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Impact of β-alanyl-L-histidine against Hypercholesterolemia.

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Research Article

ABSTRACT

β-alanyl-L-histidine, an endogenous histidine-containing dipeptide, protects protein from oxidation and glycation, which may contribute to a potential treatment for some conformational diseases including cataract. The effect of hypercholesterolemia in rabbits was tested on serum protein fractions, lactate dehydrogenase isoenzymes, total protein and glucose levels. The work was extended to estimate certain hepatic enzymes; glucose-6- phosphatase (G-6-Pase), glycogen phosphorylase (GPH), α-hydroxybutyrate dehydrogenase (α-HBDH), lactate dehydrogenase (LDH) and glycogen content. The corrective action of β-alanyl-L-histidine (carnosine) and fluvastatin were also evaluated. Twenty five adult male Newzeland rabbits were selected for this study. The animals were divided into five groups. Group 1 served as normal healthy control, group 2; hypercholesterolemic animals (fed on standard rabbit's chow with 1% cholesterol for 12 weeks), group 3; high carnosine dose (50 mg/Kg b. wt.) treated animals, group 4; low carnosine dose (25 mg/Kg b. wt.) treated ones and group 5; fluvastatin (2mg/kg b.wt.) treated group. Treatment started at the last six weeks of cholesterol feeding. Hypercholesterolemic rabbits recorded drastic changes in all parameters under investigation. β-alanyl-L-histidine has been demonstrated to provide protection against oxidative damage with respect to fluvastatin. β-alanyl-L-histidine and fluvastatin recorded an enhancement level in major parameters, where high carnosine dose recorded the most potent effect.

INTRODUCTION

In recent years, researchers have focused on the potential role of dietary antioxidants in promoting health and reducing the risk of heart disease, cancer, cataracts, and other degenerative diseases of aging, Nutrient supplement industries have rallied around the proposed benefits of antioxidant nutrients. However, recognition of the adverse health effects of high doses of antioxidant nutrients has led experts to caution against dietary supplements and recommend that such nutrients come from food sources as part of a varied diet[1].

Many biologically active substances with medicinal properties have been identified in food [2, 3]. L-carnosine (β-alanyl-L-histidine) is a biological peptide presenting many functions [4], including immunoprotective effects on human lymphocytes receptors [5] and suppresses apoptosis [6]. In addition, carnosine is a potent antioxidant where, it acts as a scavenger of hydroxyl and superoxide radicals. So, L-carnosine has been observed to have a protective effect, based on its scavenging effect of elevated free radicals in brain, kidney, liver and skeletal muscle [7]. Carnosine may therefore react with secondary lipid oxidation products rather than acting as a primary free radical scavenger [8]. It acts as a membrane stabilizer, which explains its anti-ulcer and wound healing effects [9]. Moreover, carnosine has an anti-inflammatory effect that is evident in chronic infection [10]. Moreover, [11, 12] proved that carnosine normalizes energy metabolism in bilharzial infected hamsters.

It has been demonstrated that abnormally high levels of serum cholesterol contribute to atherosclerosis and coronary artery disease. Statins, such as rosuvastatin and fluvastatin are inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase and efficiently and widely used drugs in the treatment of lipid disorders, especially hypercholesterolemia [13].Fluvastatin a synthetic hypocholesterolemic drug acts by inhibiting hydroxy methyl glutaryl Co A reductase (HMGCoA reductase); the rate limiting enzyme in cholesterol biosynthesis.

HMGCoA reduces both total and low density lipoprotein cholesterol levels [14]. The inhibition of the enzyme HMGCoA results in both down-regulation of cholesterol synthesis and the re-regulation of hepatic high affinity receptors for low density lipoproteins followed by increased catabolism of LDL cholesterol [15]. Otherwise, HMGCoA reductase inhibitors do not effect, to a significant extent, the levels and/or composition of the other major lipoprotein fractions [16]. Fluvastatin is metabolized by multiple hepatic enzymes [17]. Consequently its co-administration with other drugs does not result increased serum fluvastatin levels.

Hypercholesterolemia is closely correlated with risk of cardiovascular diseases [18]. This is mainly due to changes in feeding habits; high- fat and low-fiber diets [19]. Lipid disorders, which encompass hypercholesterolemia, hypertriglyceridemia, or their combination, and fatty liver, are prevalent worldwide [20,21]. Current approaches in reducing blood LDL-cholesterol, inhibiting cholesterol synthesis [18, 22] and blocking the absorption of dietary cholesterol [23] are of great challenge.

The aim of the present work was to evaluate the corrective action of carnosine against hypercholesterolemia in rabbits. The evaluation was done through measuring certain hepatic enzymes; representing glycolytic and glycogenolytic pathways as well as glycogen and glucose contents. Protein fractions were also taken into consideration.

MATERIALS AND METHODS

Chemicals

The chemicals used were products of different firms: Merck (Germany), Sigma (USA), and El Nasr Pharmaceutical Chemical Company (Egypt).

