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GLYCINE PROPIONYL L-CARNITINE (GPLC) AMELIORATES D-GALACTOSAMINE (D-GALN) INDUCED OXIDATIVE STRESS IN RATS BY PREVENTING LIPID PEROXIDATION AND MAINTAINING THE REDOX POTENTIAL.

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ABSTRACT

Aim: Evaluation of the role of Glycine Propionyl L-Carnitine (GPLC) in ameliorating D-Galactosamine (D-GalN) induced oxidative stress in male wistar rats. Methods: Our current study included the treatment of male wistar rats with GPLC and D-GalN. Animals were given a pretreatment of GPLC for two months at a dose of 35mg/kgBW/day after which a single dose of D-GalN (700 mg/kgBW) was administered (ip). Another group was administered with D-GalN alone. Hepatic biochemical markers of oxidative stress (ALP,AST,ALT, γ- GT ,GSH,MDA,SOD,CAT and TNF-α) were monitored after the administration of D-GalN at 0 hr, 24 hr and 48 hr in all the groups,. Results: ALP, AST, ALT and γ- GT levels in control group were measured to be 70.51 ± 1.31, 66.83 ± 1.93, 72.65 ± 1.92 and 8.90 ± 0.83 and when compared with D-GalN treated (alone) group gave $P < 0.001$. Similarly GSH, MDA, SOD, CAT and TNF-α pool in control group were measured to be 46.80 ± 0.98, 8.14 ± 0.54, 14.86 ± 0.85, 80.81 ± 1.30, 26.90 ± 0.86 and when compared with D-GalN treated group gave $P < 0.001$.On the other hand GPLC, when administered simultaneously with D-GalN, showed a significant protection against D-GalN induced damage as the values of biochemical markers when compared to the D-GalN group gave $P < 0.001$. Conclusion: GPLC is able to prevent the lipid peroxidation by ROS. Dietary supplement of GPLC could prove to be a better neutraceutical having antioxidant role against hepatotoxicants. Moreover it maintains the redox potential inside a cell due to its radical scavenging action thereby maintaining a healthy state of an individual.

KEY WORDS : Oxidative stress, biochemical marker, lipid peroxidation, neutraceutical, hepatotoxicants.

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INTRODUCTION

Carnitine is a necessary compound required during beta oxidation of fatty acids. It is needed for the mobilisation of fatty acids from cytoplasm to the inside of mitochondrial matrix for energy production (1). Availability of carnitine in greater amounts in vivo will increase the utilization of fatty acids as a fuel source, thus leading to the improved lipid profiles. It has been reported that subjects, with known disease (e.g., CVD and/or dyslipidemia), using carnitine as a supplement have noted 3 favourable results in relation to blood lipids (2-10). A significant decrease in triglycerides has been observed in subjects taking carnitine. Besides possessing potential positive effects on blood lipids, carnitine has also been shown to possess antioxidant properties in both animals (11-15) and human (16-18). These effects seem to be due to a reduction in xanthine oxidase activity (13), a free-radical scavenging activity, and/or a regulation of metabolic reactions (14). Carnitine has been used as a dietary nutrient in both animals and humans with success. Recently it has been reported that propionyl ester of L-carnitine, propionyl-L-carnitine (PLC), increases blood nitric oxide production in patients with peripheral arterial disease (19). Propionyl-L-carnitine possesses protective effects against reactive oxygen species (ROS) induced oxidation as it has been reported that it has highest affinity for carnitine acetyltransferase (14). A combination of Glycine and PLC has recently been formulated called Glycine Propionyl L-Carnitine (GPLC) in which Glycine is combined with PLC in a unique molecular bonded form. Glycine propionyl L-carnitine Hydrochloride or GPLC is a propionyl ester of carnitine that includes an additional glycine component. It has been the focus of several research studies involving human subjects to evaluate its protective effect and it was observed that GPLC supplementation resulted in increased resting (20) and stress-induced plasma nitrate/nitrite—a surrogate marker of nitric oxide production (21), improved antioxidant defense, improved high intensity training performance, and a reduction in blood lactic acid in response to repeated high intensity exersion (22). There are reports indicating that glycine independently promotes positive effects on lipid peroxidation (23, 24), vasodilation (25) and blood lipids (26, 27). It is not yet evaluated whether or not the combination of glycine and PLC would enhance synergistic effects on these parameters. Therefore, the purpose of this investigation was to determine the efficacy of GPLC to decrease lipid peroxidation, prevent oxidative stress, and improve blood lipid profiles in wistar rats administered with a hepatotoxicant D-GalN. Two experiments were carried out to verify the effects of GPLC on animal models with and without liver injury. Liver injury was induced by D-Galactosamine (D-GalN), a hepatotoxicant frequently used as a model agent because it causes diffuse hepatic necrosis in rats, with mechanism of action closely resembling that of human viral hepatitis (28). Several parameters were monitored to evaluate the biological functions of GPLC and determine their mechanisms, including hepatic biochemical markers of oxidative stress.

