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TOXICITY OF ALPHA-KETOGLUTARATE FOLLOWING 14-DAYS REPEATED ORAL ADMINISTRATION IN WISTAR RATS

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Abstract

Oral treatment of alpha-ketoglutarate (A-KG) is known to antagonise experimental cyanide poisoning in rodents. Maximum protective efficacy of A-KG has been observed at a dose of 2.0 g kg^{-1} body weight but no acute toxicity has been observed at this dose level. As a pre-clinical regulatory requirement, sub-acute toxicity of A-KG has to be determined in two different animal species, following repeated exposure by the intended route of use. The present study reports the toxicity and No Observed Adverse Effect Level (NOAEL) of A-KG following 14-days repeated oral administration at low (1.0 g kg^{-1}), middle (2.0 g kg^{-1}) and high (4.0 g kg^{-1}) doses of A-KG in Wistar rats. After termination of the exposure, animals were further observed for 7-days to assess the recovery pattern and residual effects. Clinical signs included diarrhoea at 4.0 g kg^{-1} in both the sexes and decrease in mean body weight in males. This dose also caused anaemia in females which resolved after withdrawal of treatment. In males, significant increase in absolute and relative weights of organs (adrenal, liver and kidneys) and haematological changes were observed at the end of recovery period, suggesting delayed toxic manifestations at 2.0 and 4.0 g kg^{-1} dose. However, these observations were not accompanied by any histological changes to suggest any toxicity of A-KG of clinical significance. The NOAEL of A-KG was determined as 1.0 g kg^{-1} body weight. Although, A-KG is intended to treat acute cyanide poisoning, caution on dosage should be observed during its repeated administration.

Key words: Alpha-ketoglutarate, cyanide antidote, sub-acute toxicity, rats, NOAEL.

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Abbreviations: A-KG: Alpha-ketoglutarate; **ALP**: alkaline phosphatase; **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase; **BUN**: blood urea nitrogen; **g**: gram; **Hb**: haemoglobin; **HCT**: hematocrit; **hr**: hour; **Kg**: kilogram; **MCV**: mean corpuscular volume; **MCH**: mean corpuscular haemoglobin; **MCHC**: mean corpuscular haemoglobin concentration; **ml**: millilitre; µm, micromolar; **NOAEL**: No Observed Adverse Effect Level; **PLT**: platelet count; **RBC**: erythrocyte count; **SN**: sodium nitrite; **STS**: sodium thiosulphate; **WBC**: leucocyte count.

INTRODUCTION

There are several antidotes for the treatment of cyanide poisoning but most of them suffer from serious limitations. Most commonly used methaemoglobin-generating antidotes like amyl nitrite and sodium nitrite (SN) are slow in action and cause severe cardiovascular embarrassment (18, 28). Nitrites also cause delayed toxicity and are contraindicated for victims exposed to fire smoke containing hydrogen cyanide and carbon monoxide (3, 12, 15, 16, 19, 29, 30). The treatment of SN is immediately followed by intravenous administration of sodium thiosulphate (STS). The STS enzymatically mediates the trans-sulphuration of cyanide to less toxic thiocyanate, which is eliminated through urine (1). Therapeutic problems have also been reported during the use of STS (2). Cobalt compounds like dicobalt edetate and hydroxocobalamin, which form stable metal complexes with cyanide are also popular antidotes but they have some side effects like

vomiting, urticaria, anaphylactic shock, hypotension, ventricular arrythmias, gastrointestinal problems, etc. (26). Therefore, relatively safer and more effective anti-cyanide compounds are still being explored (4).