Animals

Twenty five adult male Newzeland rabbits of similar age and weight (2.5 – 3 kg) were selected for this study. They were obtained from Animal House, Ophthalmic Institute, Giza, Egypt. Animals were kept in a controlled environment and were allowed free access of water and diet.

Composition of rabbits chow

Rabbits chow consists of 16.1% protein, 13.83% fibers, 11.38% water, 8.12% ashes, 47% carbohydrate and 3.4% fats (El-Kahira Company for Oil and Soap).

Experimental design

Duration of experiment was 12 weeks. The animals were divided into five groups each of five rabbits. Group 1 served as normal healthy control fed on standard rabbits chow. Group 2 served as hypercholesterolemic animals fed on standard rabbit's chow with 1% cholesterol [24]. Group 3and 4 served as carnosine treated animals receiving oral doses of 50 mg (high dose) and 25 mg (low dose) carnosine /kg/day, respectively [25] in the last 6 weeks of cholesterol administration. Group 5 served as fluvastatin treated group receiving oral doses of 2 mg fluvastatin /kg/day [26] in the last 6 weeks of cholesterol administration.

Sample preparations

At the end of feeding period (12 weeks), the animals were fasted overnights (12-14 h). Blood samples were taken, let to clot, centrifuged at 3000 xg for 5 minutes. Serum was separated for further determinations of total protein content and its fraction as well as lactate dehydrogenase isoenzymes.

Liver tissue was homogenized in normal physiological saline solution (0.9N NaCl) by a ratio 1:9 w/v. The homogenate was centrifuged for 5 minutes at 3000 xg at 4°C and the supernatant was used for estimation of hepatic enzymes and hepatic total protein.

Biochemical assays

Glycogen content was estimated by the method of Nicholas et al. [27] and glucose level was determined by the method of Trinder [28].

Measured colorimetrically at 503 nm. Lactate dehydrogenase isoenzyme Lactate dehydrogenase was estimated by Babson and Babson [29], where the reduction of NAD coupled with the reduction of tetrazolium salt with PMS serving as an intermediate election carrier. The resulted formazan of INT was es were carried out by the

method of Dietz and Lubrano [30]. Glucose-6-phosphatase [31] and glycogen phosphorylase [32] were determined as inorganic phosphorus released at 660 nm.

α-hydroxybutyrate dehydrogenase catalyzed the conversion of α-ketobutyrate to α-hydroxybutyrate where NADH is oxidized to NAD and the rate of decrease in absorbance is directly proportion to the enzyme activity at 365nm [33]. Serum and hepatic total protein [34]: Bradford dye react with protein in the sample resulting an increase in absorbance due to the formation of protein-Bradford complex and the developed colour was measured of 595 nm. Serum protein fractions were estimated by the method of De-Moreno et al. [35] using 10-20% gradient polyacrylamide gel electrophoresis in the presence of SDS. Individual standard protein fractions ($α_2$- macroglobulin is 180 kDa, β-galactosidase is 116 kDa, phosphorylase-b is 97.4 kDa, carbonic anhydrase is 29 kDa, β-lactoglobulin is 18.4 kDa, α- lactalbumin is 14.2 kDa and aprotinin is 6.5 kDa were used as standards [36]. The gel stained with Comassie Brilliant Blue R 250 followed with silver stain. The electrophoretic bands were expressed as percentage of the total protein content (mg/ ml). Helena France Scanner at 660 nm was used for bands detection.

RESULTS AND DISCUSSION

With respect to glycogen and glucose levels, hypercholesterolemic rabbits recorded significant increase in glycogen (291.60%), while significant decrease in glucose (26.80%) level was observed. (Table1). Treatment with high dose of carnosine improved glycogen by 142.77 % and enhanced glucose by 17.36%. Low carnosine level showed the same pattern of amelioration by 4.72 and 12.74%, respectively. In agreement with our results, Bandsma et al. [37] and Kalkan Ucar et al[38] recorded glycogen storage after hypercholesterolemia. Fluvastatin as a reference drug enhanced glycogen and glucose contents by 121.11 and 13.85% (Fig.1). Hepatic protein insignificantly increased in case of hypercholesterolemia, whereas treatment with high and low doses of carnosine as well as fluvastatin recorded improvement by 15.53, 25.14 and 10.89%, respectively (Fig.1). Serum protein content recorded significant decrease (30.93%) after hypercholesterolemia, while treatment with high or low carnosine dose and fluvastatin showed improvement by 14.32, 4.05 and 19.29%, respectively (Fig.1). Soliman et al. [39] found the same pattern of improvement after treatment of hypercholesterolemia with either carnosine or fluvastatin. Romero et al. [40] showed that increase in total protein content can be deemed as a useful index of the severity of cellular dysfunction in liver diseases. Stimulation of protein synthesis has been advanced as a contributory self healing mechanism, which accelerates the regeneration process and the production of liver [41].

Table 1: Effect of carnosine and fluvastatin on hepatic glycogen, protein and seum glucose levels of hypercholesterolemic rabbits.

