



Effect of Genistein and L-Carnitine and Their Combination on Gene Expression of Hepatocyte HMG-CoA Reductase and LDL Receptor in Experimental Nephrotic Syndrome

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Abstract

Background: Nephrotic syndrome is a disorder that leads to hyperlipidemia. L-carnitine and genistein can effect on lipid metabolism and the syndrome. In the present study, we have delved into the separate and the twin-effects of L-carnitine and genistein on the gene expressions of HMG-CoA reductase and LDL receptor in experimental nephrotic syndrome.

Methods: In this controlled experimental study, 50 male Sprague–Dawley rats were randomly divided into five groups: NC (normal-control), PC (patient-control), LC (L-carnitine), G (genistein), LCG (L-carnitine-genistein). Adriamycin was used for inducing nephrotic syndrome and the spot urine samples and urine protein-to-creatinine ratio were measured. Hepatocytic RNA was extracted and real-time PCR was used for HMG-CoA Reductase and LDL receptor gene Expression measurement.

Results: The final weight of the patients groups were lower than the NC group ($P=0.001$), and weight gain of the NC group was higher than the other groups ($P<0.001$). The proteinuria and urine protein-to-creatinine ratio showed significant differences between PC group and LC, G and LCG groups at week 7 ($P<0.001$). The expression of HMG-CoA Reductase mRNA down regulated in LC, G and LCG groups in comparison with PC group ($P<0.001$). Δ CT of LDLr mRNA showed significant differences between the PC group and the other patient groups ($P<0.001$).

Conclusion: This study shows a significant decreasing ($P<0.001$) and non-significant increasing trend in HMG-CoA Reductase and LDLr gene expression, respectively, and synergistic effect of L-carnitine and genistein on these genes in experimental nephrotic syndrome.

Keywords: Gene, Genistein, L-carnitine, LDL Receptor, Nephrotic syndrome

Introduction

The nephrotic syndrome is a kind of disorder arising from extreme protein leakage from the bloodstream into the urine because of renal damage (1). Hyperlipidemia is a disorder, which happens

alongside this disease (2). The common dyslipidemias in nephrotic syndrome are Hypercholesterolemia, increases of low-density lipoprotein-cholesterol (LDL-C) and very low-density

lipoprotein-cholesterol (VLDL-C). Consequently, these disorders in lipid metabolism result in higher risk of cardiovascular diseases that shows the importance of lipid metabolism control in this disease. Among the substances that have the capacity to control cardiovascular diseases through changes in lipid metabolism, phyto-estrogens have an important role. Food phyto-estrogens are a subgroup of flavonoids that have beneficial effects on human health. This group comprises of several subgroups of non-steroid estrogens including isoflavones and lignans having been widely distributed among plants (3). Isoflavones (genistein and daidzein) are the most significant groups to have been widely undergone study. However, these isoflavones exist only in soybeans and several other legumes (3). The beneficial effects of dietary soy protein on the serum lipid concentrations have been shown in many studies on animal and on human cells (4-7). However, it is not exactly clear yet that these effects be mediated through the isoflavones such as genistein or not.

Carnitine is bio-synthesized by two amino acids [lysine and methionine] (8), and is necessary for transporting fatty acids from cytosols to mitochondria (9). Recently, many studies have focused on the role of carnitine in lipid metabolism. It has been proven that L-carnitine (a chemical structure of carnitine) is able to bring down the levels of triglycerides within rats' tissues and plasma (10) but the mechanisms of its effects are not obvious yet.

Hydroxy Methyl Glutaryl-CoA Reductase (HMG-CoA) is the enzyme involved in synthesizing cholesterol and Low Density Lipoprotein Receptor (LDLr), which would be the LDL-C lipoprotein receptor in the liver: they are effective in the metabolic regulation of lipid and the liver cholesterol (11). Genistein gives rise to some level of LDLr gene expression (12, 13) while reducing the HMG-CoA Reductase gene expressivity (13, 14). However, rare studies have ever been conducted as for the effect of L-carnitine on HMG-CoA Reductase and LDLr; their resultant outcome is mostly paradoxical (15). The conducted investigations over the issue of the twin effect of genistein and L-carnitine on these two mentioned gene ex-

pression are extremely limited. We investigated the twin effect of genistein and L-carnitine to know if any joint pathway or synergistic effects in the pathways do exist or not.

