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Research Article

Functional Imaging Evaluation of Alpha-Ketoglutarate as Hepatoprotectant agent using ^{99m} Technetium - Mebrofenin Probe

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ABSTRACT:

Background: Alpha-ketoglutarate (AKG), an important Krebs cycle intermediate, is involved in energy metabolism. This study evaluates possible therapeutic effect of oral AKG supplementation in ameliorating xenobiotic induced hepatic dysfunction using cellular, biochemical, histopathological and *in vivo* functional imaging approaches. Carbon tetrachloride (CCl_4), a well-established experimental model for inducing liver injury was chosen as it causes toxicity by both lipid peroxidation dependent and independent pathways. ***Materials and method:*** *In vitro* effect of AKG on CCl_4 (1%) toxicity was studied in Human HepG2 cells. Cell viability was assessed using MTT assay, while changes in oxidative stress were evaluated by measuring lipid peroxidation, glutathione and LDH leakage. Comet assay was performed to evaluate any DNA damage. In subsequent study, Sprague Dawley rats were divided into three groups of 6 animals each. Group-I served as control. Group-II were administered single intraperitoneal injection of CCl_4 3ml/kg (1:1) in olive oil. Group-III received CCl_4 as in group-II followed by oral AKG @ 2gm/kg bw for one week. Serum liver enzymes, antioxidant status and histological analysis were measured. Finally, *in vivo* hepatobiliary study was also performed under gamma camera using ^{99m}Tc-Mebrofenin to determine real time functional status of rats' livers. Multiple parameters concerning hepatic mebrofenin uptake and excretion, including T_{peak} and $T_{1/2\ peak}$ were determined. ***Results*:** CCl_4 treated HepG2 cells showed cell viability of 32±2%, while AKG supplementation (10mM) improved cell viability to 69±5%. Comet assay confirmed that AKG reduced CCl_4 induced cyto-genotoxicity in HepG2 cells. There was reversal in CCl_4 induced derangement in activities of serum liver enzymes and antioxidant status ($p<0.05$) upon AKG supplementation. In functional imaging study, multiple parameters concerning hepatic mebrofenin uptake and excretion were evaluated, which showed that hepatic retention and fractional biliary excretion were significantly abnormal in CCl_4 group (T_{peak}: 3.8min ±52sec; $T_{1/2\ peak}$: 12.1min± 43sec) as compared to AKG supplemented group (T_{peak}: 2.56min±48sec; $T_{1/2peak}$: 7.53min± 23sec). Hepatic extraction fraction (HEF) was 72.18±1.8%, 41.78±4.41% and 67.98±3.86% in control, CCl_4 exposed and AKG supplemented rats respectively. ***Conclusion:*** Functional imaging study parameters were correlative to biochemical and histopathological findings. Results strongly suggest possible therapeutic role of AKG in protecting liver damage by potential hepatotoxins.

Keywords: Alpha-ketoglutarate; hepatotoxicity; oxidative stress; ^{99m}Tc-mebrofenin; functional imaging

[I] INTRODUCTION

There is enormous growing awareness of nutraceuticals as potent therapeutic supplement for hepatocellular disorders. Incidences of liver diseases are on rise, despite major scientific advances much remains desired from prescription drugs in their ability to offer protection against xenobiotic induced liver injuries, ability to stimulate liver function and/or help regenerate liver cells [1]. With significant research in the field of nutraceuticals, experimental findings suggest that these bioactive molecules not only enable cellular growth, proliferation and differentiation but also

modulate homeostasis to help cell survive various patho-physiological stressors [2]. Nutraceuticals thus have the ability to decrease disease progression, improve quality of life and prolong survival. In essence, the major theme of this paper is to evaluate the potential of Alpha-Ketoglutarate (AKG) for its hepatoprotective efficacy. The present study utilizes, apart from conventional biochemical and histopathological parameters, ^{99m}Technitium Mebrofenin Hepatobiliary imaging, for its ability to assess intervention outcome at functional level in-vivo. Alpha-Ketoglutarate (AKG), a citric acid cycle intermediary metabolite and precursor to non-essential "Alpha Ketoglutarate family" amino acids (glutamate, glutamine, proline and arginine) has been marketed as nutritional supplement.

It has also been widely used through oral and parental route in burns, surgically and chronically malnutritioned patients [3]. Studies have elucidated that in response to oxidative stress, citric acid cycle enzymes are modulated to diminish production of pro-oxidants NADH and $FADH_2$ with intracellular accumulation of AKG. AKG detoxifies H_2O2 and O_2^- (Reactive oxygen species) with concomitant formation of succinate, which in turns targets and restores cellular homeostasis and helps cell cope oxidative stress [4].

Thus endogenous AKG is not just a linking metabolite between carbohydrate and protein metabolism but also a key participant of cellular oxidative defense machinery [5] & [6]. *In vitro* and *in vivo* studies have also shown that exogenously administered AKG has the potential to protect cell from oxidative stress by improving redox homeostasis. AKG may contribute to redox state stabilization by various pathways; 1) it participates in quantitative and rapid non-enzymatic (NADPH independent) peroxidative decarboxylation [7]; 2) AKG acts as a precursor to glutamine which in turn is a precursor to GSH, a natural intra- and extracellular anti-oxidant and essential for activity of other GSH dependent antioxidant enzymes [3]; 3) AKG due to its chemical structure (alpha- keto-carboxyl group) acts as a ubiquitous collector of amino ($-NH_2$ group), detoxifies ammonia, and prevents lipid peroxidation and generation of free radicals [8]; 4) By formation of carnitine [9] and integration with malate-aspartate shuttle, AKG results in normalization of fat metabolism and causes transfer of reducing equivalent (NADH) to mitochondria with eventual generation of ATP and as a results offer protection against lipid peroxidation [10]. These experimental findings suggest that exogenous AKG may have the potential to prevent various oxidative stress induced diseased liver states.