Parameters	Normal control	Hyperchlesterolemia	Hypercholesterolemia + 50 mg carnosine	Hypercholesterolemia + 25 mg carnosine	Hypercholesterolemia + fluvastatin
Glycogen	1.80±0.18c	7.05±1.22a	4.48±0.51b	7.90±1.70a	4.87±1.92b
Glucose	88.25±9.36a	64.57±5.26c	79.89±2.90b	75.81±5.03b	76.79±4.09b
Total hepatic protein	8.63±1.04c	8.86±0.96 bc	10.2±1.05ab	11.03±1.45a	9.8±1.05abc
Total Serum protein	20.73±2.22a	14.43±1.38c	17.40±1.18b	15.27±1.42c	18.43±2.19b

- Data are means ± SD of six rabbits in each group.
- Data are expressed mg/g tissue for glycogen, glucose and protein content and mg/g tissue.
- Unshared letters beyween groups are significance value at p<0.0001.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

Glycogenolytic enzymes in hypercholesterolemia showed decrease in glycogen phosphorylase (44.7%) and glucose-6-phosphatase (67.70%) (Table2). This gives an additional support of the observed decrease in glucose level and glycogen enhancement level. Treatment with either high and low dose of carnosine improved glycogen phosphorylase by 72.89 and 86.22%, respectively, while the selected drug showed improvement by 1.78%. In case of glucose-6- Pase, treatment recorded enhancement by 54.09, 38.41 and 62.23%, respectively (Fig. 2). Yeda et al. [42] postulated the role of oxidative metabolism of excess fat fuels result in increased free radicals production. During oxidative stress, they can elicit serious sequels and widespread damage to cell constituents such as membrane lipids and proteins that lead to disturbance in enzyme levels [43].

Table 2: Effect of carnosine and fluvastatin on hepatic enzymes of hypercholesterolemic rabbits.

Parameters	Normal control	Hyperchlesterolemia	Hypercholesterolemia + 50 mg carnosine	Hypercholesterolemia + 25 mg carnosine	Hypercholesterolemia + fluvastatin
Glycogen phosphorylase	4.50 ± 1.09 [b]	2.49 ± 0.43 [c]	5.77 ± 0.81 [a]	6.37 ± 1.41 [a]	2.57 ± 0.62 [c]
Glucose-6-phosphatase	4.40 ± 0.41 [c]	7.38 ± 1.18 [a]	5.00 ± 0.28 [bc]	5.69 ± 1.21 [b]	4.51 ± 0.23 [c]
Lactate dehydrogenase	16.48 ± 1.41 [a]	8.37 ± 1.92 [b]	17.11 ± 1.72 [a]	15.69 ± 1.29 [a]	16.19 ± 2.07 [a]
α-Hydroxybutyrate dehydrogenase	18.40 ± 2.45 [a]	10.41 ± 1.95 [c]	16.66 ± 1.32 [ab]	11.44 ± 1.31 [c]	15.87 ± 2.67 [b]

- Data are means ± SD of six rabbits in each group.
- Data are expressed as µmol/min/ mg protein.
- Unshared letters beyween groups are significance value at p<0.0001.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

Table 3: Effect of carnosine and fluvastatin on lactate dehydrogenase isoenzymes of hypercholesterolemic rabbits.

Parameters	Normal control	Hyperchlesterolemia	Hypercholesterolemia + 50 mg carnosine	Hypercholesterolemia + 25 mg carnosine	Hypercholesterolemia + fluvastatin
LDH_5	6.48 ± 0.98 [a]	2.40 ± 0.98 [c]	5.27 ± 0.33 [b]	4.90 ± 0.88 [b]	7.24 ± 1.60 [a]
LDH_4	3.27 ± 0.50 [a]	1.45 ± 0.54 [b]	3.04 ± 0.71 [a]	2.90 ± 0.47 [a]	2.65 ± 0.85 [a]
LDH_3	2.86 ± 0.54 [a]	1.85 ± 0.39 [b]	3.03 ± 0.66 [a]	2.94 ± 0.30 [a]	1.99 ± 0.68 [b]
LDH_2	2.16 ± 0.92 [bc]	1.48 ± 0.36 [c]	3.16 ± 0.74 [a]	2.61 ± 0.16 [ab]	2.49 ± 1.07 [ab]
LDH_1	1.66 ± 0.72 [bc]	1.14 ± 0.37 [c]	2.57 ± 0.83 [a]	2.31 ± 0.83 [ab]	1.77 ± 0.24 [bc]

- Data are means ± SD of six rabbits in each group.
- Data of LDH fractions are expressed as µmol/min/ mg protein
- Unshared letters beyween groups are significance value at p<0.0001.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

Table 4: Effect of carnosine and fluvastatin on serum protein fractions of hypercholesterolemic rabbits.