Lack of a comprehensive study regarding this issue has caused the design of the present study with the aim of delving into the single separate and/or twin effects of genistein and L-carnitine on the HMG-CoA Reductase and LDLr gene expression among the rats afflicted by the experimental nephrotic syndrome.

Material and Methods

Animals and experimental diets

Male Sprague-Dawley rats at 8-12 weeks of age were housed individually in a room with controlled temperature (20–22 °C), humidity (55–65%) and lighting (from 0700 to 1900 h) and fed assigned experimental diet (AIN 93 M diet) (Table 1) (16). After 7 days of acclimatization, the rats were randomly divided into five groups consisting of 10 animals each with similar mean body weights (300 ± 50 g) by randomized block design method: normal control (NC), patient control (PC), L-carnitine (LC), genistein (G), L-carnitine-genistein (LCG).

Chemical reagents and Intervention

All groups received their own experimental diets during the study (8 weeks). We used carboxymethyl cellulose (CMC, Sigma Chemical Co.) as a solvent for genistein and L-carnitine. In addition, CMC was used for gavage feeding of control groups. NC group received 50 mg/kg body weight CMC by gavage for 8 weeks; PC group received CMC for 8 weeks while receiving single dose of 7.5 mg/kg body weight Adriamycin (Doxorubicin, Pharmacia Italia SPA Co.)-an antibiotic/antineoplastics drug with nephrotoxic side effect- through tail vein at the end of week 2 to induce nephrotic syndrome (17), hereafter, we keep on gavage-feeding of rats with 50 mg/kg body weight CMC for 6 weeks. LC, G and LCG groups were similar to PC except receiving 50 mg/kg body weight L-carnitine (99%, Karen Pharma & Food Supplement Co.), 50 mg/kg body

weight genistein (99%, LC-laboratories Co.) and 50 mg/kg body weight L-carnitine plus 50 mg/kg body weight genistein, respectively, that were dissolved in CMC instead of CMC.

Table1: Composition of experimental diet (AIN-93 modified diet for rodents)

Ingredient	g/kg diet
Cornstarch	465.692
Casein (>85% protein)	140.000
extrinized cornstarch (90-94% tetrasaccharides) ¹	155.000
Sucrose	100.000
Soybean oil (no additives)	40.000
Fiber ²	50.000
Mineral mix	35.000
Vitamin mix	10.000
L-Cystine	1.800
Choline bitartrate (41.1% choline) ³	2.500
Tert-butylhydroquinone	0.008
	u/kg diet
Total energy ⁴ kcal	3601.0
protein%	14.1
CHO%	75.9
fat%	10.0

¹Dyetrose (Dyets, Bethlehem, PA) and Lo-Dex 10 (American Maize, Hammond, IN) meet these specifications. An equivalent product may also be used./ ²Solka-Floc®, 200 FCC (FS&D, St. Louis, MO) or its equivalent is recommended./ ³Based on the molecular weight of the free base./ ⁴The estimate of caloric content was based on the standard physiological fuel values for protein, fat, and carbohydrate of 4, 9 and 4, respectively.

Animals were maintained ad libitum on assigned experimental diet and water during the experiment and Dietary intake and body weight were recorded every day and weekly, respectively.

This study was approved by the review board of animal ethics Tehran University of Medical Sciences and we followed the institute's guidelines in the care and use of laboratory animals.