METHODS

Reagents

GPLC was purchased from Shanghai Waseta Int'l Trading Co., Ltd., D-Galactosamine was purchased from SRL Mumbai, kits for the analysis of biochemical parameters were procured from SPAN diagnostics Gujarat, TNF-α ELISA kit was purchased from Biogenuex Ltd.

Animals and diet

30 male Wistar rats (250g of average body weight [BW]), obtained from Central Animal House Jamia Hamdard, were used in this study. Animals were ranked by initial body weight and assigned 5 groups of equal mean BWs (Fig-1). Animals were housed individually in a temperature-controlled environment with 12-hour light-dark cycle and were allowed free

access to standard laboratory food (Rat chow) and water ad libitum. The study was conducted under a project license approved by the Jamia Hamdard Animal Ethics Committee and followed institutional guidelines.

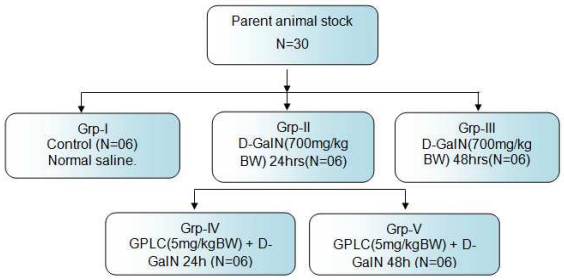

Figure 1
Grouping of animals.

Induction of liver damage

Animals were acclimatised for one week. Group-I served as control and was administered normal saline. Animals from Grp-II and III received a single dose of D-Galactosamine (ip) (700 mg/kg of BW). After 24 hrs of D-GalN administration, animals from Grp-II were sacrificed by cervical dislocation (decapitation) under proper anaesthesia and livers were dissected out, blotted, and weighed. Part of the liver was immediately immersed in 10% (wt/vol) buffered formalin fixative for subsequent embedding in paraffin. Another part was homogenised and used for biochemical analysis. Similarly after 48 hrs of D-GalN treatment animals were sacrificed from Grp-III and processed as mentioned earlier.

Pre-treatment with GPLC

GPLC was given as pre treatment in a dose of 35mg/day/kg BW as standardised. Animals from Grp-IV and V received GPLC for one month and a single dose of D-GalN (700mg/kg BW) was given on 31th day. After 24h and 48h of D-GalN administration, animals from Grp-IV and V were sacrificed by decapitation and processed as mentioned earlier.

Preparation of tissue homogenate

Liver tissue was quickly placed in ice cold normal saline, perfused with normal saline solution to remove blood cells, blotted on filter paper and frozen at -80°C. The frozen tissues were cut into small pieces and homogenized in cold PBS (pH7.4) (1ml/g of tissue), centrifuged at 4000 rpm for 15 min and temperature 4°C. The supernatant was pipette out and used for biochemical analysis.

AST, ALT, ALP and γ-GT assay

AST, ALT, ALP and γ-GT activities were determined by using commercially available reagent kits (SPAN diagnostics Ltd. Surat Guj.). AST, ALT and ALP were performed according to the method described (29, 30) and γ-GT was performed by the carboxy substrate method.The assays were performed according to the manufacturer's guidelines.

GSH and Malonaldehyde (MDA)

Total GSH and MDA were measured according to the method described earlier (31, 32).

SOD and CAT

SOD activity was measured as described previously (33). Briefly the assay mixture contained 2.87 ml of Tris HCl buffer (50mM, pH 8.5), 25 µl of pyrogallol (24 mM in 10mM HCl) and 100µl of PMS in a total volume of 3ml. The enzyme activity was measured at 420nm and expressed as Umg^{-1} protein. One unit of enzyme is defined as the enzyme activity that inhibits auto oxidation of pyrogallol by 50%.

Catalase activity was measured by the method as described (34). The reaction mixture consisted of 2ml phosphate buffer (0.1M, pH

7.4), 0.95 ml hydrogen peroxide (0.019M) and 0.05 ml PMS in a final volume of 3ml. Changes in absorbance were recorded at 240nm. Catalase activity was calculated as nmol H_2O_2 consumed min^{-1} mg^{-1} protein.

TNF-α

TNF-α concentrations were determined using ELISA kit {method as described previously (35)}, conducted according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was done by Tukey-Kramer test. Data is represented as mean and standard deviation for various sample groups. Significant difference $P<0.001$ was determined by analysis of variance (ANOVA) when value of 'q' is greater than 4.046.