Serum protein fractions	Normal control	Hyperchlesterolemia	Hypercholesterolemia + 50 mg carnosine	Hypercholesterolemia + 25 mg carnosine	Hypercholesterolemia + fluvastatin
KDa 180	1.45±0.31b	0.82±0.20c	0.63±0.13c	1.24±0.02b	1.92±0.59a
KDa 116	1.69±0.26a	1.70±0.40a	0.94±0.19b	1.50±0.16a	1.47±0.16a
KDa 97.4	1.03±0.32d	1.89±0.21bc	2.38±0.44a	1.55±0.24c	2.07±0.29ab
KDa 66	9.91±1.49a	4.43±1.25d	6.22±1.06c	5.31±0.72cd	8.03±0.68b
KDa 48.5	0.75±0.16d	1.12±0.33c	0.58±0.16d	2.66±0.25a	2.01±0.32b
KDa 29	0.41±0.10c	0.50±0.19c	0.73±0.18ab	0.56±0.21bc	0.84±0.20a
KDa 18.4	1.54±0.31a	0.66±0.20c	0.93±0.21b	0.33±0.06d	0.82±0.18bc
KDa 14.2	1.08±0.17a	1.27±0.26a	0.60±0.26bc	0.45±0.05c	0.64±0.09b
KDa 6.5	0.72±0.19ab	0.59±0.14b	0.79±0.08a	0.56±0.07b	0.69±0.11ab

- Data are means ± SD of six rabbits in each group.
- Serum protein fractions are expressed in µg/µl of serum.
- Unshared letters beyween groups are significance value at p<0.0001.
- Statistical analysis is carried out using one way analysis of variance; Costat Computer Program.

Concerning glycolytic enzymes, hypercholesterolemia significantly decreased LDH by 49.20% and HBDH by 43.42% (Table 2). The decrease in enzyme activities was attributed to the increase ROSs and oxidative stress that affect cell membrane fluidity and fragility leading to enzymes leakage [44, 45]. Increased ROSs also increased "NADH/NAD" ratio causing shift to the left in the equilibrium of the oxidoreductive couple lactate-pyruvate resulting in hyperlactacidaemia [46]. The extracellular release of LDH and HBDH [47] could lead to liver lowering activities. Treatments with either the two doses of carnosine or fluvastatin recorded improvement by 53.03, 44.42 and 47.45% for LDH and 33.97, 5.60 and 29.67% for HBDH (Fig. 2).

LDH isoenzymes in hypercholesterolemic rabbits recorded decrease in LDH_5, LDH_4 and LDH_3 subunits by 62.96m 55, 66 and 35.31%, respectively, while LDH_2 and LDH_1 not significantly decreased (Table 3). Carnosine (high and low dose) and fluvastatin showed improvement by 44.29, 38.58 and 74.69% for LDH_5, while LDH_4 improved by 63.91, 44.34 and 36.70%. LDH_3 ameliorated by 41.26, 38.11 and 4.89%, respectively (Fig.3). LDH_2 enhanced by 77.78, 31.48 and 46.76% and LDH_1 improved by 86.14, 70.48 and 37.95% after treatment with either the two carnosine doses or fluvastatin, respectively (Fig.3). In the present study, LDH_5 is expressed as the main dominating subunits and it is specific for liver disease. This observation found support in the statement of Varley et al. [48]. The same authors mentioned an increase in serum LDH_5 in case of liver disease, even the serum total LDH activity remained within normal limits. $LDH_1;$ the myocardial subunit also affected by hypercholesteroleima, which confirmed the observed decrease in HBDH; the lactate dehydrogenase -1- isoenzyme and give an additional support of myocardial dysfunction associated with hypercholesteroplemia [49].