In present study, AKG is assessed, *in vitro* and *in vivo*, in one of the most frequently applied and well validated CCl_4 induced experimental model of hepatotoxicity. CCl_4 is metabolized by mixed function oxidases CYP (2E1, 2B1, 2B2) to form highly reactive radical trichloromethyl (CCl_3). CCl_3* initiates two distinct pathways of cellular damage 1) halo alkylation of crucial cellular molecules such as nucleic acid, protein, lipids or/and 2) CCl_3* gets oxidized to form trichloromethyl peroxy radical (CCl_3*COO) causing lipid peroxidation, generation of reactive aldehyde and oxygen species, alteration of reduced or oxidized glutathione and derangement in calcium homeostasis.Apart from hepatocytes, non-parenchymal (stellate, kupffer, macrophages) cells of liver release cytokines (TNF-α, IL-6, IL-10, TGF-α/β) in response to CCl_4 intoxication. Thus, CCl_4 toxicity is a multifactorial process with several possible pathogenic pathways, with haloalkylation and lipid peroxidation being the predominant one. Intervention with agents having anti-oxidant, mitogenic, calcium homeostasis maintaining, nucleic acid methylation restoration properties are likely to alleviate CCl_4 induced hepatotoxicity.

Conventionally, evaluation of any test intervention in hepatotoxic experimental models is assessed on parameters which reflect hepatocellular injury (serum transaminases level, histopathology), synthetic activity (serum albumin, prothrombin time) and excretory function (serum bilirubin). As these parameters are static and analytic in nature,

they as such are of limited value to assess real time functional capacity/reserve of liver.

Hepatobilliary scintigraphy (HBS) is a radionuclide imaging modality utilized for evaluation of hepatocellular function and patency of biliary system. Quantification of functional capacity of liver using HBS has been successfully performed in rats previously [11]. Earlier studies have reported significant co-relation between HBS with biochemical and histopathological parameters [12]. In our study parameters to assess physiological functioning of hepatocytes in-vivo were obtained using radio-tracer ^{99m}Tc-Mebrofenin (^{99m}Tc-MEB). This radiotracer has high specificity for hepatocytes (98%), does not undergo metabolism or conjugation, is actively excreted from hepatocytes and shows rapid hepatobiliary transit with no enteric absorption [13].^{99m}Tc MEB measures both uptake and excretory function of liver. Uptake takes place along basolateral membrane of hepatocytes, with no specific receptors, identifiably through salt and organic anion transporter (OATP-1&2).

Excretion along canicular membrane occurs through multi drug resistance protein (MRP 1&3). In acute liver injuries production of various cytokines (IL-6 and TNF-α) are upregulated. These pro-inflammatory mediators are known to cause downregulation of transporters and thereby affect the hepatic ^{99m}Tc-MEB handling [14]. We have assessed hepatic MEB handling by measuring 1) Hepatic extraction fraction (HEF), for quantification of uptake; signifying viable parenchymal liver cell mass, 2) Time to half of peak activity ($T_{1/2\ peak}$), for quantification of excretion; signifying hepatonecro-inflammatory activity. The present study therefore utilizes both structural and functional outcomes parameters for assessing AKG as a hepato-protectant.

[II] MATERIALS AND METHODS

2.1. Chemicals and Reagents

Alpha-ketoglutarate (AKG) was procured from (Fluka Chemika, Buchs, Switzerland), Carbon tetrachloride (CCl_4) (Merck, Mumbai, India). HepG2 cells were obtained from National Center for Cell Sciences, Pune, India. Assay kits for detection of Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Albumin and Total bilirubin (Biovision, India), Lipid peroxidation, Thiobarbituric acid reactive substances (TBARS), Superoxide Dimutase (SOD), CAT (Catalase), Glutathione peroxidase (GPX) and reduced Glutathione (GSH) assay were procured from (Calibiochem, Merck, India) and the kit for Lactate Dehyrogenase (LDH) assay was procured from (Abcam, India). All other chemicals were of analytical grade.

2.2. In vitro studies

2.2.1 Maintenance of HepG2 cells

The human hepato-cellular carcinoma cell line, HepG2, was procured from National Centre for Cell Sciences (Pune, India). Cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% Penicillin (100 U/ml) and Streptomycin (50 U/ml). Cultures were maintained at 37ºC supplied with 5% CO2 and 95% air in a humidified incubator (Thermo Scientific Steri-cycle, India). Sub-culturing was done periodically using 0.25% Trypsin-EDTA solution after every three days.

2.2.2 Cell viability and LDH leakage, MDA assay, GSH assay and GPX Assay

Cell viability was observed using tetrazolium dye colorimetric test (MTT assay) [15]. HepG2 cells were collected and seeded in 96-well plates at a density of $5x10^3$ cells/well, cultured in 96 well plates for 24 h. For cytotoxicity evaluation, the cells were incubated with variable doses of AKG (1mM-150mM) for 24 h.

