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Sub-acute inhalation toxicity study of submicronic alpha-ketoglutarate respiratory formulation

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Abstract

Alpha Ketoglutaric Acid (AKG), one of two ketone derivatives of glutaric acid is an important biological compound that serves as a natural scavenger of ammonium ion, facilitating its conversion to amino acids and protein. AKG inhalation has potential therapeutic role against ammonia-induced structural and inflammatory changes in the lungs. In the present study sub-acute inhalation toxicity of a novel submicronic AKG respiratory formulation was evaluated in Sprague Dawley rats. Sub-acute inhalation toxicity study of novel AKG respiratory formulation at doses, 1, 3 and 5% was conducted as per Schedule-'Y' guidelines of ICMR, India. Hematological, serum biochemical and lung toxicity biomarkers in bronchoalveolar lavage (BAL) fluid were determined. Histopathological analysis of lung tissues and other vital organs was carried out to observe any microscopic changes. Hematology, serum biochemistry and lung toxicity biomarkers in BAL fluid revealed no adverse effects of AKG inhalation except for a slight increase in levels of BAL fluid protein. At autopsy, no histopathological changes in major vital organs, including the lungs were observed. The safety evaluation data suggest that aerosols of submicronic AKG respiratory formulation are safe for inhalation and could be developed as a potential therapeutic option against lung injuries induced through chemical toxicants such as ammonia.

Keywords: Industrial chemicals; alpha-ketoglutaric acid; sub-micronic aerosols; inhalation toxicity; BAL fluid analysis.

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1. Introduction

Inhalation exposure to toxic industrial chemicals such as ammonia causes chemical burns of the skin, eyes, and especially mucous membranes of mouth, pharynx, and respiratory tract.[1] Inhalation toxicity is particularly important considering the fact that lungs are one of the most susceptible organs because of their high vasculature, large surface area and direct interaction with atmosphere.[2] Currently, there is no specific pharmacologic approach for mitigating the effects of ammonia and other such inhalable toxicants on the lung. High-dose glucocorticoids as anti-inflammatory drugs, alone or in combination with other immunosuppressive agents are presently prescribed, although there is a high incidence of their adverse effects.[3] Trials of high-dose steroid therapy have also failed to show any improvement in mortality rate of patients suffering from acute lung injuries.[4] Since a toxicological event of such nature, whether accidental or intentional is usually unpredictable, adequate preparedness is of utmost importance. This has necessitated development of novel, more effective antidote formulations that are also cost-effective to ensure their availability at mass scale. As significant amount of toxicant reaches directly to the lungs upon inhalation, a delivery system that traps or neutralizes the toxicant at the point of entry would be especially beneficial.

Alpha-ketoglutaric acid (AKG), one of two ketone

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derivatives of glutaric acid is a key intermediate in the Krebs's cycle and is considered as a crucial molecule in transmembrane amino acid transport and protein metabolism.[5] AKG also serves as a natural scavenger of ammonium ions, facilitating their conversion to amino acids and protein, thereby reducing the level of ammonium ions in the body. Over the last few years AKG is being pursued widely as a cyanide antidote, wherein it interacts with cyanide to yield non-toxic cyanohydrin intermediates.[6,7] However, whereas cyanide is a lethal cellular poison, ammonia exposure is usually not fatal. A large number of people may be exposed during such an accident, becoming severely symptomatic and requiring immediate treatment. We feel that the natural scavenging action of AKG for ammonium ions can therefore be exploited to develop it into a potential antidote formulation against ammonia induced lung toxicity.

Our proof-of-concept studies using inhalable AKG have shown promising results against ammonia induced oxidative stress and acute lung injuries in animal models.[8] The present work focuses on preclinical evaluation of a novel sub-micronic respiratory formulation of AKG by carrying out sub-acute inhalation toxicity studies in experimental animals as per Schedule-'Y' guidelines of Indian Council for Medical Research (ICMR), India. The rationale is based on previous studies reported from our lab and elsewhere, which have shown that submicron sized drug particles get deposited deeper into lung parenchyma as compared to micronized drugs, thereby increasing their potential efficacy.[9,10]

2. Materials and Methods

2.1 Chemicals

AKG was procured from Sigma Chemical Company (St. Louis, Missouri, USA). All other chemicals were of analytical grade and were purchased from Merck India Ltd.