Serum protein electrophoresis measures specific protein in the blood to identify some diseases. Protein provides energy and is needed for the body to generate new cells, maintain and rebuild muscles, carry other nutrients and support the immune system [50]. Blood serum contains two major protein groups; albumin and globulin which carry substances through the blood stream. More than half of the protein in blood serum is albumin. In our study 180 kDa (α2- macroglobulin), 66 kDa albumin, 18.4 kDa (β-lactoglobulin) and 6.5 kDa (aprotinin) significantly decreased with 43.44, 55.29, 57.14 and 18.05 kDa %, respectively as a result of hypercholesterolemia (Table 4 and Fig. 4 &5). Therefore, these four fractions may be considered as markers in hypercholesterolemic condition. Treatment with 50 or 25 mg carnosine or fluvastatin show enhancement with different degrees. α- macroglobulin enhanced by 13.11, 28.96 and 11.03%, while albumin improved by 18.06, 8.88 and 36.32%, respectively. β-lactoglobulin ameliourated by 17.53, 21.43 and 10.39%, whereas aprotinin enhanced by 8.33, 4.17 and 13.89%, respectively. In parallel with our results Steiner et al. [51] reported changes in several sets of proteins associated with cellular stress.
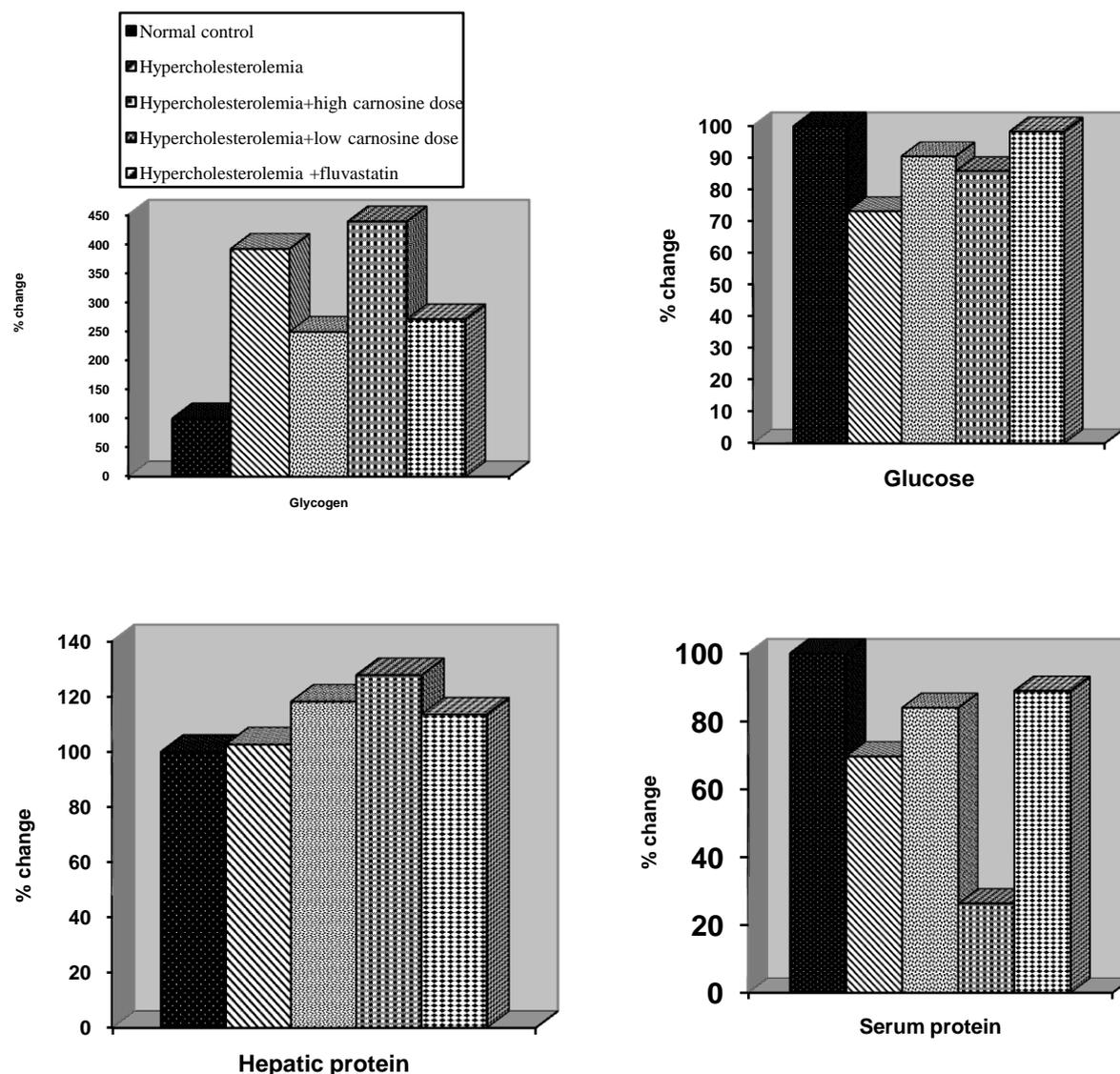
Figure 1: Percentage changes of glycogen, glucose and total protein contents in hypercholesterolemic treated rats.

These above mentioned corrective action of carnosine on all tested parameters might be attributed to many corrective abilities of the dipeptide. Carnosine proved to present a universal buffer action combating many stressful conditions [52]. The dipeptide scavenged ROS and protected protein modification mediated by peroxyl radical generated in lipid peroxidation [53]. In addition, Hipkiss and Chana [54] proved the ability of carnosine to protect the already formed proteins against inactivation. Carnosine also decreased protein denaturation and played a role in the disposal of glycated protein in the affected tissue [55]. An additional reported function of carnosine was the induction of dose-dependent vascular relaxation independent of endothelium [56]. This relaxation could be of utmost important in combating deleterious vascular insults of hypercholesterolemia. One could conclude that the stress had been minimized, even completely combated by the histidine dipeptide.