Extensive studies have shown that alpha-ketoglutarate (A-KG), a keto-acid could be an effective oral treatment for cyanide poisoning (6, 9). Oral administration of A-KG has also been reported to antagonise the toxic effects of several cyanogens (10). Presently, A-KG is envisaged as prophylaxis for fire fighters, occupational exposures or rescue operations in contaminated areas and as therapy for suicidal or accidental cases, casualties of smoke inhalation or as an antidote for chemical warfare agent (7). The carbonyl group of A-KG is known to interact with cyanide, a highly reactive nucleophile to form less toxic cyanohydrin complex (21). Also, A-KG is sold over the counter as a nutritional supplement to bolster the citric acid cycle and improve various metabolic disorders. Additionally, A-KG is known to neutralise the toxic effects of nitrogenous chemicals like ammonium compounds, amines, hydrazines etc. (23). The use of A-KG salts in clinical nutrition and metabolic care is well established (14). Maximum antidotal efficacy of A-KG against acute experimental cyanide poisoning has been observed at a dose of 2.0 g kg^{-1} body weight (6, 9). Although, acute oral administration of 2.0 g kg^{-1} A-KG did not produce any toxicity in rats (8), its intraperitoneal administration at 4.0 g kg^{-1} dose caused certain physiological changes (11). Protective efficacy of repeated administration of A-KG has also been reported against acute and sub-acute cyanide poisoning (5, 27). However, no controlled trials or safety studies have been carried out so far to determine the safety limits of A-KG following repeated exposure, considering the fact that there could be several instances where A-KG could be instituted repeatedly, particularly against occupational or dietary cyanide exposures (27). As per the pre-clinical regulatory requirement for a new drug entity, 2-4 weeks toxicity data following repeated administration of agents by the intended route of exposure are warranted. These data have to be generated at low dose, intermediate dose and high dose in both the sexes of two mammalian species, preferably one rodent and one non-rodent. The present study reports the 14 days sub-acute oral toxicity and the No Observed Adverse Effect Level (NOAEL) of A-KG in rats.

MATERIALS AND METHODS

Chemicals and reagents

Alpha-ketoglutarate (alpha-ketoglutaric acid. disodium salt; A-KG; >99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other analytical grade chemicals and reagents were obtained from Merck India Ltd. (Mumbai).

Animals

A total of 40 male and 40 female Wistar rats (8 weeks old) were obtained from the Animal Breeding Facility of Jai Research Foundation (JRF), Valvada, India and were acclimatised for 5 days prior to the experiment. Animals were maintained on sterile rice husk in polypropylene cages and fed on rat pellet feed (Amrut brand, manufactured by Pranav Agro Industries Ltd., Pune, India) and charcoal filtered, UV sterilised water (Aquaguard water filter system, India) *ad libitum*. Rats were maintained in an environment-controlled room (temperature: 19-24^{o}C; relative humidity: 64-66% and photoperiod: 12 hr light and 12 hr dark cycle). This study was assigned to JRF by Defence R&D Establishment (DRDE), Gwalior, India (Study No. DRDE-P1-2003/ Task-8). The study protocol was approved by the JRF's ethical committee on animal experimentations.

Treatment

The animals were randomised and equally distributed into 4 groups of 10 rats each as follows: (G1) vehicle control, (G2) A-KG 1.0 g kg^{-1} (low dose), (G3) A-KG 2.0 g kg^{-1} (middle dose) and (G4) A-KG 4.0 g kg^{-1} (high dose). All the groups comprising of first 5 animals were considered as terminal group and the remaining 5 as recovery group. Solution of A-KG was prepared daily in double distilled water, immediately prior to oral administration in rats by gavages at a dose volume of 10 ml kg^{-1} body weight. The control animals received equivalent amount of water. Animals were treated daily for 14 days and on the 15th day, blood and urine samples were collected from the first 5 animals in each group. Thereafter, animals were sacrificed by carbon dioxide asphyxiation. The removed organs were weighed and processed for histological examination. The remaining 5 animals in each group were further kept under observation for 7 days to assess the recovery pattern and residual effects, and were sacrificed on the 22nd day for various biochemical, haematological and histological examinations. The animals were daily observed for feed consumption, various signs and symptoms (skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, autonomic and central nervous system, somatic-motor activity, behavioural pattern and general changes), mortality, morbidity and weight gain.