Sample preparation

During the study, urine samples were collected (spot urine method) at the end of weeks 2, 3 and 7 and measured urine total protein and protein-to-creatinine ratio from spot urine samples. Pyrogallol-Red/Colorimetric End Point method and Taussky et al. (18) & Owen et al. (19), modified

method were used for measuring of protein and creatinine, respectively. After 8 weeks, the animals were sacrificed after overnight fasting by bleeding from the abdominal aorta under diethyl ether anesthesia. Blood was collected from abdominal aorta and livers were surgically excised. The blood was allowed to coagulate and centrifuged at 1,100 rpm for 15 minutes, and serum was stored at -20 °C until albumin analysis (salt fractionation method). Liver tissues were collected and immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

RNA Extraction from Liver and cDNA Synthesis

Cytoplasmic RNA was extracted and purified using RNeasy Plus Mini Kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocol. Quantity and purity of extracted RNA was checked by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA). A ratio of A 260/280 between 1.9–2.1 was taken into account as pure RNA (Fig. 1). Single-strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen).

Real-time PCR for Gene Expression

PCR primers for HMG-CoA Reductase and LDLr genes and 18-S gene (as housekeeping) were designed by Primer express 3 software (Applied Biosystem, Foster city, CA, USA) and purchased from Metabion (Table 2).

PCR reactions were briefly as follows (20, 21); PCR proceeded in special optical tubes in 48-reaction plates (MicroAmp Optical, ABI) with 20 µl reaction mixture containing 10µl Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster city, CA, USA), 7µl DEPC treated water, 0.5µl forward primer, 0.5µl reverse primer and 2µl cDNA as template. The wells were sealed with optical adhesive film (Applied Biosystem, Foster city, CA, USA), and the plate was centrifuged for a few seconds at high speed. Amplification conditions were performed using the standard two-step run protocol; step 1: 10s at 95 °C, step 2: 40 cycles of 15s at 95 °C plus 1s at 60 °C.

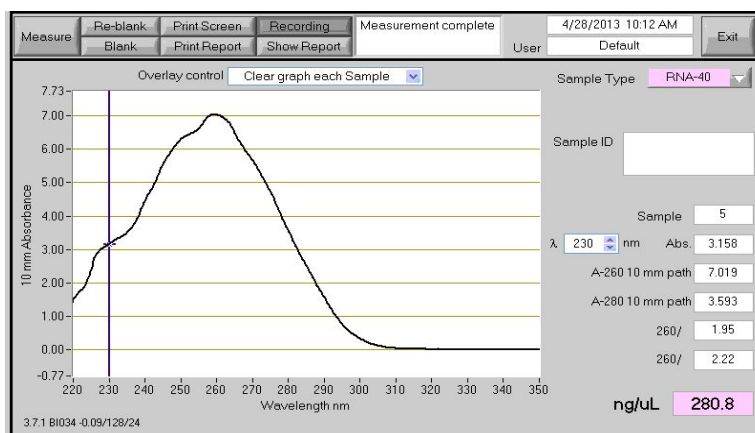


Fig. 1: NanoDrop spectrophotometer of the extracted RNA

After completion of amplification cycles, melt curve was generated to verify if a single gene product had been amplified. For each gene, mRNA expression level was normalized to the level of 18-S. The fold changes of genes expression were computed using the comparative Ct ($2^{-\Delta\Delta C_t}$) method.

Statistical analysis

Statistical analysis was done using SPSS 18.0 for windows (Chicago, IL, USA). Normality of all data was checked by Kolmogorov-Smirnov test and histogram. Data was expressed as mean \pm SD. Comparison of quantitative variables between different groups were evaluated by one-way ANO-

VA, followed by post hoc Scheffé test. P -value < 0.05 was considered statistically significant.

Results

Body weight and food intake

As shown in Table 3, there were not any statistically significant differences between groups in initial weight, but final weight and weight gain of the patient groups was significantly less than the NC group ($P < 0.05$). The PC group did not show any statistically significant differences compared with LC, G and LCG groups in all these variables (Table 3).