Fluvastatin treated rabbits also recorded improvement percentages by variable degrees. These results found support in Steiner et al. [51] research using fluvastatin as a lipid lowering agents, proved the statin down-regulating role of cholestrerol concentration and also through its antioxidant properties [44,45, 57]. However, Steiner et al. [51] stated that long term treatment by statin lead to disturbance in enzyme activities. Thus, fluvastatin beneficial action for treating hypercholestrolemia much lagged behind that of carnosine,
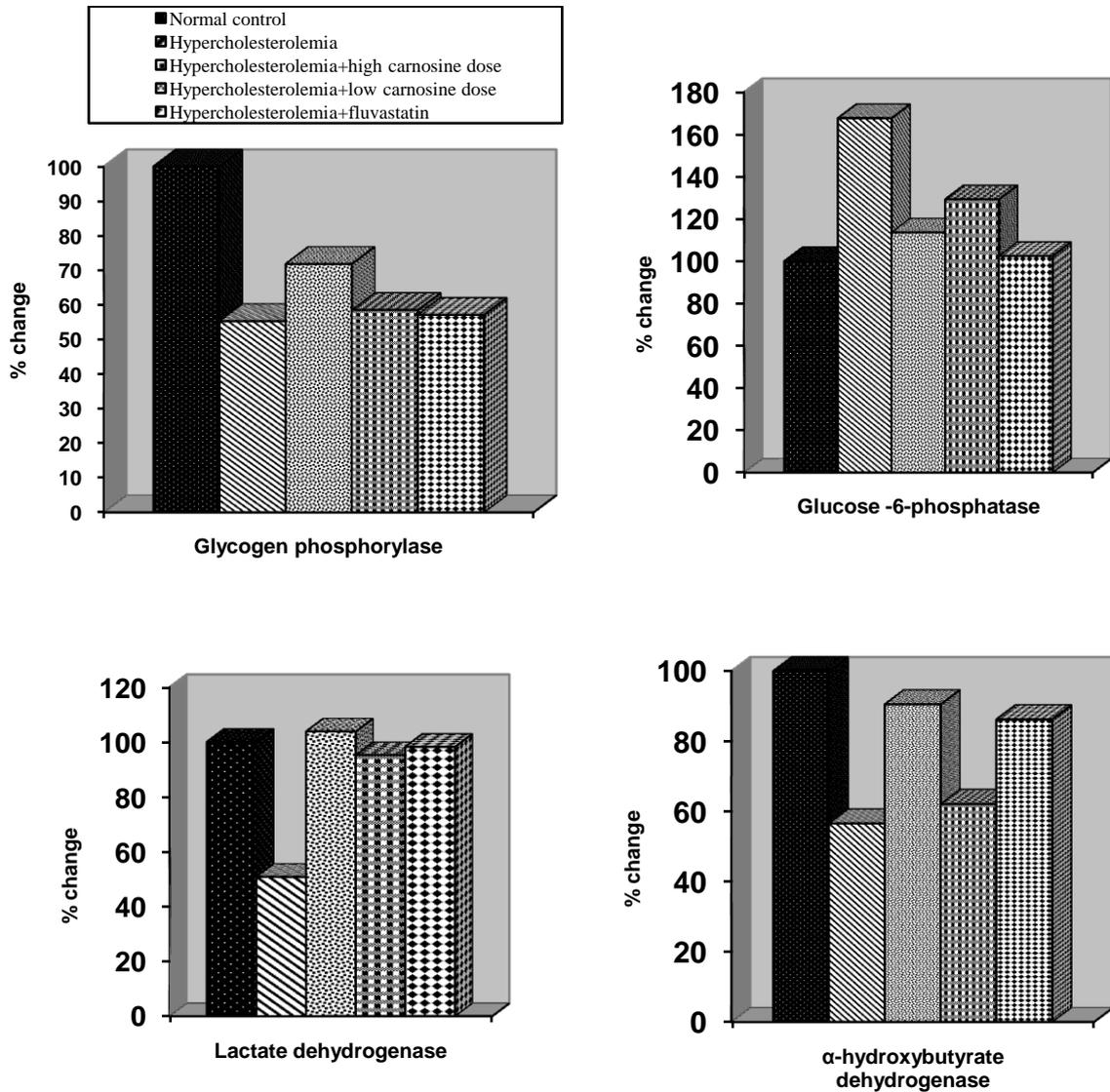
