Assessing Nutraceutical Alpha Ketoglutarate for Its Effectiveness: $^{99m}$Technetium-Mebrofenin Nuclear Imaging in CCl$_4$ Induced Hepatotoxic Animal Model

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Abstract---Alpha Ketoglutarate (AKG), the active metabolite nutraceutical have been available in market and has been demonstrated to have anti-oxidant properties previously. Newer metabolic functioning of this novel metabolite have been under covered recently in microbial studies, indicating there is more to α-KG, and it might be an evolutionary molecule and a common linking metabolite which acts to help cell survive when it is exposed to various stressors. The current study assess its hepatoprotective effectiveness in a well-established CCl$_4$ induced acute hepatotoxic animal model, which are now known to have both lipid peroxidation dependent and independent pathways causing liver injury. Parameters assessed included, Biochemical (Liver functioning tests, oxidative stress, antioxidant levels) and histopathological assessment. $^{99m}$Tc-Mebrofenin Nuclear (functional imaging) of animals were done, it provides objective validation of other parameters assessed, provides in-vivo quantification of liver functioning and parameters assessed can quantify oxidative and inflammatory pathways to liver injury and intervention response distinctly.

Keywords---Alpha-Ketoglutarate (AKG), Carbon Tetrachloride (CCl$_4$), Hepatotoxicity, $^{99m}$Technetium-Mebrofenin

I. INTRODUCTION

In past decade or so nutraceutical have generated great deal of consumer interest, largely on account of reported beneficial potential of these novel bioactives to treat chronic conditions with fewer to no side effect profile. Scientific research supporting the biological activities of these nutraceuticals in, in-vitro and in-vivo experimental models lack stringent validating criterion. Also inability to uncover the most active metabolite among the various polyphenols; scarce and inconclusive human trials; lack of long term studies, have led low confidence and criticism from the clinical fraternity [1]. Thus there is a need for stringent scientific validation of prophylactic and therapeutic utility of these class of agents.

Alpha-ketoglutarate (AKG), non-essential amino acid, have been marketed for some time now with various reported benefits, most important being its anti-ageing and anabolic properties which have been ascribed largely to its antioxidant properties. Biochemically AKG has been described for its ability to convert ammonia to non-toxic amino acids like glutamine, non-enzymatic oxidative decarboxylation of hydrogen peroxide and as such suppresses free radical generation and prevention of lipids peroxidative damage [2]. AKG is shown to inhibit oxidative stress induced by H$_2$O$_2$ in erythrocytes and cultured neurons [3], [4]. In addition to its protective activity on redox homeostasis, AKG is shown to have chelating properties by forming, active complexes with iron in brain homogenates [5]. As such AKG is known to have anti-oxidant properties. In past AKG has been shown to be effective as an antidote against cyanide poisoning [6], reducing oxidative stress caused by sodium valproate [7], ethanol [8], and ammonium acetate [2]. More novel roles that AKG might have in a cell than merely having antioxidant properties are being under-covered. Recent mechanistic studies showed newer molecular mechanisms of this metabolite. α-KG inhibits ATP synthase and leads to reduced ATP content, decreased oxygen consumption and increased autophagy, in both C. elegans and mammalian cells and also shown to prevent mtDNA damage. Thus α-KG might be an evolutionary molecule and a common linking metabolite which acts to help cell survive when it is exposed to various stressors [9].