2.2 Experimental Animals

Male Sprague Dawley rats (220-230g), obtained from Experimental Animal Facility of the institute were used for the study. All animal experiments were approved by Institutional Animal Ethics Committee duly constituted for the purpose (INM/IAEC/2009/IX) and confirmed to general national guidelines on the care and use of laboratory animals. The animals were housed in polypropylene cages and were kept in a room maintained at 25±2^{0}C with a 12h light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory animal feed (Golden Feed Laboratory, Delhi, India) and water *ad libitum*.

2.3 Preparation of AKG respiratory formulation

AKG respiratory formulation was prepared in ethanol and saline solution in a ratio of 30:70 by the method reported from our laboratory earlier.[11] Ethanol was added to produce submicronic aerosol particles and to increase rate of drug output. The particle size distribution of generated aerosols was validated by particle size analyzer (Lasair II, Particle size measuring systems Inc, USA). The size of AKG aerosols delivered to animals after nebulization through nebulizer-spacer assembly was in the range of 300-500nm. A whole body exposure chamber (**Figure 1**) for aerosol inhalation was used in conjunction with an indigenously developed spacer ('Anukool'). Dose and volume of nebulization solution were chosen on basis of previous such work with AKG and other nebulized compounds.[9,12]

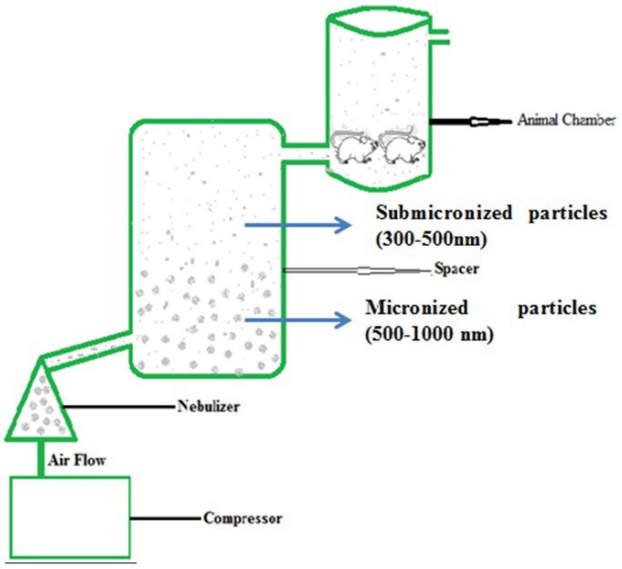


Figure 1. Schematic diagram of inhalation assembly used for aerosol inhalation of AKG respiratory formulation(s).

2.4 Validation of aerosol delivery into lungs: In vivo organ distribution study

In order to confirm that the text compound, i.e., AKG was delivered to the lungs, gamma scintigraphy based

radiometry technique was used to assess its concentration in lungs and other organs post-inhalation. Radiometry techniques have been routinely used in our laboratory to generate important *in vitro* and *in vivo* pharmacodynamic and pharmacokinetic data with respect to a number of potential drug candidates.[13,14]

AKG was radiolabeled with Technetium-99m (Tc-99m) with high radiolabeling efficiency and biological stability by the method described previously from our lab.[6,11] A 3ml solution of AKG respiratory formulation mixed with tracer quantity of Tc-99m-labeled AKG (37MBq) was nebulized to three groups of 6 rats each. One group of rats were sacrificed at 0.5, 2 and 4h respectively and radioactive counts corresponding to drug concentration in lungs, stomach, intestine and blood were counted using well-type gamma spectrometer (Capintec, USA) as per previously established method.[15]

2.5 Sub-acute toxicity study design

Twenty-four rats were randomly divided into four groups of six animals each. The animals of group-I served as control and received no treatment. Group-II, III, and IV animals received aerosols of AKG respiratory formulation in concentration of 1, 3 and 5% respectively twice a day for 20min up to 5 weeks through whole body inhalation assembly. The experimental protocol adhered to Schedule-'Y' guidelines of ICMR, India. All animals were observed twice daily for any morbidity/ mortality and detailed physical examinations were performed weekly. Changes in body weight gain, food and water intake were recorded and local injuries were studied during dosing period. Morphological parameters of toxicity i.e., mucous membrane (nasal secretion), eye irritation, tear secretion, excessive blinking, salivation, cyanosis, lethargy, pyloerection (ruffled fur), paralysis, skin irritation, edema, erythema, respiratory rate, sleeping habits as well as directed and non-directed movements within the cage were observed throughout the experiment.