Figure 2: Percentage changes of hepatic enzymes in hypercholesterolemic treated rats.
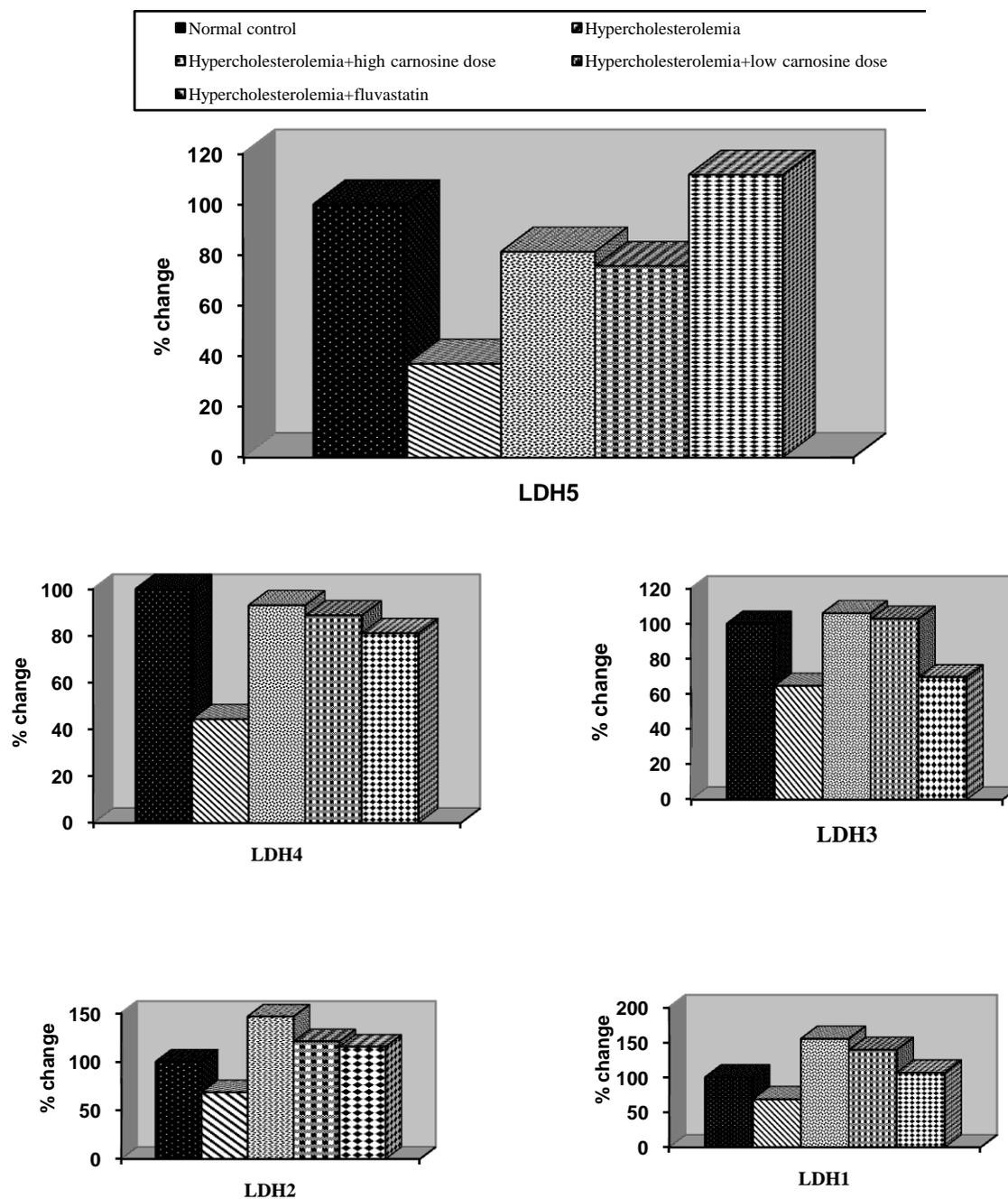
Figure 3:Percentage changes of lactate dehydrogenase enzymes in hypercholesterolemic treated rats.
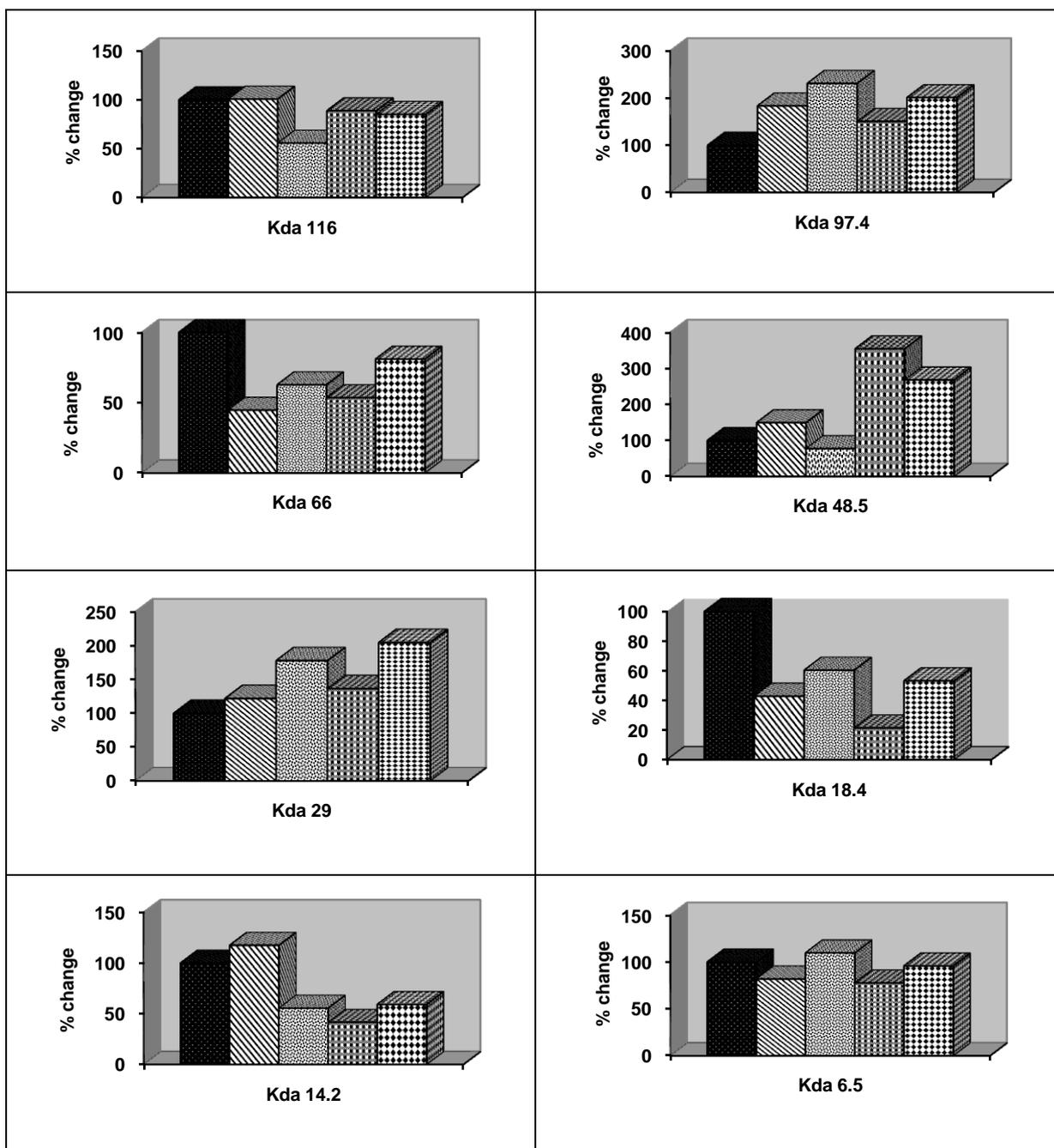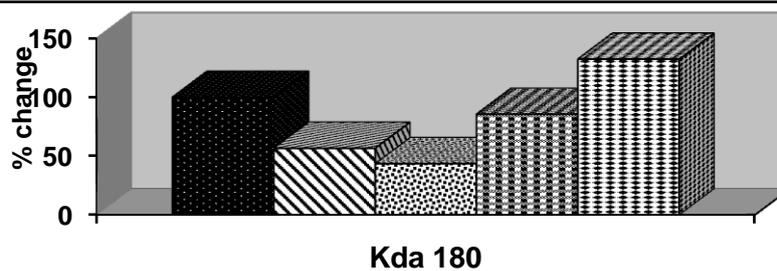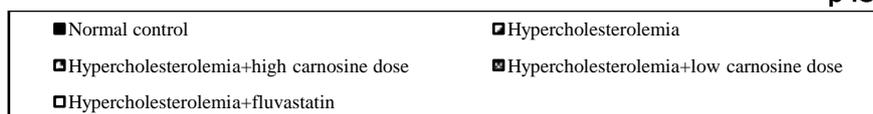
Figure 4: Percentage changes of serum protein fractions.
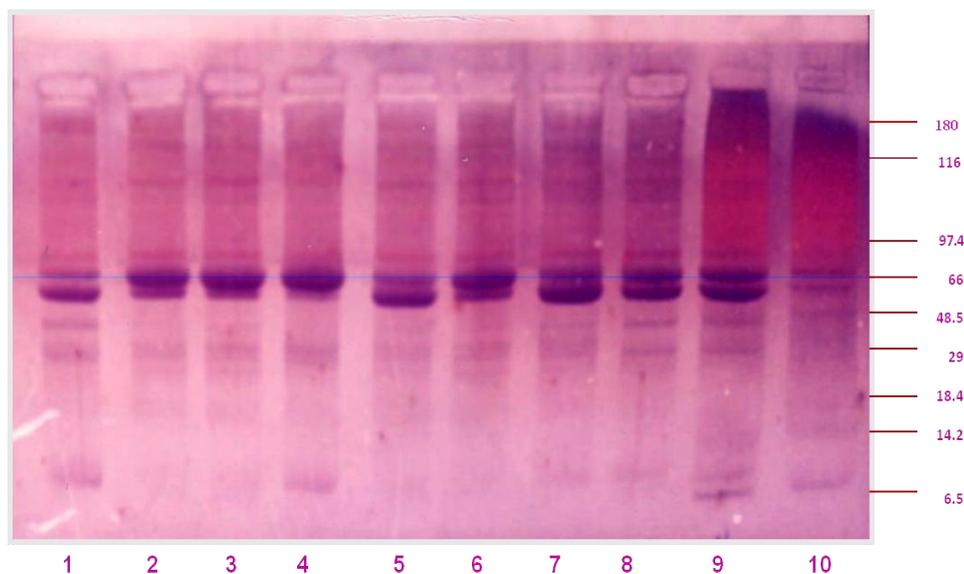
Figure 5: Electrophoretic profile of serum protein on SDS-polyacrylamide gel electrophoresis"SDS-PAGE"

Lane 1: Normal control

Lane 2, 3: Hypercholesterolemia

Lane 4, 5:Hypercholesterolemia treated with 50 mg carnosine

Lane 6, 7:Hypercholesterolemia treated with 25 mg carnosine

Lane 8, 9:Hypercholesterolemia treated with fluvastatin

Lane 10:Standard marker protein

CONCLUSION

Carnosine exerts a therapeutic role against metabolic disorders associated by hypercholesterolemia. It improved glycolytic and glycogenolytic machineries as well as the protein contents and showed more potent effect than fluvastatin.

ACKNOWLEDGEMENT

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