Carbon- tetrachloride (CCl$_4$) is a well-known potent hepatotoxin, which has been used since seventies to create in-vivo models of acute and chronic liver injuries. The critical mechanism and signaling pathways for pathogenesis in well perfused liver and isolated or cultured hepatocytes have been elucidated [10]. Now there is a growing body of evidence to suggest that CCl$_4$ toxicity is, rather a multifactorial process, unlike as previously thought. Earlier, much of CCl$_4$ hepatotoxicity, had been attributed to its activation by mitochondrial cytochrome P450 (CYP) 2E1, and very marginally by other CYPs (CYP2B and CYP3A), resulting in formation of trichloromethyl (CCl$_3$) and trichloromethylperoxy radical (CCl$_3$OO$^\cdot$). Both free radicals being highly reactive species cause subsequent lipid peroxidation, resulting in cellular and intra-cellular membrane...
lysis, leading to hepatocytic necrosis [10]-[13]. However, more recent studies have shown that, lipid peroxidation-independent mechanisms, also seem to be involved in CCl4 induced hepatotoxicity. CCl4 intoxication induced a severe decrease in mitochondrial respiratory chain complex IV activity; mitochondrial DNA (mtDNA) alterations, reduced glutathione (GSH) depletion, decreased aconitate activity and macromolecular ultrastructural alterations [13]. As such CCl4 induced liver injury is not merely a free radical reactive species hepatotoxicity model. Studies in rats treated with CCl4 showed that cytokines (TNF-α and IL-6) caused perturbation in bile acid and organic anion transporters [14] which was objectively demonstrated by 99mTc-Mebrofenin hepatobiliary scan in a carbon tetrachloride-induced liver injury model [15].

In the current study, the animal model of CCl4-induced acute liver injury is employed, meanwhile apart from serum transaminases, free radical, antioxidant, mitochondrial and histopathological parameters, in-vivo physiological liver functioning is assessed using 99mTc-Mebrofenin nuclear (functional) imaging modality. It provides better objective validation to other parameters assessed as serum level of various substances produced by the liver, including albumin, bilirubin, alkaline phosphatase, and transaminase are indirect indicators of liver function whereas measurement of radioactivity emitted from the liver serves as a direct indicator of the physiological status of the hepatocyte [16].

Also, since a previous 99mTc-Mebrofenin hepatobiliary scan [15], in a carbon tetrachloride–induced liver injury model, showed derangement, which were described to be cytokine mediated rather than oxidative stress mediated. This modality parameter was included in the study.

II. MATERIALS AND METHODS

A. Ethics Statement

All the procedures and care managed to the animals has been approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) regulations and the study protocol was approved by the Institutional Animal Ethics Committee of the Institute (INM/IAEC/2009/06/009), INMAS, DRDO, and Delhi, India.

B. Chemicals

Alpha Ketoglutarate (AKG) (Fluka chemika, buchs, Switzerland) and CCl4 (Merck Ltd, India), Glutathione peroxidase kit and Glutathione reductase assay kit were procured from (Calibiochem, Merck, India), Lipid peroxidation (TBARS) colometric Assay kit, Superoxide Dimutase (SOD) Activity Assay kit, Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline Phosphate Assay Kit, Albumin and Total Bilirubin kits were procured from (Biovision, India). All other chemicals used were of highest purity grade commercially available.

C. Experimental Animals

Male Sprague Dawley rats weighing (200 to 250 g) were obtained from the animal house of Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, Delhi. All the animals were housed in polypropylene cages and were administered food (Hindustan Lever Ltd, Mumbai, India) and water ad libitum in the experimental animal care facility.

D. Experimental Protocol

AKG toxicity Study: The animals (n=6, each group) were administered variable doses of AKG, Group1 (G1): 0mg/kg, Group2 (G2): 1gm/kg, Group3 (G3): 2gm/kg, Group 4(G4): 4gm/kg orally by gavage for 2 weeks. Animals were observed for mortality and morbidity. Clinical symptoms and body weight were recorded daily throughout the study period. At the end of treatment animals were sacrificed.

Hepatotoxicity Model: For liver injury model, single injection CCl4 was injected intraperitoneally at 2ml/kg body weight with and without orally administered AKG (2gm/kg bodyweight) for one week.