Table 2: Primer sequences for real-time PCR

Gene	Sequence	Length	TM	CG%
HMG-CoA Reductase	Forward 5'-GTGGGAACGGTGACACTTA-3'	19	49.47	53
	Reverse 5'-CTTCAAATTTTGGGCACTCA-3'	20	49.14	40
LDLr	Forward 5'-CTGGCGGTAGACTGGATC-3'	18	50.97	61
	Reverse 5'-CAATCTGTCCAGTACATGAAGC-3'	22	52.39	45
18-S	Forward 5'-CCATCCAATCGGTAGTAGC-3'	19	49.61	53
	Reverse 5'-GTAACCCGTGTAACCCCAT-3'	20	50.05	50

Table 3: Body weight and food intake in rat fed experimental diets for 8 weeks

Variable	NC group	PC group	LC group	G group	LCG group	Pvalue ^a
Initial weight (g)	296.50 \pm 25.50	291.50 \pm 28.19	294.77 \pm 23.62	303.22 \pm 34.23	312.00 \pm 26.25	0.501
Final weight (g)	355.30 \pm 18.43	283.30 \pm 34.13 ^b	277.88 \pm 48.73 ^b	283.44 \pm 47.47 ^b	263.14 \pm 67.88 ^b	0.001
Weight gain (g/day)	1.04 \pm 0.45	-0.14 \pm 0.50 ^b	-0.36 \pm 0.83 ^b	-0.34 \pm 0.58 ^b	-0.87 \pm 0.77 ^b	0.00
Food intake (g/day)	20.36 \pm 0.73	16.73 \pm 0.49 ^b	16.36 \pm 1.16 ^b	15.33 \pm 1.10 ^b	15.16 \pm 0.40 ^b	0.00
Protein Intake (g/day)	2.55 \pm 0.09	2.10 \pm 0.06 ^b	2.05 \pm 0.14 ^b	1.92 \pm 0.14 ^b	1.90 \pm 0.05 ^b	0.00
Energy intake (Kcal/day)	73.34 \pm 2.65	60.25 \pm 1.77 ^b	58.93 \pm 4.20 ^b	55.21 \pm 3.98 ^b	54.61 \pm 1.45 ^b	0.00

Values are means \pm SD./ ^aOne-way ANOVA between groups./ ^bValues for PC, LC, G and LCG groups are significantly different compared with NC group by post hoc Scheffé test at $P < 0.05$.

Serum albumin, Proteinuria and protein-to-creatinine ratio

There were not any statistical significant difference between groups in proteinuria and urine pro-

tein-to-creatinine ratio at week 2, but the differences were significant between PC group and LC, G and LCG groups at week 7 (Table 4).

Table 4: Proteinuria and protein-to-creatinine in experimental groups at weeks 2, 3 and 7 and serum albumin

Variable	NC group	PC group	LC group	G group	LCG group	Pvalue ^a
Week 2: Proteinuria (mg/dl)	107.70±4.92	111.85±5.92	102.00±48.74	105.11±28.94	98.00±9.38	0.877
Week 3: Proteinuria (mg/dl)	107.50±6.36	671.14±180.28 ^b	273.00±99.73 ^c	384.66±237.18 ^b	210.25±131.77 ^c	0.00
Week 7: Proteinuria (mg/dl)	103.90±22.65	1864.00±298.27 ^b	328.50±27.87 ^c	680.88±348.82 ^{bc}	400.25±120.13 ^c	0.00
Week 2: Urine protein-to-creatinine ratio	2.96±1.26	3.96±0.71	1.85±0.28	3.74±1.82	3.67±1.47	0.104
Week 3: Urine protein-to-creatinine ratio	3.39±1.12	29.98±7.41 ^b	18.70±30.71	14.38±7.92	5.88±3.71 ^c	0.001
Week 7: Urine protein-to-creatinine ratio	3.87±1.30	71.48±17.20 ^b	22.97±3.19 ^c	38.11±19.59 ^{bc}	20.67±21.28 ^c	0.00
Serum albumin	3.63±0.25	1.32±0.34 ^b	2.73±0.23 ^{bc}	1.47±0.63 ^b	2.86±1.35 ^c	0.00

Values are means±SD.