For cytoprotective activity, HepG2 cells (5×10^3 cells per well) were maintained in culture media containing 1% CCl_4 (6 h) in the presence or absence of optimized dose of AKG (24 h). At the end of the drug treatment period, to each well 20µL of MTT solution (5mg/mL) was added and incubated at 37°C for 3 hours. The culture medium in each well was then replaced with 100 µL of DMSO and the purple formazon crystals were dissolved by mild shaking. The absorbance of the solution was measured at 570 nm

spectrophotometrically (Bio-Quant, USA) and the percentage cell viability was calculated.

2.2.3 Measurement of LDH leakage, MDA assay, GSH assay and GPX Assay in HepG2 cells

The HepG2 cells after the treatment with CCl_4 and AKG were harvested and sonicated with buffer to obtain cell homogenate. The leakage of LDH leakage, MDA assay, GSH assay and GPX Assay was estimated in accordance with the manufacturer's protocol.

2.2.4 Morphological analysis: HepG2 cells (5×10^3 cells per well of a 6-well plate) were maintained in culture media containing 1% CCl_4 (6 hrs) in the presence or absence of AKG (24 hrs). At the end of incubation period, cells were fixed in 4% paraformaldehyde for 10 min, mounted in glycerin and examined under inverted photomicroscope (Olympus CKX41, Japan) at 100X magnification.

2.2.5 Comet assay

The cells were treated as above. At the end of incubation the cells were harvested and used for comet assay analysis. The comet assay was performed by following the protocol of Singh et al 1990; [16]. The assay is based on the principle that in an electric field small DNA molecules migrates faster than large DNA molecules and the damaged and fragmented DNA moves faster and appears like a 'comet tail', while the undamaged DNA as 'Head' in the electrophoresis unit. The extent of DNA migration depends directly on the DNA damage present in the cells [17]. SYBR green dye was used to stain the DNA. DNA damage was expressed as Olive tail moment. The tail moment was given as the % of DNA in tail multiplied by the length between the center of the head and tail [18]. Results were given as mean±SEM.

2.3 In vivo experiments

2.3.1 Experimental animals

Male Sprague Dawley rats (weighing 200 to 250 g) were obtained from the Experimental Animal Facility, INMAS, DRDO, Delhi and housed in polypropylene cages and provided food (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee of the Institute (INM/IAEC/2009/06/009) duly constituted for the purpose. After one week of acclimatization, animals were divided into three groups (n=6). The control animals were given olive oil as vehicle. The liver toxicity was induced by injecting a single intraperitoneal injection of CCl_4 3ml/kg (1:1) in olive oil (selected on the basis of previous works reported). To the last group, AKG (2gm/kg bodyweight) by oral gavage was given for one week along with CCl_4 injection. All other experimental conditions were identical with a 12/12 h light/dark cycle at 21±2°C. At the end of dosing schedule, the animals were subjected to gamma scintigraphy assisted functional imaging study. At the end of functional imaging study, the animals were sacrificed and blood samples were collected for subsequent biochemical estimations. Portion of the liver tissue was stored in formaldehyde solution for histopathological examination and in liquid nitrogen determining antioxidant status.

2.4 Hepatic Functional Imaging

2.4.1 Radioligand

^{99m}Tc-N-(-3-bromo-2,4,6 trimethylacetanitride) Immunodiacetic acid (Mebrofenin) is a commonly used hepatic imaging radiopharmaceutical and was therefore selected for the study.

2.4.2 Gamma Camera Design

For ^{99m}Technetium-Mebrofenin Hepatobiliary Scintigraphy (^{99m}Tc-MEB-HBS) in rats, a dual head gamma camera (Symbia, T2, SPECT/CT Siemens) dedicated for research facility and equipped with low energy, high resolution, parallel-hole collimator interfaced to a computer was used. A mechanical support was designed in which animal could be fixed for standardizing orientation and position of animal for image acquisition.

*2.4.3 Image Acquisition Parameters***:**

Scintigraphy was performed after an overnight fast to minimize the effect of food on hepatic blood and bile flow. Animals were sedated with ketamine/Xylazine (50mg/kg & 5mg/kg respectively). Once sedated a bolus of 40 MBq (in 0.3ml of saline) of ^{99m}Technetium-Mebrofenin

(BRIT, Mumbai, India) was injected intravenously into rat tail. Images were acquired immediately upon injection. Protocol for dynamic acquisition of images was adopted to accommodate faster metabolic rates in rats, as had been used in previous studies. Anterior images were acquired for 30 min (5 sec/frame for 10 min & 60 sec/frame for 20 min). A 15% window centered on 140KeV ^{99m}Tc peak in a 128×128 matrix at a zoom factor of 2 was used.