E. Nuclear Medicine Procedures

Radiopharmaceutical, 99mTc-Mebrofenin (MEB)

99mTc-sodium pertechnetate (BRITS, Mumbai, India), 185–222 MBq in 3 mL normal saline, was mixed with Mebrofenin (MEB) according to the manufacturer’s instructions. This class of agent has gamma ray emitting properties of 99mTc which are acquired using gamma cameras. Mechanism of uptake of 99mTc-MEB by the hepatocyte is similar to those of organic anions such as bile acids or bilirubin [17]. It has high first pass extraction with hepatic uptake of 99mTc-MEB being 98% and it strongly resists displacement by a high bilirubin level [18]. Unlike bilirubin, 99mTc-MEB is secreted into bile canaliculi in their native state, without undergoing any conjugation [19]. Thus it serves as an ideal in tracer for in-vivo quantification of hepatic functioning and delineation of hepatobiliary tree.

The dose not taken up by the liver is excreted through urine.

F. Image Acquisition: Gamma Camera

A gamma camera (Siemens) equipped with a low-energy, high-resolution, parallel-hole collimator and interfaced to a computer, was used for image acquisition. A 20% window centered on 140 keV was used for energy discrimination. Dorsal images were acquired for 60 min using a 64 × 64 × 16 matrix at a zoom factor of 2. Images were acquired for 1s per frame for 2 min, followed by 1 min per frame for 58 min.

G. Image Processing: Workstation

Data were analyzed using a conventional nuclear medicine workstation (Pegasys; ADAC Laboratories). Regions of interest were drawn over the liver, and hepatic time–activity curves were generated. The curves generated over the liver provide a measure of the rapidity of uptake and excretion of
The time at which maximal hepatic activity of 99mTc-MEB occurred (T_{peak}) as well as the time required for peak 99mTc-MEB activity to decrease by 50% (T_{1/2 peak}) were determined.

Management and husbandry conditions were identical in all groups with 12/12 h light/dark cycle at 21±2°C. Food and water were provided ad libitum. At the end of the experiment animals were sacrificed and blood samples were collected and serum was separated by centrifugation at 2000×g for 10 min and stored for biochemical estimations. Portions of liver tissue was placed in formaldehyde solution for routine histopathological examination and one portion in liquid nitrogen for antioxidant status.

**H. Measurement of Serum Parameters and Antioxidants**

Leakage of intracellular enzymes as alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) [20], Total Bilirubin and Albumin were determined by using semi-automatic biochemical analyzer (Krish Bio medicals, Delhi) using the commercially available kits according to the manufacturer’s details.10% homogenate of liver was prepared by using IKA T25 digital Ultra Turrax at 4ºC in 0.15 M KCl. The homogenate was centrifuged (12,000 rpm for 45 min at 4°C) in Remi cooling centrifuge to remove the debris and supernatant was used for enzyme assay. Lipid Peroxidation, MDA was evaluated by the thiobarbituric reactive oxygen substances (TBARS) by the method of Ohkawa [21]. The final concentration of MDA was expressed as nm/100g tissue. Catalase (CAT) activity was estimated by the method of Beers and Seizer [22] and is expressed as n moles of H$_2$O$_2$ decomposed/min/mg protein. Superoxide dismutase (SOD) activity was estimated by the method of Marklund [23] and is expressed as units/min/mg protein. Total reduced glutathione (GSH) was determined by the method of Ellman [24] and Glutathione peroxidase was determined by the method of Rotruck [25].

**I. Histopathology Analysis of Liver**

Tissues were processed by standard histopathological procedure and microscopic changes in liver were observed. Liver tissues were fixed in 10% formalin and embedded in paraffin and 5 µm size sections were cut. The sections were deparaffinized using xylene and ethanol and then washed with phosphate buffer saline (PBS) and with permeabilization solution (0.1 M citrate, 0.1% Triton X-100). The deparaffinized sections were stained with hematoxylin and eosin. Tissue histology was observed under the fluorescence microscope (Olympus BX 60).