Haematology

The animals were fasted over night prior to blood collection. Blood was collected from the orbital sinus plexus under ether anaesthesia and haematological analysis viz. leucocyte count (WBC), erythrocyte count (RBC), haemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT) were performed on an automated haematological analyser (Sysmex K-1000, USA). Clotting time and differential leucocyte count were measured manually by standard procedures.

Clinical chemistry

Serum alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), chloride, cholesterol, creatinine, glucose, phosphorous (inorganic), total bilirubin, total proteins and blood urea nitrogen (BUN) were measured on automated HITACHI 902 (Japan). Potassium and sodium were estimated on flame photometer (Systronics 128, India), and calcium by Erba-Chem 5 plus (USA).

Urinalysis

The urine samples were collected from all the animals by placing them in stainless steel metabolism cages for 3 hr. The parameters analysed were appearance and colour, volume, sediment, specific gravity, pH, blood, glucose, bilirubin, ketone, protein and urobilinogen using URS-9 brand reagent strips (Teco Diagnostics, USA).

Pathology

All the animals were euthanised by carbon dioxide and subjected to complete necropsy under the supervision of a veterinary pathologist. Testes, ovaries and epididymides were fixed in Bouin's solution, eyes in Davidson's fixative and remaining organs (adrenals, brain (including cerebrum, cerebellum and medulla/pons), caecum, duodenum, oesophagus, female mammary gland, ileum, kidneys, lungs, oviducts, peripheral nerve (sciatic nerve), prostate, salivary glands, skeletal muscle, spinal cord (cervical, mid-thoracic and lumbar), sternum with bone marrow, thymus, trachea, uterus, all gross lesions, aorta, colon, heart, jejunum, liver, lymph node, pancreas, pituitary, rectum, seminal vesicles, skin, spleen, stomach, thyroid/parathyroid, urinary bladder and vagina in 10% neutral formalin. Absolute weights of adrenals, brain, uterus, ovaries, testes, epididymides, heart, liver, kidneys, thymus and spleen were recorded immediately after dissection and the relative weights were calculated. After fixation, the tissues were dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax. Multiple sections from each block were prepared at 4-5 µm thickness and stained with haematoxylin and eosin (20).

Statistics

All the parameters characterised by continuous data such as body weight, feed consumption, organ weight, relative organ weight, haematological, clinical chemistry and urine analysis were subjected to Bartlett's test to meet homogeneity of variance before conducting one-way Analysis of Variance (ANOVA) and Dunnett's test where the data did not meet the homogeneity of variance, Student's t-test was performed to calculate significance. Other than control, all the test groups were compared with each other. The results were expressed as mean ± SE and the statistical significance was estimated at 1% and 5% level. Data analysis was performed employing Sigma Stat software (Jandel Scientific Inc., USA).

RESULTS

Oral treatment of 1.0 (low dose), 2.0 (middle dose) and 4.0 g kg^{-1} (high dose) A-KG for 14 days did not cause any mortality but animals receiving the highest dose exhibited diarrhoea during the second week in both sexes. No other clinical signs were observed during the experimental period. At high dose, male rats revealed significant decrease ($p \leq 0.05$) in mean body weight which were 242.3, 243.7 and 271.7 g on 6, 7 and 13 days, respectively as compared to 256.6, 256.0 and 289.1 g, respectively on corresponding days in control animals. The feed consumption also decreased in low (week 1 and 2) and middle dose (week 1), as compared to control group (data not shown).

