g and 4°C.
- Gather the supernatant and promptly employ it for subsequent examinations (such as ELISA) or store it by freezing at -80°C. Keep the cellular residue for the assessment of lung cell influx (10).

Protein measurement

The collected bronchoalveolar lavage fluid (BALF) was immediately placed in a centrifuge for 15 minutes at 1500 rpm. The supernatant was isolated and preserved within sterile Eppendorf tubes at -80°C. The quantification of total protein content in the cell-free BALF was carried out utilizing a BCA protein assay kit provided by Biobasic (Catalog number: SK3021), employing bovine serum albumin as the reference standard. Subsequently, the samples were assessed by a microplate reader at a wavelength of 562 nm (11). Add 25 microliters of the sample and standards along with 200 microliters of the prepared working solution to the tube. Homogenize the mixture and incubate it in a Bain-Marie at 60 degrees Celsius for one hour. Subsequently, measure the absorbance at a wavelength between 570 and 620 nm to calculate the protein content.

Cytology / Number of granulocytes predominantly neutrophil analysis of percentage of granulocytes

Lung histopathology

To prepare histological sections of the mice, a lethal dose of pentobarbital sodium was administered to induce deep anesthesia. Once the footpad - pinch response was no longer observed, 1 mL of 10% paraformaldehyde was introduced into the lungs through a tracheal catheter, and the lungs were fixed in 10% formalin. Afterward, cutting into 5 mm slices in thickness were acquired and subjected to staining with hematoxylin and eosin (H&E), periodic Acid - Schiff (PAS), and Masson's trichrome. This was done to evaluate the presence of inflammatory cell infiltration, the extent of goblet cell hyperplasia, and the distribution of collagen fibers, respectively. A pathologist, who was unaware of the study details, analyzed the histopathological changes, and images were captured using a Labomed Lx500 light microscope. Each field was sequentially evaluated, and a predetermined severity scale ranging from 0 to 10 was employed for scoring each parameter.

Spleen culture evaluation by vitality assessments / cytokine assay / MTT

- Administer a fatal dose of barbiturate anesthetic (120 mg / kg) to administer euthanasia to the mouse via intraperitoneal injection using a 26 G needle.
- Before making any incisions, immerse the entire mouse in 70% ethanol. Aseptically remove the spleen and place it in a distinct 15 ml tube holding 5 ml of ice - cold RPMI / FBS (RPMI with 2% FBS).
- To acquire a single cell suspension from the spleen, position the organ (s) amidst two aseptic 100 µm cellular sieves in a culture dish holding 2 ml of chilled RPMI /

FBS or BSS / FBS. Utilize the piston of a 1 ml syringe to delicately crush the organ (s) until it is finely fragmented.

- Transfer the cell suspension into a 15 ml tube and wash meshes of the cell strainer with cold RPMI / FBS or BSS / FBS. Merge the remaining cell suspension and introduce it into the identical 15- ml tube. Centrifuge at 453 x g for 5 minutes at 4°C and then remove the supernatant.
- Quantify the cell density per milliliter by employing a hemocytometer for cell counting. Approximately 1 × 106 cells per well were added to a 6- well culture.
- Introduce 3 ml of growth medium into the designated wells. Place the plates in an incubator set at 37°C with 5% CO_2 for duration of 3 hours.

MTT assay protocol

- The MTT is assay is employed to evaluate cellular metabolic function, acting as an indicator of cell viability, proliferation, and cytotoxicity. This colorimetric method hinges on the conversion of the yellow tetrazolium salt (3- (4, 5- dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide or MTT) into purple formazan crystals by cells displaying active metabolism. Living cells contain NAD (P) H- dependent oxidoreductase enzymes that facilitate the reduction of MTT to formazan. A darker solution indicates a higher number of living and metabolically active cells. (Alternatively, due to the lipophilic nature and net positive charge of MTT, it can penetrate the cell membrane and, within living cells, undergo reduction by mitochondrial enzymes or cell plasma enzymes including oxidoreductases, dehydrogenases, oxidases, and peroxidases, utilizing NADH, NADPH, succinate, or pyruvate.) This conversion leads to the formation of water-insoluble formazan.
- Remove media from cell cultures. Spleen cells were cultured at a concentration of 1 x 106 cells / ml, and 100 µl of sterile MTT component with an ultimate concentration of 5 mg / ml in PBS (Sigma) was introduced into every well.
- Place 100 µL of media without serum and 50 µL of MTT solution into each well.
- Incubate the plate at a temperature of 37°C for duration of 4 hours.
- Wrap the plate in foil. After 4 hours of incubation at 37°C, the wells were washed, and 100 µl of DMSO (Sigma) was added to the cultures, thoroughly mixing to dissolve the dark blue formazan crystals.
- Measure the absorbance at a wavelength of 570 nm. The plate must be read within a time frame of 1 hour after adding DMSO.