**IV. RESULTS**

**A. AKG Toxicity Study**

After 2 weeks effects of AKG on serum ALT, AST and ALP activities in rats treated with variable doses of AKG, Group1 (G1): 0mg/kg, Group2 (G2): 1gm/kg, Group3 (G3): 2gm/kg, Group 4(G4): 4gm/kg were evaluated. The clinical biochemistry analysis was performed at the end of exposure did not revealed any significant changes in any of the observed parameter however high dose terminal group animals revealed minor elevations in the level of ALT, AST and ALP. The administration of alpha Ketoglutarate at dose levels of 4g/kg body weight revealed significantly decreased mean body weight (data not shown). No mortality has been seen at these doses. No significant alterations has occured in the LFT at lower doses except elevations in ALP at high dose (Fig 1).

**Fig. 1** Effect of variable doses of AKG on serum enzyme activities (G1: control, 0gm/kg, G2: low dose, 1gm/kg, G3: mid dose, 2gm/kg, G4: high dose, 4gm/kg). Data is expressed as Mean ±SD, p<0.05

**B. Effect of AKG on CCl$_4$ Induced Liver Injury**

The effect of AKG on liver injury administered by CCl$_4$ were examined. After 24 hrs there was a remarkable increment in ALT and AST levels while ALP and Bilirubin levels were not altered significantly. The oral administration of AKG attenuated the serum elevations and hence suggesting curative effect (Fig 2. & 3).

**Fig. 2** Effect of AKG on serum enzyme activities in CCl$_4$ induced hepatotoxicity in rats. Data are expressed as Mean ±SD; n=6, **p<0.01, when compared to control, ##p<0.01, when compared to CCl$_4$ group
Effect of AKG on Albumin and Bilirubin in CCl4 induced hepatotoxicity in rats. Data are expressed as Mean ±SD; n=6, *p<0.01, when compared to control, **p<0.01, when compared to CCl4 group.

C. Effect of AKG on Hepatic Antioxidant Levels

The levels of liver MDA, as product of lipid peroxidation, SOD showed increment in CCl4 treatment group which are significantly higher than the control group animals. While AKG treatment reversed the alterations. In CCl4 treatment group there is a significant reduction of hepatic GSH and Catalase as compared to normal control group. AKG supplementation elevated the GSH and Catalase activity as compared to the CCl4 group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl4</th>
<th>CCl4+AKG</th>
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<tbody>
<tr>
<td>TBARS (nM/100g tissue)</td>
<td>0.92±0.23</td>
<td>2.34±0.20*</td>
<td>1.34±0.17**</td>
</tr>
<tr>
<td>SOD, Superoxide Dismutase (50% inhibition of NBT reaction/min/mg protein)</td>
<td>5.78±0.61</td>
<td>3.02±0.34*</td>
<td>4.02±0.39**</td>
</tr>
<tr>
<td>Catalase (μmoles/min/mg protein)</td>
<td>69.03±3.6</td>
<td>39.7±4.3*</td>
<td>59.45±5.40**</td>
</tr>
<tr>
<td>Glutathione Peroxidase (μg of GSH consumed/min/mg protein)</td>
<td>6.23±0.14</td>
<td>4.52±0.12*</td>
<td>5.01±0.17**</td>
</tr>
<tr>
<td>Reduced Glutathione (mg/g tissue)</td>
<td>44.83±4.8*</td>
<td>20.82±5.67*</td>
<td>32.04±7.82*</td>
</tr>
</tbody>
</table>

Mean ±SD; n=6 ; * p<0.05, when compared to control, **p<0.01, when compared to CCl4 group

D. Histopathology Analysis of Liver Tissues

The histopathological examination showed that treatment with CCl4 caused typical centrilobular hepatocytic steatosis (both macrovesicular and microvesicular) and cagulative necrosis especially in the periporal hepatocytes. While the AKG treated animal tissues showed almost normal histological profile of hepatocytes with mild congestion. Thus, histological examination clearly demonstrated the protection of AKG against CCl4 hepatotoxicity.