Table 1. Summary of haematology values in male rats of recovery group

Parameters	**Control**	**A-KG**		
		1.0 g kg^{-1}	**2.0 g kg^{-1}**	**4.0 g kg^{-1}**
WBC count (10^3/µL)	15.8±1.56	19.8±3.59	25.1±2.89	27.7±3.21* (75%↑)
RBC count (10^6/µL)	7.8±0.18	7.7±0.16	7.5±1.20	7.3±0.34
Hb (g/dL)	16.5±0.29	16.1±0.21	15.3±0.37	15.0±0.68
HCT (%)	45.8±0.54	44.7±0.37	42.6±0.47** (7%↓)	42.9±0.44** (6%↓)
MCV (fL)	58.6±1.10	58.5±1.17	57.0±0.65	59.8±2.97
MCH (pg)	21.0±0.33	21.0±0.24	20.4±0.29	20.7±0.24
MCHC (g/dL)	35.9±0.30	36.0±0.42	35.8±0.63	34.8±1.42
Platelet (10^3/µL)	880±78.8	801±52.3	817±48.8	847±70.7
Clotting time (sec)	96.0±6.50	84.0±11.2	90.0±9.49	90.0±9.5
Lymphocyte (%)	82.0±2.52	83.0±1.65	83.0±2.66	84.0±4.18
Neutrophil (%)	17.0±2.38	16.0±1.79	16.0±2.53	15.0±4.23
Monocyte (%)	1.0±0.32	1.0±0.58	1.0±0.25	1.0±0.38
Basophil (%)	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Eosinophil (%)	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00

Values are mean ± SE (n=5). *Significantly different from control at $p \leq 0.05$ and **significantly different from control at $p \leq 0.01$.

Table 2. Summary of clinical chemistry values in female rats of recovery group

Parameters	**Control**	**A-KG**		
		1.0 g kg^{-1}	**2.0 g kg^{-1}**	**4.0 g kg^{-1}**
Glucose (mg/dL)	94.8±2.57	111.1±7.93	117.7±3.47* (24%↑)	114.3±6.38
ALT (IU/L)	50.8±2.08	44.9±2.36	46.2±4.78	43.4±3.05
AST (IU/L)	197.4±7.99	181.4±18.13	170.9±6.53	171.6±11.01
Total bilirubin (mg/dL)	0.14±0.013	0.11±0.013	0.12±0.013	0.09±0.013* (36%↓)
Creatinine (mg/dL)	0.32±0.008	0.30±0.004	0.35±0.027	0.29±0.013
BUN (mg/dL)	20.5±0.64	20.6±1.82	21.2±1.45	16.4±1.48
Total protein (g/dL)	7.48±0.076	7.19±0.165	7.42±0.125	7.27±0.206
Albumin (g/dL)	3.91±0.027	3.90±0.044	3.93±0.072	3.74±0.103
Calcium (mg/dL)	10.5±0.031	10.3±0.054* (2%↓)	10.2±0.098	10.3±0.174
Phosphorus (mg/dL)	5.97±0.29	6.56±0.27	6.43±0.38	6.98±0.19
Cholesterol (mg/dL)	85.9±2.54	77.7±4.69	69.6±5.31* (19%↓)	67.7±3.47* (21%↓)
ALP (IU/L)	213.1±16.2	200.4±21.9	185.2±15.9	177.1±19.7
Sodium (mEq/L)	121.3±0.59	121.1±3.29	121.2±1.25	121.1±1.14
Potassium (mEq/L)	3.8±0.036	3.7±0.081	3.6±0.067	3.6±0.098
Chloride (mEq/L)	103.8±3.97	107.5±2.43	101.3±7.90	103.3±3.85

Values are mean ± SE (n=5). *Significantly different from control at $p \leq 0.05$.

No haematological changes were observed in male rats 14 days after treatment with various doses of A-KG. However, at the end of recovery period, significant increase ($p \leq 0.05$) in WBC count (high dose) and decrease ($p \leq 0.01$) in HCT (middle and high dose) were observed (Table 1). In female rats, statistically significant decrease ($p \leq 0.01$) in RBC count in all the treatment groups and Hb and HCT in high dose group was observed at terminal period. An increase in MCV (high dose) was also observed. These changes, however, resolved after withdrawal of treatment (data not shown).