^aOne-way ANOVA between groups.

^bValues for PC, LC, G and LCG groups are significantly different compared with NC group by post hoc Scheffé test at $P<0.05$.

^cValues for LC, G and LCG groups are significantly different compared with PC group by post hoc Scheffé test at $P<0.05$.

HMG-COA Reductase and LDLr Expression in hepatocyte-Extracted mRNA

Expression of HMG-COA Reductase mRNA between the LC, G, LCG groups and the PC group had statistically significant differences ($P<0.001$, Table 5). L-carnitine and genistein could down-regulate HMG-COA Reductase and twin-effects of them had a synergistic effect,

however the differences between the LC, G, LCG groups were not statistically significant. As to the relative gene expression of LDLr, significant differences were not observed between the groups, but there were statistically significant differences between LC, G, LCG groups and PC group in ΔCT ($P<0.001$, Table 5).

Table 5: ΔCT and mean of HMG-COA Reductase and LDLr gene expression in hepatocytes

Variable	NC group	PC group	LC group	G group	LCG group	Pvalue ^a
ΔCT of HMG-COA Reductase	14.52±1.72	13.29±0.48	13.32±1.94	13.14±1.96	12.68±2.38	0.371
Mean of gene expression of HMG-COA Reductase	1.41±1.32	2.66±0.88	0.78±0.61 ^c	0.24±0.20 ^{bc}	0.23±0.15 ^{bc}	0.000
ΔCT of LDLr	-0.16±0.48	3.31±1.88 ^b	-0.33±0.66 ^c	-0.82±1.24 ^c	-1.14±2.21 ^c	0.000
Mean of gene expression of LDLr	1.24±0.83	0.19±0.08 ^b	0.89±0.67	1.09±0.49	0.74±0.34	0.035

Data are reported as means ± SD. ΔCT = CT of target gene – CT of 18S.

^aOne-way ANOVA between groups./ ^bValues for PC, LC, G and LCG groups are significantly different compared with NC group by post hoc Scheffé test at $P<0.05$./ ^cValues for LC, G and LCG groups are significantly different compared with PC group by post hoc Scheffé test at $P<0.05$.

Discussion

Over the separate effects of genistein and L-carnitine, we have studied their cumulative twin ef-

fects, as well, in Sprague-Dawley rats. As shown in Table 3, the final weight in addition to the weight gain have been lower and negative in all patient groups in comparison with the NC group; the rea-

sonable cause for this phenomenon has been rats' affliction by the nephrotic syndrome which has consequently followed by reductions in food and energy intakes among these groups. In spite of receiving less protein in the patient groups as compared with the NC group, the proteinuria and the urine protein-to-creatinine ratio have been higher in the patient groups versus the NC group, as brought out in Table 4: this indicates their having been caught by the nephrotic syndrome and the protein tissue lysis leading to higher weight loss among these groups, although such increase amongst a number of patient groups has not been statistically significant.

Genistein Effect on LDLr and HMG-CoA Reductase Gene Expression

Insulin and glucagon are key controllers for the matter of biosynthesizing cholesterol and triglyceride within the liver: they exert their biological controlling influence through some changes in the level of activity and/or expressing gene levels involved in the synthesizing and/or absorbing cholesterol; the same influence can also be exerted by means of synthesizing fatty acids. Such genes transcription controlling would be accomplished through the means of a family of transcription factors by the name of SREBP (22). Genistein possesses the capability to increase the glucagon to insulin ratio (23), thus giving rise to some increase not only in the level of SREBP-2 expression, but also in its activity (24). SREBP-2 would preferably hook on to those promoters of the number of genes involved in absorbing and biosynthesizing cholesterol [including HMG-CoA Reductase and (LDLr)-LDL recipient] (11). In fact, the category of isoflavones present in soy protein can activate SREBP-2 so that, as a result, the serum cholesterol clearance could well increase (25). When the cholesterol level comes down in the cells belonging to animals fed with soy protein, the mature nuclear SREBP-2 form content increases by up to the level of 119% in comparison with the rats dieted on casein (26) which in itself would lead to some increase in the level of LDLr gene expression (27). Borradaile et al. have also conducted a study on the human liver cells

(HepG2) to show that genistein causes increase in the levels of LDLr mRNA by 3 to 6 times; this means that they increase the absorption and the lysis of marked LDLs statistically significantly (13). All the same, it should not be far from notice that such effects might actually be independent of the insulin to glucagon ratio (28).