Animals were sacrificed and dissected after 24h of last exposure. Haematological analysis [viz., red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and platelets counts] was done using an automatic hematological analyzer (Cobas Integra 400 Plus, Roche Diagnostic, Mannheim, Germany) and compared with controls. Difference in biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP), cholesterol, triglycerides and glucose was also studied using an automated analyzer (Roche, Hitachi 912 Model, Mannheim, Germany). Tissue samples of different vital organs (viz., lungs, liver, kidney, heart and spleen) were collected and preserved in 10% buffered formalin solution for histopathology analysis to observe any microscopic changes. Histopathological changes in lungs and respiratory route were specifically observed.

2.6 Analysis of lung toxicity biomarkers in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) fluid was collected as per previously reported method.[8] BAL fluid was centrifuged (300g, 10 min) to collect acellular supernatant, which was stored at −20°C. The supernatant was used for estimation of total protein concentration, enzymatic parameters, viz. Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), Superoxide dismutase (SOD), Catalase (CAT), reduced glutathione and lipid peroxidation by already established methods.[16-22] Total cell counts in BAL fluid were also measured by the method of Sole et. al.[23] All determinations were made in triplicate.

3. Statistical analysis

Numerical data are expressed as mean ± SD. Body weight, change in body weight, food consumption, clinical pathology, and organ weight data were analyzed using student's t-test to determine inter group differences. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Kramer multiple comparisons test. Minimum criterion for statistical significance was set at $p < 0.05$ for all comparisons.

4. Results

4.1 Validation of aerosol delivery into lungs: In vivo organ distribution study

Organ distribution data of inhaled AKG respiratory formulation in rats is shown in **Table 1**. The experiment confirmed that significant amount of sub-micronic AKG reached the lungs and therefore would be available for local therapeutic action. It is well documented in literature that when a drug is nebulized through any spacer device, approximately 60-70 per cent of the drug is not available for inhalation since it gets stuck on the spacer walls and only

30-40 per cent drug is actually inhaled.[24] Present results also corroborated this fact wherein only 29.84±1.67 per cent of the total nebulized dose was inhaled by animals. Of the inhaled dose, nearly one-third amount reached the lungs while majority of remaining dose went into the stomach from where it was absorbed into the blood and got distributed with time.

4.2 Gross pathology

General health, change in body weight, and food and water consumption remained similar in three test groups with no significant difference with respect to control group. No other symptoms like hair shedding, pyrexia, dyspnoea, or change in heart rate were observed. Normal eating and movement post-nebulization were taken as evidence for no gross cardiovascular strain on the animals. There were no behavioral changes in terms of directional and non-directional movements within the cage.

4.3 Hematological and serum biochemistry analysis

There were no treatment-related changes in hematological parameters between control and treated groups, indicating that the nebulized AKG respiratory formulation, in spite of reaching into the blood in substantial amount are not toxic to circulating red cells, nor interfered with their production and that of platelets. The values of RBCs, WBCs, Hb, HCT, MCV, MCH, MCHC, and platelets were found to be within normal range in treated animal groups and there were no significant changes ($p > 0.05$) as compared to the control animals.

Further, there were no significant alterations in the levels of AST, ALT and ALP, suggesting that sub-acute administration of AKG submicronic particles did not alter hepatocyte function and normal metabolism of the animals. Changes in other parameters like glucose, protein and triglycerides were also non-significant when compared with control.

4.4 Macroscopic examination and vital organ/ body weights ratio analysis

No macroscopic changes were observed in experimental animals on scheduled necropsy. Organ/body weight ratios of vital organs were comparable with control, suggesting there were no adverse effects of AKG inhalation on animals.

4.5 Lung toxicity biomarkers in BAL fluid

Total cell counts, total protein, lactate dehydrogenase, superoxide dismutase, malonaldehyde and alkaline phosphatase observed in BAL fluid of animals in test groups showed no significant change with respect to control indicating safety of the developed inhalable formulation. Total protein concentration was however marginally increased in Group-IV animals treated with 5% AKG respiratory formulation (**Table 2**).

4.6 Histopathological analysis

Histopathalogical analysis of vital organs (heart, kidneys, liver, and spleen) showed no adverse effects related to AKG inhalation. Histopathology of lungs of treated animals revealed no significant inflammatory cell infiltration, no interstitial and alveolar edema, vascular congestion and alveolar collapse **(Figure 2).** Bronchial passages and alveolar spaces were within normal limit even at highest concentration group (Group-IV). No polymorphonuclear leucocytes in terminal bronchiole were observed in lungs of inhaled rats.