Clinical chemistry analysis conducted at the end of the exposure and recovery period did not reveal any treatment related changes in male rats (data not shown). However, during the recovery phase, female rats exhibited increased levels of glucose (middle dose) and decreased levels of bilirubin (high dose) and cholesterol (middle and high dose) as compared to control animals ($p \leq 0.05$). No other significant changes were observed (Table 2).

Urinalysis performed during the experimental period did not indicate any treatment related changes (data not shown).

No statistically significant changes were observed in the absolute and relative organ weights of the animals sacrificed at the end of 14-day treatment period in either sex (data not shown). However, in the recovery group of male rats, absolute weight of adrenal, liver ($p \leq 0.01$) and kidney ($p \leq 0.05$) were significantly increased in mid dose and adrenals ($p \leq 0.01$) and liver ($p \leq 0.05$) in high dose groups (Table 3). Table 4 shows the relative organ weight in male rats of the recovery group. The data show increase in relative organ weight of adrenal ($p \leq 0.05$), kidneys and liver ($p \leq 0.01$) at corresponding time points as compared to control. Also, increased relative organ weight of kidney was evident at high dose of A-KG. A significant increase ($p \leq 0.05$) in absolute kidney weight of female rats in high dose group with corresponding increase in relative kidney weight was also observed at similar time point (data not shown).

Visceral examination revealed lesion in spleen (splenomegaly/enlargement, atrophy) and kidney (congestion) in both the sexes of control and treated rats. Additionally, female rats showed lesions in lungs (pneumonic foci), liver (whitish deposits), and uterus (hydrometra). Microscopic examination of different organs revealed varying degree of pathological changes in both terminal and recovery groups. However, these changes were also observed in the control rats as well (data not shown).

Table 3. Summary of terminal body weight and organ weight (g) in male rats of recovery group

Body weight/ Organs	**Control**	**A-KG**		
		1.0 g kg^{-1}	**2.0 g kg^{-1}**	**4.0 g kg^{-1}**
Body weight	306.0±9.5	294.6±15.9	304.6±5.5	292.6±1.3
Adrenals	0.049±0.002	0.057±0.004	0.065±0.002** (33%↑)	0.068±0.002** (39% ↑)
Testes	2.94±0.106	2.79±0.094	2.76±0.072	2.53±0.375
Epididymides	0.877±0.029	0.860±0.030	0.905±0.036	0.825±0.086
Kidneys	2.64±0.099	2.66±0.091	2.97±0.074* (13%↑)	2.79±0.033
Brain	1.96±0.056	2.00±0.047	1.95±0.025	1.93±0.058
Heart	1.19±0.056	1.04±0.059	1.19±0.063	1.09±0.037
Spleen	1.49±0.183	1.47±0.188	1.79±0.142	2.06±0.417
Liver	11.28±0.559	12.92±0.657	13.84±0.516** (23%↑)	13.39±0.196* (19% ↑)
Thymus	0.507±0.055	0.411±0.027	0.410±0.046	0.414±0.011

Values are mean ± SE (n=5). *Significantly different from control at p≤0.05 and **significantly different from control at p≤0.01.

Table 4. Summary of relative organ weight (%) in male rats of recovery group

Organs	**Control**	**A-KG**		
		1.0 g kg^{-1}	**2.0 g kg^{-1}**	**4.0 g kg^{-1}**
Adrenals	0.016±0.001	0.020±0.002	0.022±0.001* (38% ↑)	0.023±0.001* (44% ↑)
Testes	0.968±0.065	0.963±0.078	0.906±0.014	0.866±0.127
Epididymides	0.288±0.016	0.294±0.015	0.297±0.012	0.281±0.026
Kidneys	0.861±0.009	0.907±0.026	0.975±0.015** (13% ↑)	0.953±0.013** (11% ↑)
Brain	0.642±0.021	0.686±0.033	0.640±0.012	0.659±0.025
Heart	0.389±0.001	0.353±0.012	0.390±0.021	0.372±0.012
Spleen	0.486±0.055	0.500±0.061	0.591±0.050	0.709±0.149
Liver	3.68±0.104	4.39±0.112** (19%↑)	4.54±0.133** (23% ↑)	4.58±0.042** (24% ↑)
Thymus	0.166±0.017	0.141±0.009	0.135±0.016	0.141±0.003

Values are mean ± SE (n=5). *Significantly different from control at p≤0.05 and **significantly different from control at p≤0.01.