RESULTS

Animals intoxicated with D-GalN showed considerable alteration in biochemical markers of oxidative stress. An elevation in level of ALT, AST, ALP and γ-GT was observed when compared with the control (Table-1). Pretreatment with GPLC conferred a significant protection against D-GalN induced damage. GPLC was able to strengthen the antioxidant defence system of the body. No significant elevation was observed when GPLC treated animals were administered with D-GalN. Elevation in lipid peroxidation was observed in animals intoxicated with D-GalN as the level of MDA showed a considerable increase in those animals which were treated with D-GalN. Elevation in the level of MDA showed a paralleled significant reduction in GSH (Glutathione) (Table-2). Pre-treatment with GPLC showed a significant protection when animals were administered with D-GalN. Glutathione dependent anti-peroxidative enzymes, SOD and CAT, showed a significant decrease in D-GalN intoxicated subjects as compared to the normal ones. Treatment with GPLC conferred protection against the damage by D-GalN (Table-3). TNF-α being a marker of apoptosis showed a significant increase in animals administered with D-GalN. Prior treatment with GPLC gave protection against the cell death induced by D-GalN as can be seen from the Table-4. GPLC was able to ameliorate the effect of D-GalN.

Table-1

Effect of GPLC and D-GalN on the activities of liver marker enzymes. P < 0.001, a = significant difference compared to control group, b = significant difference compared to D-GalN group.

S.No	AST(U/L)	ALT(U/L)	ALP(U/L)	γ-GT(U/L)
Control	70.51 ± 1.31	66.83 ± 1.93	72.65 ± 1.92	8.90 ± 0.83
D-GalN(700mg/kg BW) 24hrs	580.07 ± 1.89[a]	505.37 ±2.63[a]	270.94 ± 3.45[a]	28.44 ± 1.19[a]
D-GalN(700mg/kg BW) 48hrs	648.45±1.49[a,b]	567.27±1.49[a,b]	330.77 ±6.79[a,b]	38.30 ± 1.32[a,b]
GPLC(35mg/kgBW) + D-GalN 24h	77.61 ± 1.77[b]	77.12 ± 2.02[b]	82.28 ± 2.33[b]	9.58 ± 0.95[b]
GPLC(35mg/kgBW) + D-GalN 48h	89.72± 1.76[b,]	83.93 ± 2.30[b]	89.74 ± 2.54[b]	10.69 ± 1.12[b]

Table- 2

Effect of GPLC and D-GalN on the activities of liver marker enzymes GSH and MDA. P < 0.001, a = significant difference compared to control group, b = significant difference compared to D-Ga1N group.

S.No	GSH(mg/g)	MDA(nmol/g)
Control	46.80 ± 0.98	26.90 ± 0.86
D-GalN(700mg/kg BW) 24hrs	24.31 ± 0.70[a]	51.9 5± 1.00[a]
D-GalN(700mg/kg BW) 48hrs	17.66 ± 0.56[a]	62.32 ± 1.54[a]
GPLC(35mg/kgBW) + D-GalN 24h	46.18 ± 0.36[b]	27.75 ± 1.11[b]
GPLC(35mg/kgBW) + D-GalN 48h	45.79 ± 0.33[b]	28.44 ± 1.12[b]

Table- 3
Effect of GPLC and D-GalN on the activities of liver marker enzymes-SOD and CAT. P value < 0.001, a = significant difference compared to control group, b = significant difference compared to D-Ga1N group.

S.No	SOD(U mg^{-1} of protein)	CAT(U mg^{-1} of protein)
Control	14.86 ± 0.85	80.81 ± 1.30
D-GalN(700mg/kg BW) 24hrs	8.62 ± 0.59	50.82 ± 0.98
D-GalN(700mg/kg BW) 48hrs	5.79 ± 0.35	32.61 ± 0.68
GPLC(35mg/kgBW) + D-GalN 24h	14.15 ± 0.71	79.09 ± 0.87
GPLC(35mg/kgBW) + D-GalN 48h	13.58 ± 0.66	78.32 ± 0.72

Table- 4
Effect of GPLC and D-GalN on the activities of apoptotic marker TNF-α. Significant P value < 0.001,a = significant difference compared to control group, b = significant difference compared to D-Ga1N group.