Table 1. *In vivo* organ distribution study of submicronic alpha-ketoglutaric acid (AKG) respiratory formulation post inhalation. Data are expressed as Mean ± S.D (n=6)

Organ of interest	Percentage of inhaled AKG at different time intervals post-nebulization		
	0.5h	2h	4h
Total Lungs	9.87 ± 0.35	6.014 ± 0.29	3.852 ± 0.11
(a) Left lung	4.25 ± 0.13	2.42 ± 0.06	1.62 ± 0.05
(b) Right lung	5.62 ± 0.22	3.59 ± 0.23	2.232 ± 0.06
Stomach	16.52 ± 1.24	5.54 ± 0.12	1.54 ± 0.06
Intestine	1.85 ± 0.04	2.24 ± 0.58	2.58 ± 0.06
Blood	1.6 ± 0.04	5.5 ± 0.14	3.25 ± 0.15
Total	**29.84 ± 1.67**	**25.308 ± 1.13**	**15.074 ± 0.38**

Table 2. Effect of inhalation of submicronic alpha-ketoglutaric acid (AKG) respiratory formulation on BAL fluid parameters

Lung toxicity biomarkers in BAL fluid	Groups			
	Group I	Group II	Group III	Group IV
Total protein	16.40 ± 1.82	16.65 ± 1.86	17.25 ± 1.94	17.45 ± 2.12
Total cell counts	0.056 ± 0.006	0.058 ± 0.007	0.057 ± 0.006	0.062 ± 0.008
Lactate dehydrogenase	6.62 ± 0.78	6.82 ± 0.88	6.78 ± 0.83	6.82 ± 0.88
Superoxide dismutase	12.34 ± 1.36	12.76 ± 1.35	12.86 ± 1.48	13.16 ± 1.45
Lipid peroxidation	2.54 ± 0.345	2.43 ± 0.36	2.48 ± 0.40	2.63 ± 0.52
Catalase	17.44 ± 1.42	17.70 ± 1.48	16.10 ± 1.54	16.46 ± 1.56
Alkaline phosphatase	43.35 ± 5.40	45.40 ±4.70	47.45 ± 6.90	50.30 ± 6.80
Reduced glutathione	11.38 ± 1.53	11.10 ± 1.66	10.93 ± 1.60	10.98 ± 1.66

Group I: Control; Group II: 1% AKG; Group III: 3% AKG; Group IV: 5% AKG respiratory formulation inhalation
Total cell counts: million cells/mL; Total protein: mg/dL Lactate dehydrogenase: unit/mL; Alkaline phosphatase: unit/L; Superoxide dismutase: unit/mg protein; Malondialdehyde: µmol MDA/mL; Catalase: µmol H_2O_2 consumed/min/mg protein
All the values are mean ± SD; (n=6); $p < 0.05$.