2.4.4 Image analysis

Scintigraphic data were processed on a Pegasys workstation (ADAC laboratory). Region of interest (ROI) were drawn around liver (organ of interest), heart and large mediastinum vessels and (Blood pool) and over total field of view (total activity). ROI were drawn on threshold based algorithm, with 20% of maximum liver value in summed image of first 2.5 min. Hepatic Extraction Fraction (HEF), a parameter to assess hepatic uptake were calculated based to protocol described earlier and modified for use of ^{99m}Tc-MEB-HEF [19]. HEF is expressed as activity accumulated in liver at percentage of total activity injected per unit time over a specified duration of time. This specified duration of time in which these calculations are made should represent a phase of homogenous distribution of ^{99m}Tc-MEB in blood and before rapid phase of hepatic excretion [20]. This provides a true quantification of uptake because it takes into account the fluxes between systemic and hepatic blood pool and hepatocyte uptake. In present study this duration of time was between 30 sec to 120 sec post injection, as it is before the average T_{peak} of normal values. Along with HEF, T_{peak}-time to reach maximum hepatic uptake and $T_{1/2\ peak}$-time for hepatic uptake to reduce by 50%, were calculated. For this a second liver ROI was drawn excluding large bile ducts and superimposing bowel loops; from which time-activity curve were generated and T_{peak} and $T_{1/2\ peak}$ were calculated [21].

2.5 Measurement of conventional liver function markers and effect on antioxidant status

Intracellular enzymes - Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Total bilirubin and Albumin were determined by using semi-automatic biochemical analyzer (Krish Bio medicals, Delhi) using commercially available kits. The frozen liver tissue was thawed homogenate of liver was prepared in ice cold saline by using tissue homogenizer (IKA T25 Digital Ultra Turrax). The homogenate was centrifuged in cooling centrifuge to remove the debris and supernatant was used for enzyme assay. Lipid peroxidation and activities of antioxidant enzymes, viz., Catalase (CAT), Superoxide dismutase (SOD), Total reduced Glutathione (GSH) and Glutathione peroxidase were measured using commercially available kits and in according with manufacturer's instructions.

2.6 Histopathological analysis

Tissues were processed by standard histopathological procedure. 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 light microscope (Olympus BX 60) and photographed.

2.7 Statistical analysis

Commercial computer packages were used for data analysis (GraphPad prism version 5, San Diego, California, USA and Microsoft Excel version 15 (Microsoft Corporation, New York, USA). All data are presented as mean ± SEM. All statistical tests were two tailed and difference were evaluated at the 5% level of significance.

[III] RESULTS

3.1. *In vitro studies*

3.1.1: Cytoprotective potential of AKG in CCl_4 treated HepG2 Cells

Cytoprotective assessment of AKG at the dose levels 1mM-150mM was done to establish the safety and optimum dose**.** AKG exhibited maximum cell viability at 5mM-10mM conc. (157-

158%). Even at higher doses AKG did not exhibit any significant cytotoxicity. HepG2 cells exposed to CCl_4 (1%) showed a decreased percentage of cells viability (32%) as compared to normal control, indicating injury to HepG2 cells. The results showed that AKG (10mM) supplementation in CCl_4 treated cells resulted in an increased cell proliferation as evidenced by an increase in cell viability (69 %).

***3**.1.2: Effect of AKG on LDH leakage, MDA assay and GSH assay and GP_X in HepG2 cells*

The formation of MDA content in the cells is measured by thiobarbituric acid reactive substances (TBARS), the final metabolites of peroxidized polyunsaturated fatty acids, have been used as a marker of lipid peroxidation. In comparison to control group, the treatment of HepG2 with CCl_4 led to more MDA formation indicating the oxidation of lipids. The MDA content in the CCl_4 group (1.85±0.15 mmol/L/g proteins, $p<0.01$) is much higher than the control group (0.53±0.12 mmol/L/g proteins). It could be seen that the AKG treatment (0.78±0.10 mmol/L/g proteins) prevented the MDA formation. The GSH content in the cells serves as the index of redox status of cells. The treatment to CCl_4 significantly decreased the GSH content in the cells (20±8.1, $p<0.05$) indicating oxidative injury.

The treatment with AKG (45±4.9) significantly provided cytoprotective effect against this depletion, comparing to the CCl_4 group which are nearly close to the control cells (55±6.3). The CCl_4 exposed cells showed more (34±4.2, $p<0.05$) LDH leakage into the culture medium as compared the control group (4.06±0.7). The treatment of AKG (7±1.6) significantly diminished the LDH leakage in the culture media. The CCl_4 exposed cells showed significantly higher level of GPx activity (191±7.87, $p<0.05$) as compared to control group (87±2.16). The AKG treatment had induced markedly reduction in the enzyme activities of GP_X (119±5.35). The results clearly showed the AKG exhibits antioxidants properties and had shown its protective effect against CCl_4 induced lipid peroxidation, GSH depletion and leakage of LDH and GP_X activities (Fig 1).

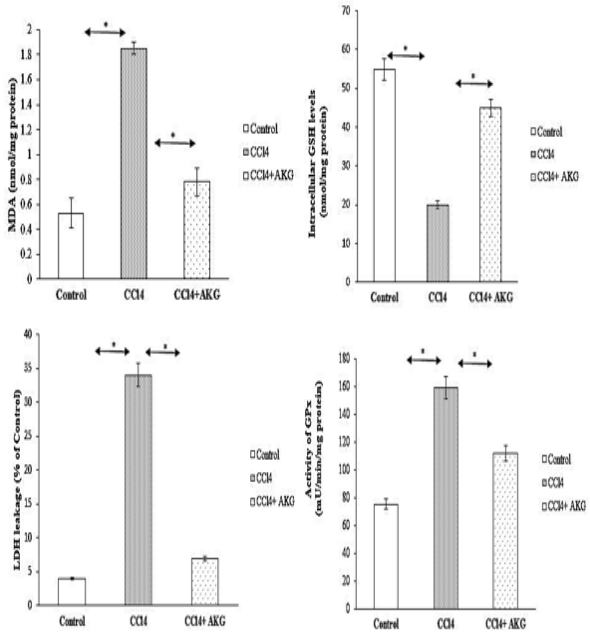

Fig 1: The effect of AKG on MDA, GSH assay, LDH leakage and GPX activities on HepG2 cells administered with CCl_4.