E. 99mTc-Mebrofenin (MEB) Handling

In control group rats, 99mTc-mebrofenin showed prompt uptake, uniform distribution and efficient tracer accumulation with rapid tracer clearance from the liver. For 300 microcurie of injected dose, peak counts stood at (400+/−120 counts/sec) with Tpeak (148+/−40 sec) and T 1/2 peak (424+/−56 sec) for the control group. In CCl4 treatment group rats, 99mTc-mebrofenin did not showed delayed tracer uptake Tpeak (223+/−52 sec; p not significant versus normal control), liver tracer clearance, though was markedly delayed in CCl4-treated rats T 1/2 peak (1017+/−130 sec; p significant versus normal control) and showed less efficient tracer accumulation; peak counts stood at (200+/−70 counts/sec). AKG, intervention in CCl4 group, significantly reverted back the deranged parameters. Peak counts stood at (430+/−126 counts/sec) with Tpeak (153+/−48 sec) and T 1/2 peak (443+/−70 sec) for the AKG intervention group.
**Fig. 5** MEBroFEN IN IMAGING SHOWS KINETICS OF HEPATIC MEBroFEN IN UPTAKE AND EXCRETION IN REPRESENTATIVE RATS.

**Fig. 5A.** Control rat shows prompt mebrofenin clearance from liver.

**Fig. 5B** CCl₄ rat shows considerably longer retention of mebrofenin activity in liver.

**Fig. 5C.** CCl₄ + AKG shows prompt mebrofenin clearance from liver.
V. DISCUSSION

The administration of AKG at higher doses revealed significantly decrease mean body weight and decreasing trend of mean body weight throughout the experiment. High concentration of AKG (4gm/kg b.w.) led diarrhea to animals while No observed adverse effect level (NOAEL) for AKG at lower doses is seen over two weeks experiment period.

The present study was conducted to evaluate the curative effect of AKG against CCl₄ induced hepatotoxicity in rat. This study gives some scientific evidences on the effect of AKG on enzymatic, antioxidant status, histological observations and nuclear functional imaging study. CCl₄ is used as hepatotoxic agent in animals for establishing acute and chronic hepatic injury models and to study the hepato-curate action of plants and other compounds [26], [27]. CCl₄ induced hepatic damage are related to its conversion into reactive toxic metabolites [28]. The mechanism of hepatic injury by CCl₄ involves lipid peroxidation of membrane bound fatty acids which result in destructing the cell membrane and the intracellular organelles of the hepatocyte [29]. Serum hepatobiliary enzymes such as AST, ALT and ALP are present in high concentrations in the liver under normal conditions and the elevations in serum enzymes indicates the hepatocytes membrane damage [30]. The present data revealed that serum AST, ALT, ALP and Bilirubin were significantly increased and albumin decreased in rats intoxicated with CCl₄ in comparison with control group. The treatment of AKG significantly ameliorated the toxic effects. Recent microbial studies on AKG had shed newer light to its antioxidant properties, are in agreement with our result.