S.No	TNF-α(pg/dl)
Control	8.14 ± 0.54
D-GalN(700mg/kg BW) 24hrs	22.92 ± 0.94
D-GalN(700mg/kg BW) 48hrs	36.72 ± 0.70
GPLC(35mg/kgBW) + D-GalN 24h	9.11 ± 0.73
GPLC(35mg/kgBW) + D-GalN 48h	9.76 ± 0.65

DISCUSSION

In our current study we assessed the relationship between GPLC supplementation and hepatic biochemical markers of oxidative stress. There are earlier reports which reveal the protective effect of GPLC against oxidative stress-mediated hepatic injury induced by intense aerobic exercise (36) but this effect has not been demonstrated in relation to D-GalN-induced liver injury. In our study, GPLC administration resulted in a significant change in various biochemical parameters in rats with and without D-GalN-induced liver injury. In experiment 1, we examined the effects of GPLC treatment on 7-week-old male wistar rats without acute liver injury. Biochemical analysis showed that GPLC supplementation significantly maintained ALT, ALP and AST activity, which shows its promising hepatoprotective effect. Another important protective prospectus of GPLC is the GSH concentration in the liver. GSH (L-g-glutamyl-cysteinyl- glycine) is a major antioxidant present in a cell which is able to play a vital role of eliminating peroxides and other oxidants. It has been seen to plays a prominent role in the detoxification and antioxidation of exogenous and endogenous compounds, as well as the maintaining intracellular redox status (37). There are ample reports on the importance of GSH in the detoxification of chemically reactive metabolites as drug induced toxicity has been observed after GSH depletion (38). In our first experiment, hepatic GSH levels were found to be nearly same in the groups receiving GPLC as compared to the control group. It is known that moderate oxidation of GSH can cause the formation of glutathione disulfide (GSSG), and that GR catalyzes the reduction of GSSG back to GSH to maintain adequate levels of cellular GSH (39). Another antioxidant enzyme CAT also plays a vital role to protect a cell from the oxidative damage These results strongly suggest that GPLC administration for four weeks promoted antioxidant activity in the liver by maintaining hepatic GSH levels. The second experiment was conducted using a hepatitis animal model to further clarify the hepatoprotective effects of GPLC administration. In experiment 2 liver damage was induced in which rats were injected with D-GalN (700mg/kg BW), an amino sugar that gets selectively metabolized by hepatocytes and causes hepatotoxicity. There have been early studies

which show that D-GalN induces diffused hepatic necrosis after a single intraperitonial administration, the mechanisms of this effect has not yet been fully elucidated. It has been proposed that D-GalN induced damage is due to the potential of D-GalN to decrease the uridine 5-triphosphate concentration in the hepatocytes, which in turn leads to inhibition of mRNA and protein synthesis, ultimately leading to liver cell necrosis (40-42). There have been early studies which reveal that endotoxins are involved in the induction of this hepatic injury and promote the secretion of proinflammatory cytokines (41). Another suggested mechanism of D-GalN-induced hepatic injury is through reactive oxygen species (ROS) produced by activated hepatic macro- phages (43) and it has been reported that D-GalN injection leads to liver damage by promoting reactions that generate ROS or oxidative stress (44). Although the detailed mechanisms following D-GalN injection are still under investigation, it is likely that liver injury occurs as a result of a combination of the events described above.

The results of experiment 2 showed detrimental effects of D-GalN on metabolic liver function, as indicated by increased activities of ALT, AST, ALP, **γ-**GT and MDA for the D-GalN-injected control group (CG). Increases in the activities of ALT and AST have previously been reported after D-GalN exposure. (45,46). In contrast, these enzymatic activities were reduced by 76% (ALT), 81% (LDH), 77% (AST), and 28% (ALP) in the D-GalN-injected group that received GPLC supplementation, which exhibited levels similar to the control group. These results indicate that GPLC supplementation prevented the histopathological alterations and increases in enzymatic activity normally induced by D-GalN, demonstrating a significant hepatoprotective effect of GPLC in this model of hepatic damage.

CAT and GSH are the key defense players which are needed for the cleavage of ROS. It was observed that GPLC supplementation does not result in an alteration in the concentration of GSH and CAT as compared to the normal group. D-GalN administration resulted in a significant decline in CAT and GSH which agrees with the earlier studies. TNF-α inflammatory mediator has been reported to get increased in a condition of oxidative stress (47). TNF-α mediates a cascade which leads to the apoptosis. In our study we monitored an increase in the level of TNF-α which is in agreement with the above study. GPLS supplemented groups does not show any significant elevation in the levels of TNF-α which shows that GPLC confers a significant protection against the damage induced by D-GalN.

CONCLUSION

In conclusion, this study shows that GPLC possesses the potential to serve as a safe and better hepatoprotective agent and helps to maintain the normal functions of liver following injury. Although GPLC shows promise for the amelioration of liver injury in rats, additional work needs to be carried on to evaluate its more health benefits. This is the study to notice the antioxidant benefits with combined PLC and glycine supplementation in healthy animal subjects. GPLC can prove to be a better neutraceutical having a multitude of health benefits if taken as a supplement.

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