Values are given as mean± SEM, Average of 3 replicates. MDA (Malonaldehyde) is expressed as mmol/L MDA/g proteins, GSH (reduced glutathione) is expressed as mg GSH/g proteins and LDH (Lactate dehydrogensase) is expressed as the ratio of decrease in absorbance per min in test group to the ratio of control group, GPX (Glutathione peroxidase) is expressed as mU GPX/mg protein. *$p<0.05$ when compared with control and AKG treatment.

3.1.3: Effect of AKG on Morphology of HepG2 Cells

After the drug treatment, micro-photographs of the cells were taken. HepG2 cells usually grow in colonies and are adherent to the substrate. Their normal morphology includes indistinct cell boundaries and generally display epithelial cell-like morphology. Exposure to 1% CCl_4 disturbed cell morphology, conversion of inherent shape to round shape indicating swelling, asymmetrical plasma membrane, decrease in adherent property and cells had secreted debris and formed blebs. AKG treatment significantly ameliorated the morphological changes induced by CCl_4 exposure. The results have been depicted in Fig 2.

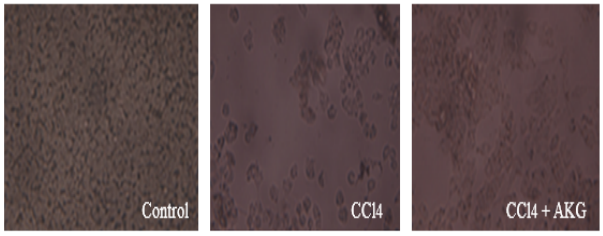

Figure 2: Effect of AKG in ameliorating the CCl_4 induced toxicity on morphological characteristics of HepG2 cells. The cells were photographed under inverted light microscopy (magnification, 100×)

3.1.4: Comet Assay

The level of DNA damage was scored by comet assay analysis. Succeeding single-cell electrophoresis, lengths of the comets (Olive Tail Moments, (OTM) depend on toxicant's exposure, with longer tails indicating more DNA damage. Figure 4 shows representative comet assay pictographs after CCl_4 exposure with and without AKG supplementation. Untreated control cells showed no tails (1.2 OTM±0.21 SEM). OTM were evident after CCl_4 exposure (13.8 OTM±1.14

SEM), however, cells incubated with AKG (6.02 OTM±1.1 SEM) showed substantially smaller tail compared to CCl_4 exposed cells as shown in Fig 3.

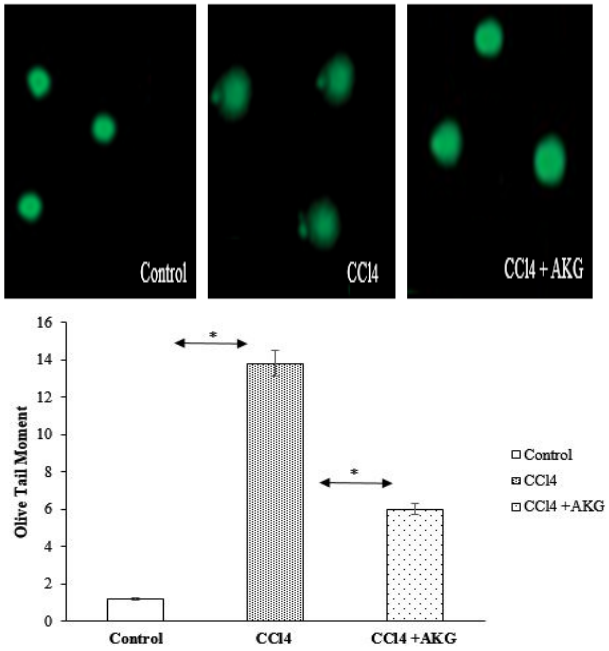

Figure 3: Comet assay analysis for evaluating the genotoxicity of CCl_4 and the ameliorative effect of AKG treatment on CCl_4 induced DNA damage.

3.2 In vivo experiments

3.2.1: Effect of AKG on CCl_4 Induced Liver Injury in Rats

AKG administration showed a protective effect against CCl_4 induced liver injury (Table 1). There was a remarkable increase in ALT, AST and ALP levels in CCl_4 exposed group while Albumin and bilirubin levels were not significantly altered. Oral administration of AKG attenuated the elevation in enzymatic levels, suggesting protective effect of AKG.