Oxidative stress is a prominent feature in the pathophysiology in acute liver disease [31]. Oxidative stress occurs when reactive oxygen species (ROS) are not adequately neutralized by cellular antioxidant defense mechanisms [32]. Thiobarbituric acid reactive substances (TBARS), the final metabolites of peroxidized polyunsaturated fatty acids, have been used as biomarkers of oxidative stress [33] Antioxidant defense was evaluated by measuring glutathione (reduced GSH), glutathione peroxidase (GSHpx),superoxide dismutase(SOD) and catalase (CAT) [34]. In the present study CCl₄ treated rats have significantly higher levels of TBARS and significantly lower antioxidant levels. This is in agreement with CCl₄ induced hepatotoxic model [35].In hepatocytes CCl₄ undergo bioactivation by mitochondrial cytochrome P450 (CYP)2E1, resulting in formation of trichloromethyl (CCl₃) and trichloromethyl peroxy radical (CCl₃OO). Both free radicals being highly reactive species cause subsequent lipid peroxidation, as evident by significantly higher levels of TBARS, resulting in cellular and intra-cellular membrane lysis, leading to hepatocytic necrosis and decrease in hepatic antioxidant enzymes. The decreased concentration of antioxidants in CCl₄ liver may be due to NADPH reduction or their utilization in the exclusion of free radicals. The present study reveals that treatment with AKG has a critical role in abrogating the oxidative stress-mediated toxicity of CCl₄. CCl₄-induced raised TBARS and reduced anti-oxidant levels were significantly reverted to control levels with co-administration of AKG. These are in general agreement with previous study in which, administration of AKG prevented the rise in the TBARS and hydroperoxides levels in rats treated with sodium valproate. The likely explanation to these may be that AKG offer protection against oxidative damages, by participating in the non-enzymatic, NADPH independent oxidative decarboxylation in the hydrogen peroxide [7]. Ammonia is known to inhibit antioxidant enzymes, AKG by acting as a scavenger of amino groups transforms the ammonia to nontoxic amino acids like glutamate or glutamine [36]. AKG is also known be involved in GSH synthesis [37]. AKG as a strong ammonia and phosphate binding factor may indirectly stabilize redox state in organism [38]. The present study shows that AKG offers protection against oxidative damage caused by CCl₄.

99mTc-mebrofenin hepatobiliary scintigraphy is widely used for assessing overall physiological liver function and assessing hepatobiliary tree for the transit of bile. The two functional parameters measured as integral part of hepatobiliary imaging are T₁/₂ peak and T₁/₂ peak. Tpeak Represents the efficacy of mebrofenin uptake by hepatocytes and T₁/₂ peak reflects the dynamics between extraction, retention and clearance of biliary mebrofenin [39]. Thus Tpeak and T₁/₂ peak represents mebrofenin handling which involves the hepatocyte transporters. The specific transporters involved in the hepatic excretion of 99mTc-mebrofenin is not established. An earlier study of 99mTc-mebrofenin hepatobiliary scintigraphy in CCl₄ animal hepatotoxic model, observed no significant change in Tpeak with significantly delayed T₁/₂ peak. The study opinioned that CCl₄ induced -inflammatory cytokine (TNF-α, IL-6) mediated the downregulation of receptors such as ATP–dependent proteins the bile canalicular transporters like, bile salt excretory pump (BSEP) and the multidrug resistance–related protein the basolateral exporters like MRP1 and MRP3. As such the downregulation of cytokine-regulated exporters resulted in significantly delayed T₁/₂ peak without significantly affecting Tpeak. Thus Tpeak and T₁/₂ peak represented the inflammatory cytokine mediated hepatic injury and not the direct oxidative hepatic necrosis. Mebrofenin accumulation (total radioactivity counts/sec at Tpeak), represents functional parenchymal liver cell mass [40]. Thus depletion of parenchymal liver cell mass, as in CCl₄– induced peroxidative hepatic necrosis, causes reduced peak mebrofenin accumulation.

In the present study, CCl₄-induced hepatotoxic group, showed reduced peak mebrofenin accumulation (indicative of oxidative hepatic necrosis), significantly delayed T₁/₂ peak with no significant delay in Tpeak (indicative of inflammatory cytokine mediated hepatocyte injury).The result were in agreement with two previous studies [41]. Treatment with exogenous AKG significantly reverted back such abnormal parameters. Thus AKG showed hepatoprotective effect as a
potent antioxidant as well as prevented cytokine mediated inflammatory pathogenesis. Molecular underpinnings of its function in this regard needs to be explored further. It protective role in other inflammatory pathologies might be a potential research avenue.

VI. COMPETING INTEREST

The authors declare that they have no competing interest.

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AUTHOR’S CONTRIBUTION

LM carried out experimental design, blood and liver sampling, statistical analysis and participated in drafting the manuscript. AK carried out nuclear medicine procedures. All authors read and approved the final manuscript.

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