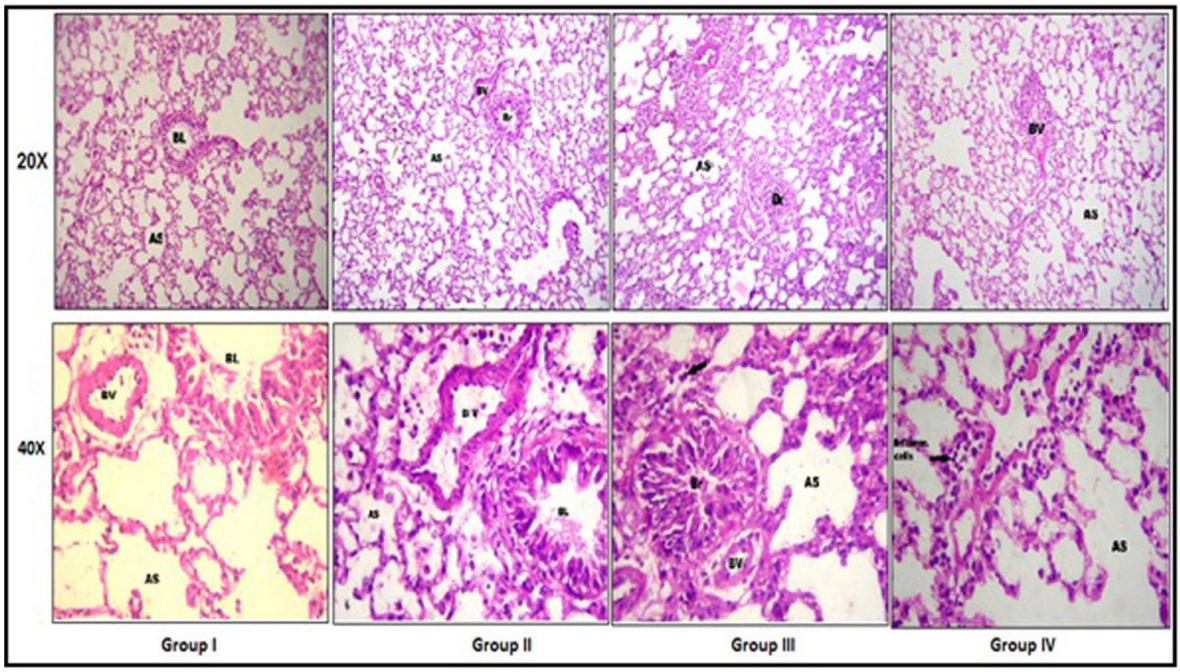


Figure 2. Comparison of photomicrographs of lungs of control and AKG treated animals showing terminal bronchiole (BL), blood vessel (BV) and alveolar spaces (AS) at 20X and 40X. Group I: Control; Group II: 1% AKG inhalation; Group III: 3% AKG inhalation; Group IV: 5% AKG inhalation.

5. Discussion

In the present study submicronic respiratory formulation of AKG was investigated for its subacute inhalation toxicity. The formulation is being developed as an antidote for inhaled chemical toxicants like ammonia. Present work aimed at fulfilling the mandatory task of generating animal safety data prior to carrying out phase-1 human trials.

A whole body exposure chamber was used for inhalation of drug aerosols as it provided an exposure environment in which the test subjects were unrestrained and could move about freely within cages. The system also provided efficient exposure to a large numbers of animals at the same time, and is very useful for studies where daily exposure duration may be as much as 24h. Since the animals left are unrestrained, the experimental procedure does not result is any significant physical

stress to the animal, which may otherwise affect the experimental findings to a variable extent.[8,12] However, the designed system also leads to oral and dermal exposure of the test compound. Any such exposure in the present study with no adverse effects was taken as an added confirmation of the safety of AKG aerosols. The assessment of toxicity in inhalation studies is similar to that used for toxicology studies conducted for any other route of drug administration, and includes analysis of body weight, food consumption, clinical signs of toxicity, clinical pathology, gross necropsy and histopathology. In the present study, since there was no mortality or adverse effects on any of the physiological, hematological and biochemical parameters, it can be inferred that sub-micronic AKG respiratory formulation is non-toxic at designated doses.

Collection and analysis of BAL fluid has become an established technique to study cellular and soluble components of the lower respiratory tract.[25] Studies of cells and proteinacious substances in lung washings provide an insight into the effects of any particular inhalation agent on lung biochemistry. BAL fluid analysis was therefore done to ascertain any AKG inhalation induced lung toxicity. It is a minimally invasive procedure that offers an opportunity to investigate intra-alveolar alterations associated with lung diseases and a valuable tool for studying immune and inflammatory mechanisms in pulmonary disorders.[25,26] There were also no indications of nonspecific immune responses, as indicated by normal ALP and LDH activity in BAL fluid.

The non-toxic effect of sub-micronic AKG inhalation on lung tissue was further validated by histopathological assessment as there were no morphological changes observed in alveolar macrophages. Lungs of both control and treated animals showed normal architecture, suggesting no microscopic changes and morphological disturbances at all dose levels. In fact, lungs of AKG-treated rats did not develop any edema or alveolitis although they showed concentration dependent perivascular inflammation. Non-significant accumulation of inflammatory cells in alveolar spaces, normal alveoli and bronchioles confirmed no significant lung injuries in AKG inhaled animal groups.

6. Conclusion

On the basis of hematological, serum biochemistry and bronchoalveolar lavage studies, the developed AKG respiratory fluid formulation was found to be safe. The formulation can potentially be used for delivering sub-micronic AKG particles deeper into the lung parenchyma and alveolar region for better therapeutic efficacy against chemical toxicants' induced lung injuries, such as ammonia.

7. Conflicts of interest

The authors report no conflicts of interest.

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