[Table-1]. Antioxidant activity of AKG on CCl_4 induced liver damage in rats

Parameters	Control	CCl_4	CCl_4+AKG
ALT (U/L)	48±5.08	133.5±6.10*	70±3.9**
AST(U/L)	62±3.74	276±14.8*	71.3±5.02**
ALP (U/L)	76.6±4.67	107±3.5*	85±2.5**
Albumin gm/dl)	3.7±0.32	2± 0.14	2.73±0.63
Bilirubin (mg/dl)	0.8±0.104	1±0.17	0.6±.012

Table-1: Data are expressed as Mean ±SD; n=6, *significantly different from control group, **p<0.05, when compared with CCl_4 group

3.2.2: Effect of AKG on Hepatic Antioxidant Levels

Table 2 represents the results confirming the hepatoprotective role of AKG in terms of maintaining the hepatic antioxidant levels. Levels of liver MDA, a product of lipid peroxidation, showed increment in CCl_4 exposed group. Treatment with AKG reversed the alterations. Moreover, in CCl_4 exposed group there was a significant reduction of hepatic SOD, GSH and Catalase activity as compared to control animals. AKG supplementation resulted in significant elevation in the GSH and Catalase activities as compared to the CCl_4 group.

[Table-2]. Antioxidant activity of AKG on CCl_4 induced liver damage in rats

Parameters	Control	CCl_4	CCl_4+AKG
LPO	4.55±0.25	13.7±0.78*,**	6.43±0.430$^{**,\#}$
SOD	83.3±2.35	42.2±1.73*,**	74.16±2.6$^{\#,\#\#}$
CAT	166±44.5	51.1±2.11*,**	160.1±6.36$^{**,\#}$
GP_X	323.6±6.10	194.18±4.2*,**	285.6±4.3$^{**,\#}$

Table: 2. Data are expressed as Mean ±SD; n=6 , *Control compared with CCl_4 , **p<0.001, $^{\#}$ CCl_4 treated group compared with AKG, $^{\#\#}$p<0.01, LPO (nmol of MDA/mg protein, SOD (Kat/g protein), CAT (nmol of

H_2O_2 decomposed/min/mg protein), GP_X (nmol of GSH oxidized/min/mg protein)

3.2.3: Histopathology Analysis of Liver Tissues

The histopathological examination showed that exposure to CCl_4 caused increased fatty degeneration, cytoplasmic vacuolization, typical centribular hepatocytic steatosis (both macrovesicular and microvesicular) and coagulative necrosis, especially in the periportal hepatocytes. AKG supplementation resulted in almost normal histological profile of hepatocytes with mild congestion, can be seen in Fig 4.

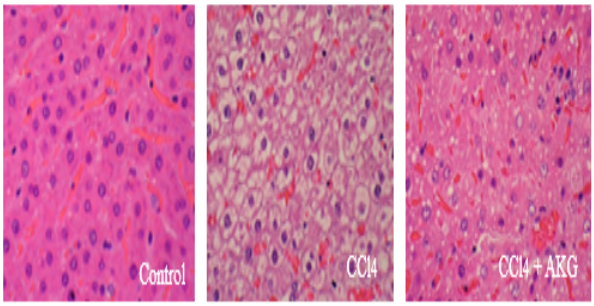

Figure 4: A: Control liver showing normal histological appearances; B: CCl_4 treated rats showing coagulative necrosis and formation of vacuoles; C: AKG treatment group rats showing normal histological profile of hepatocytes. (Magnification 400X)

3.2.4 In vivo functional imaging study using ^{99m}Tc-Mebrofenin (^{99m}Tc-MEB)

In control group rats, ^{99m}Tc-mebrofenin showed prompt uptake, homogenous and efficient tracer accumulation with rapid tracer clearance from the liver. For 40MBq of ^{99m}Tc-MEB injected, Hepatic Extraction Factor (HEF) stood at (72.18±1.80) with T_{peak} (2.41 min±40 sec) and $T_{1/2\ peak}$ (6.87±46 sec) in control group. In contrast, CCl_4 treatment group rats, ^{99m}Tc-mebrofenin did not show significantly delayed tracer uptake T_{peak} (3.8min ±52 sec; p not significant versus normal control), liver tracer clearance, though was markedly delayed in CCl_4 treated rats $T_{1/2\ peak}$ (12.1 min± 43 sec; p significant versus normal control) and showed less efficient tracer accumulation; HEF (41.78±4.41). In comparison, AKG intervention in CCl_4 group, significantly reverted back the deranged parameters. HEF stood at (67.98±3.86) with T_{peak} (2.56 min±48 sec) and $T_{1/2\ peak}$ (7.53min± 23 sec) for the AKG intervention group (Table3, Figure 5).

[Table-3]. Hepatic Mebrofenin Handling

^{99m}Tc-Mebrofenin	Control	CCl_4	CCl_4+AKG
(HEF %)	72.18±1.80	41.78±4.41*	67.98±3.86**
T_{peak} (min)	2.41±40	3.8 ±52	2.56 ±48**
$T_{1/2\ peak}$ (min)	6.87±46	12.1± 43 *	7.53 ± 23**

Table 3: Data are expressed as Mean ±SD; n=6, *significantly different from control group, **p<0.05, when compared with CCl_4 group

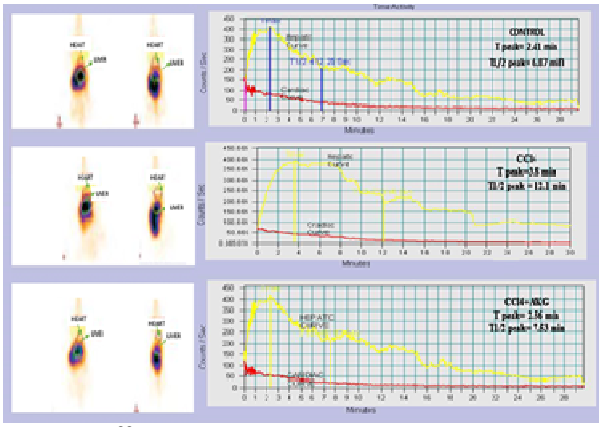

Figure 5: ^{99m}Tc Mebrofenin functional imaging uptake (T_{peak}) and clearance ($T_{1/2\ peak}$) of representative rat each from control, CCl_4 and AKG treatment. Control shows prompt mebrofenin activity clearance from liver. CCl_4 group shows considerably delayed mebrofenin activity clearance from liver. CCl_4 + AKG group shows significant improvement in mebrofenin activity clearance from liver as compared to CCl_4 group.