DISCUSSION

Significant research efforts have been expended in the recent past to develop A-KG as an oral treatment for cyanide poisoning (6, 7, 9, 10). Acute oral administration of A-KG at a dose (2.0 g kg^{-1} body weight) conferring maximum protection did not produce any toxicity in rats (8). However, its intraperitoneal administration at a higher dose (4.0 g kg^{-1}) caused certain physiological changes (11). Considering the fact that A-KG could be administered repeatedly to treat acute or sub-acute cyanide poisoning (5, 27), the present study was designed to assess the sub-acute toxicity of A-KG given orally in rats.

In the present study, male rats showed significant decrease in mean body weight only on three different days during 14-days of A-KG exposure, but no persistent decrease or change during the recovery period was observed. Also, no decrease in the mean body weight was observed in female rats. The transient loss in body weight due to high dose of A-KG can be attributed to loss of appetite accompanied by diarrhoea during the early phase of exposure. We have earlier reported that rats given an acute oral or intraperitoneal dose of A-KG (>2.0 g kg^{-1}) experienced diarrhoea (8, 11). This kind of drug-induced osmotic diarrhoea is well known (17, 25). Arginine-A-KG and ornithine-A-KG are other forms of A-KG which are widely used in clinical nutrition without any discernible adverse

effects, except diarrhoea (13, 22). Similarly, the haematological changes observed in the present study were inconsistent and did not correlate with the histology of bone marrow. Also, the changes were not uniform in both the sexes of rat, and therefore, cannot be concluded as anaemia. The changes in clinical biochemistry and urine were also inconsistent and not suggestive of any major disorder. These observations corroborate with our previous studies (8, 11). Change in thymus weight could be incidental. Significantly increased absolute weight and relative weights of organs (adrenal, liver and kidney) and haematological changes observed during the end of recovery period in male rats, indicated effects of A-KG after withdrawal of test substance. Although, there was a two-factor difference between successive dose levels, poor dose response relationship was observed in altered parameters and also histology of corresponding organs did not reveal any evidence for the observed effects. Lesions recorded in different organs were recorded either both in control and treatment groups at comparable level or only in few animals without a consistent pattern and were in conformity with the historical control data of JRF (24). Thus, it may be inferred that different changes recorded in various organs of rats (control and treated groups) were spontaneous or incidental in nature and unrelated with test substance or different sexes. This is also supported by the fact that no such changes in relative organ weight were observed in previous acute studies (8, 11).

To summarise, repeated oral administration of A-KG for a period of 14 days produced diarrhoea in rats, particularly in high dose. The decrease in body weight of male rats, haematological changes in female rats were only incidental and did not correlate with histological observations. Also, most of the effects were found to be reversible after withdrawal of treatment. Indeed, in male rats, significant increase in absolute and relative weight of organs (adrenal, liver and kidneys) and haematological changes observed at the end of recovery period could be delayed toxic manifestations of A-KG, particularly at mid and high dose. However, these alterations did not reveal any concurrent change in histological profile of respective organs. At 1.0 g kg^{-1} (low dose) no adverse effects were observed in any of the parameters and therefore, NOAEL for A-KG for 14-day oral exposure in Wistar rats was determined as 1.0 g kg^{-1} body weight. The study suggests that caution on A-KG dosage should be observed during its repeated administration for the management of long-term cyanide poisoning.

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