The isoflavones' effect, especially that of genistein, on HMG-CoA Reductase turns out to be paradoxical. There are a number of studies indicating that genistein could give rise to increase in LDLr gene expression to be followed by HMG-CoA Reductase gene expression within the human DLD-1 cancerous colon cells (12, 29).

Our own investigation has shown that genistein causes some reduction in HMG-CoA Reductase gene expression: this is in contrast with the PC group. Such reduction was even greater than that of the L-carnitine recipient group, despite the fact that the said difference did not prove statistically significant (Table 5). Moreover, the LDLr expression in the group receiving genistein was actually higher as compared with the PC group, and even: as contrasted with L-carnitine; however, these differences were not statistically significant, though the Δ -CT difference in between the G group and the PC group turned out to be statistically significant (Table 5).

L-carnitine Effect on LDLr and HMG-CoA Reductase Gene Expression

Dyslipidemia is generally common among patients with renal ailments, specifically among those afflicted by the nephrotic syndrome. Nearly 40 percent of those patients undergoing haemodialysis are gripped by some sort of dyslipidemia: the majority of them are afflicted by hyperlipidemia type IV and plasma HDL-C reduced levels (30). The progressing and advancement of dyslipidemia among such patients are themselves under the influence of a number of various parameters such as carnitine shortage, leading to actual disorders in the process of lipid metabolism (31, 32). The carnitine can significantly bring down the levels of rats' tissues and plasma triglyceride (33). Still, and overall systematic review would indicate that carnitine has no statistically significant effects on tri-

glyceride, cholesterol, and their probable derivatives (34). The carnitine supplement has turned out to be effective in adjusting the lipid profile among those patients whose level of triglyceride has been above the 200 mg/dl or the HDL-C level has actually been below 35 mg/dl (35).

In the present study, L-carnitine could well have caused some statistically significant decrease in HMG-CoA Reductase gene expressivity in comparison with the PC group in spite of the fact that such reducing effect has not been up to the group receiving genistein. In addition, the LDLr expression because of receiving L-carnitine went on the increase in comparison with the PC group: although such an increase was not statistically significant, the difference in Δ -CT did prove significant (Table 5).

Very limited studies are available regarding the effect of carnitine on the gene expressions of the two genes of HMG-CoA Reductase and LDLr. Evaluation has been made in Mondola's et al. investigation into the effect of carnitine on cholesterol metabolism and HMG-CoA Reductase activity in rats' liver cells where it has been shown that L-carnitine could well rein in the HMG-CoA Reductase activity and also increase the bonding of LDL on two the liver cells (15).

The Twin Effect of Genistein and Carnitine on LDLr and HMG-CoA Reductase Gene Expression

In our study, the synergistic effect of genistein and L-carnitine on the expressivity of both genes have actually been observed, although this effect turned out to be statistically significant nearly as far as comparison was made in between HMG-CoA Reductase vs. the PC group. Δ -CT gene LDLr, too, showed of itself some significant synergistic effect (Table 5). Similar studies concerning the twin effect of genistein and carnitine on these two genes' expression are skimpy to such a degree that their availability would prove difficult.

Conclusion

A 6-week gavaging with L-carnitine and genistein increased the HMG-CoA Reductase and LDLr

gene expression and had synergistic effects in rats with nephrotic syndrome; however, the changes were statistically significant only for HMG-CoA reductase. It seems genistein is more effective on these gene expressions in comparison with L-carnitine. These finding could warrant future studies to determine the therapeutic effects of these supplements on nephrotic syndrome and lipid metabolism management.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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