[IV] DISCUSSION

Essence of the current study is to accumulate some experimental support of therapeutic effect of AKG, for its, cytoprotective and hepatoprotective potential. We have assessed both *in-vitro* and *in-vivo* model using CCl_4 induced liver injury in HepG2 cells and Sprague dawley rats respectively. ^{99m}Tc Mebrofenin functional imaging of liver was carried to assess therapeutic effect at physiological level of liver in-vivo to negate the role of various cofounding factor that have significant role in vivo. Human hepatoblastoma derived cell line (HepG2) exhibit most of the cellular features of normal

human hepatocytes making them preferential model for studying xenobiotic induced liver toxicities [22]. In present study firstly we investigated the effect of AKG on cellular proliferation is attributed to the fact that HepG2 cell lines were seeded in media containing AKG and compared to controls (HepG2 cells seeded in AKG devoid media). Cells seeded on 10mM of AKG containing media (out of studied range 1- 150mM of AKG) showed a maximal increment of above 150% on MTT assay when compared to the control (Fig 1). In another investigation on effects of exogenous AKG on cellular growth and proliferation, AKG was observed to have caused metabolic activation and thus enhanced proliferation of cultured fibroblasts and chondrocytes [23]. AKG mediated cellular proliferation can be attributed to the fact that AKG, a citric acid cycle intermediate serves as a source of energy for the cells [24], removes toxic metabolites (eg ammonia) from the media in which cell as are being cultured [25] deaminates/transaminates ammonia released into media of cultured HepG2 cells to glutamate/glutamine [26], which then undergoes aerobic oxidation generating cellular energy with resultant lesser glucose consumption and reduced oxidative stress [27]. With experimental findings suggesting that AKG has cellular proliferative effect, its anti-oxidants and cyto-geno-protective effects were assessed in CCl_4 induced HepG2 cell toxicity model.

CCl_4 is catabolised to reactive metabolites, trichloromethyl (CCl3) and trichloromethyl peroxy ($^{\cdot}$OOCCl3) free radicals, which induces lipid peroxidation and thereby damages cellular and oraganelle lipid membranes causing swelling and necrosis of cells with release of cystolic enzymes as AST,ALT and LDH, making it highly useful experimental model to investigate anti-oxidant mediated cyto-protection [28].

In present study incubation of HepG2 cells with CCl_4 resulted in significant lipid peroxidation (elevated TBARS), reduced anti-oxidant enzymes (depleted peroxidases GP_X), glutathione (GSH) depletion and DNA damage (Fig 1,3). GSH depletion makes cell more vulnerable to reactive oxygen species (ROS), free radicals and reactive metabolites resulting in deranged calcium homeostasis ultimately causing cell membrane and disruption and cytotoxicity [29]. CCl_4 induced cytotoxicity in HepG2 cells, in current study were assessed on parameters MTT assay, cytosolic enzymes in medium, microscopic observation; showed reduced cell viability, LDH leakage in media, deranged cellular morphology respectively. In present study when AKG was simultaneously supplemented to CCl_4 exposed HepG2 cells, it significantly improved the endpoints of assessed parameters. The presented results showed that AKG supplementation improved redox homeostasis (diminished TBARS redox markers, elevated peroxidases GP_X, higher GSH concentration) and demonstrated cyto-protection (raised cellular viability on MTT assay, reduced LDH level in medium and alleviation of deranged cellular morphology) (Fig 2). Comet assay showed that AKG reduced CCl_4 induced genotoxicity. Olive tail moment is used as a parameters to reflect DNA damage remained same as in control cells (Fig 3). The in-vitro investigation using HepG2 cells, demonstrated that AKG prevents CCl_4 induced oxidative stress and cyto-geno-toxicity. In this model, because end points are assessed in co-incubation of AKG with toxicant, AKG mediated effects reflects the direct anti-oxidant and enzyme modulatory action of AKG [30]. It is likely that AKG administration caused higher concentration of reduced glutathione (GSH), as AKG is a glutamine precursor for which GSH is formed [31]. AKG positively modulates the activity of peroxidases (GPx), antioxidant enzymes, which is presence of GSH scavenges, hydrogen peroxide and lipid peroxides into water and oxidised glutathione [32].

To assess if proliferative/ anti-oxidative properties of AKG as assessed in-vitro can be translated in physiological milieu and whether AKG can offer hepato-protection, AKG was studied in a CCl_4 induced hepatotoxic invivo model. CCl_4 induced hepatotoxicity is a good model to study hepatic injury because elementary lesions and pathogenic

pathways in vivo replicate those seen in most liver diseases [33]. Administration of CCl_4 is known to cause free radical mediated oxidative stress and induce tissue injury. Oxidative stress has been recognised to be the involved etiology of various chronic diseases including liver diseases [34].