Stablish of acute model

To confirm the model, after the desired time three mice were randomly selected and euthanized with the overdose of pentobarbital sodium (Sigma Aldrich, Louis, USA) (120 mg / kg), and their different organs (e.g., lung, spleen) were excised for histopathological analysis. Histopathology findings revealed that, in the group of acute model induced with CEES analogue, pathological changes in the form of the presence of edema (Fig 1A), disruption of the alveolar epithelial surface (Fig 1B) and infiltration of leukocytes (Fig 1C) are shown in figure 1. Exposure to CEES significantly increased cytokines TNF-α, IL-17, IFN-γ and IL-10 in BALF, spleen culture supernatant, intraperitoneal lavage and serum compared to the control group. Protein levels in BALF (Bronchoalveolar Lavage Fluid), nitric oxide and the number of peritoneal macrophages in the peritoneal cavity were increased in acute model mice treated with CEES. The results show that lymphocyte viability was decreased in all cases where mice were induced with CEES.

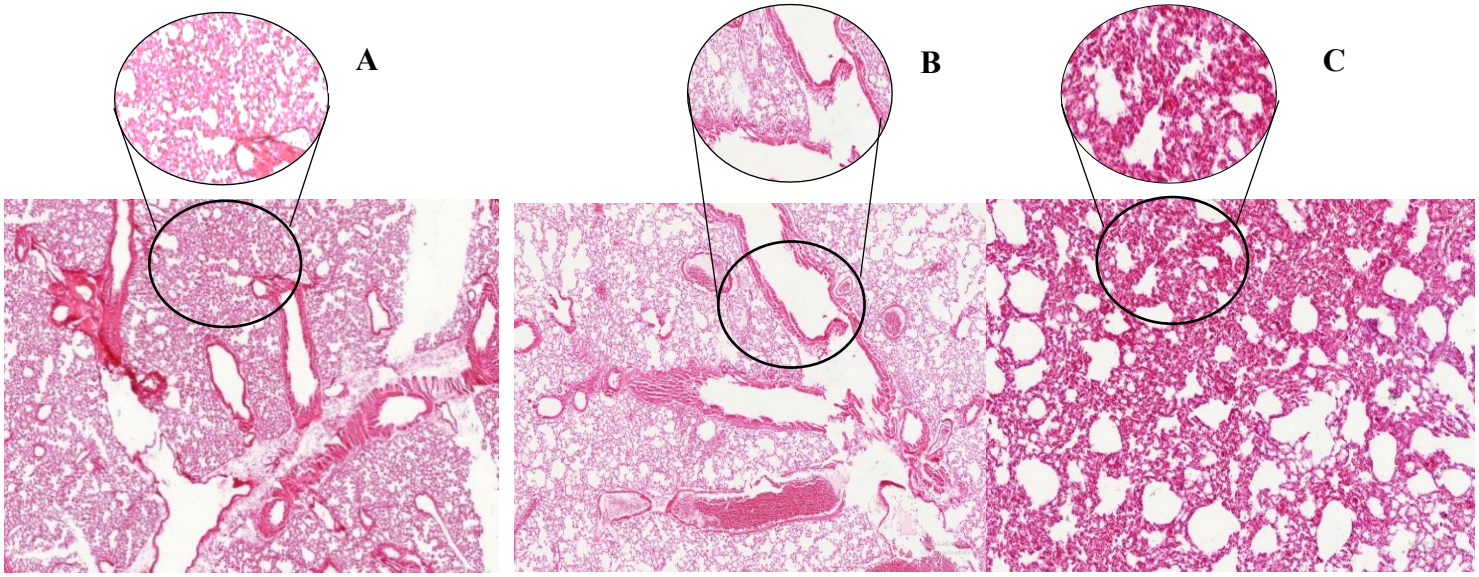

FIGURE 1. Pathological complications observed in the lung tissue of mice receiving 10 mg/kg CEES (H&E, 100x)

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Not declared.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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