A co-ordinated action of cascade of anti-oxidants is critical for neutralisation of free radicals. The potential cascade of anti-oxidant in hepatocytes includes, SOD, CAT, GSH and GP_X. SOD detoxify highly reactive superoxides to hydrogen peroxide, which is turns gets neutralise by CAT and GP_X into H_2O. GSH is a potent inter/intra-cellular antioxidant compound. Also CAT and GPX requires the presence of reduced glutathione (GSH) for their activity [35]. In present study administration of CCl_4 caused significantly reduced activities of anti-oxidant enzyme SOD, CAT, GP_X and GSH (Table 2). Previous studies have also reported that CCl_4 reduces activities of anti-oxidant enzymes and causes hepatotoxicity [36]. On other hand low administration of AKG prevented toxic effects of CCl_4 by restoring anti-oxidant enzymes and glutathione. Anti-oxidant properties of AKG can be attributed to its ability to participate in non-enzymatic (NADPH independent) oxidative decarboxylation of H_2O_2 and its ability to acts as energy level substrate metabolite [37]. AKG is precursor of glutathione, which in terms is a precursor to GSH, are natural anti-oxidant besides glutathione in itself is a mitochondrial substrate involve in protects cell from oxidative stress [31]. Also constitutive hyperammonia is a well-known complication of acute and chronic neural diseases. Ammonia is known to inhibit anti-oxidant enzymes by activating NMDA receptors on NO synthase, which in term enhances production of NO and other thiobarituric acid positive compounds resulting in oxidative stress, there are several other studies on generation of free radicals by ammonia [38]. AKG being natural ubiquitous collector of ammonia (NH2 group) detoxifies ammonia to glutamic acid. Whereas AKG ameliorated the liver injury by its potent anti-oxidant free radical scavenging properties, its cytoprotective role was further confirmed by assaying markers of structural and functional hepatocellular damage.

Generation of high concentration of free radicals may exceed the capacity of antioxidant defence mechanism. Free radicals induced lipid per-oxidation (a critical factor in hepatic injuries) of mitochondrial and cellular membrane resulting in loss of structural and functional hepatocyte integrity [36]. Lipid peroxidation of plasma membrane leads to malonaldehyde formation (MDA). Estimation of MDA level are based on its chemical reactivity with thiobarbituric acid (TBARS) [39]. Indicators for structural integrity assessed in present study are serum levels of hepatocellular cytosolic enzymes (ALT, AST and ALP), functional integrity was assessed by hepatic metabolism (levels of serum total bilirubin) and hepatic biosynthesis by (levels of serum albumin). CCl_4 treatment showed significant increase in activities of hepatocellular cytosolic enzymes in serum, raised total Bilirubin and reduced levels of albumin indicating CCl_4 induced hepatotoxicty secondary to lipid peroxidation (raised TBARS) [40]. Co-treatment with AKG had significantly reduced TBARS and normalised the deranged assessed parameters. This is in agreement with accepted view that restoration of these parameters indicate stabilization of plasma membrane and regeneration of liver hepatocytes. It is likely that the cyto-protective effect of AKG might be due to ability of AKG to undergo very rapid and highly quantitative and per-oxidative decarboxylation owing to alpha keto carboxyl group. Thus AKG is able to protect CCl_4 induced hepato-toxicity by its intrinsic ameliorative properties. Beneficial effects of AKG are further confirmed by histopathological studies. It supported the results noted in serum analysis and provided direct evidence of the possibility of AKG being able to minimize disruption of structure of hepatocytes and accelerate hepatic regeneration.

Liver function was assessed in-vivo using ^{99m}Tc Mebrofenin Hepatobiliary Scintigraphy (^{99m}Tc-HBS). Functional evaluation of liver involves assessing the vital physiological processes of

hepatocytes viz. uptake, metabolism, conjugation and excretion. The two scintigraphic parameters that have great value in assessing these physiological process are; Hepatic Excretion Fraction (HEF) which represents uptake and measures first pass efficiency and Excretion half time ($T_{1/2\ peak}$) which represents intracellular transit and excretion. Well validated scintigraphic acquisition and interpretation protocol of ^{99m}-Tc MEB-HBS were followed to determine functional capacity of liver with and without intervention. There was little inter-subject variability and high reproducibility. In CCl_4 induced hepatotoxic rats, ^{99m}Tc MEB hepatic handling was markedly impaired (Table 3). Hepatic Excretion Fraction (HEF) was decreased with prolongation of time required for peak hepatic uptake (T_{peak}) and delayed excretion half time ($T_{1/2\ peak}$). These derangements in ^{99m}-Tc MEB handing were related closely to hepatic injury as reflected by morphologically apparent abnormalities in hepatocytes and increased serum transaminases. Reduction in hepatic extraction efficiency was secondly to loss of variable hepatocytes mass (Necrosis). $T_{1/2\ peak}$ was significantly delayed due to downregulation of excretory transporters by cytokines (IL-6 & TNF-α) released from activated kupffer cells (Hepato-necro inflammation) and impaired ATP generation caused by CCl_4 intoxication. AKG owing to its anti-oxidant and free radical scavenging properties, showed statistical significant improvement in these assessed scintigraphic parameters.

These findings and our result suggest that endogenous AKG would be useful in alleviating oxidative stress induced diseased liver states in general.

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