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Treatment of High Fat Diet-Induced Hyperhomocysteinemia with Pyridoxine, Cinnamon and Pitavastatin in Young Male Albino Rats

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ABSTRACT

Introduction: Hyperhomocysteinemia refers to elevated serum homocysteine (HCY) and is a strong but modifiable risk factor for the development of coronary artery diseases owing to its notoriety to directly and indirectly damage vessel walls causing atherosclerosis. **Aims & Objectives:** To compare the effects of treatment with pyridoxine (PYR), cinnamon (ACCE) and pitavastatin (PIT) in high-fat diet (HFD)-induced hyperhomocysteinemia in rats. **Place and duration of study:** This study was conducted at Shaikh Zayed Hospital and UHS, Lahore for the duration of 60 days. **Material & Methods:** 60 albino male rats (6 weeks of age) were divided into 10 groups equally (Group 1: control). Group 2 (dietary preventive) and Groups 3-10 (therapeutic) were induced using HFD for 30 days (HFD continued throughout). Only Group 2 was later reverted to normal lab diet. They were treated for 30 days, after induction, orally, once a day: Group 3 (PIT 0.3mg/kg); Group 4 (PYR 18mg/kg), Group 5 (ACCE 200mg/kg), Group 6 (PIT 0.3mg/kg + PYR 18mg/kg), Group 7 (PYR 18mg/kg + ACCE 200mg/kg), Group 8 (PIT 0.3mg/kg + ACCE 200mg/kg), Group 9 (PIT 0.3mg/kg + PYR 18mg/kg + ACCE 200mg/kg) and Group 10 (PIT 0.15mg/kg + PYR 9mg/kg + ACCE 100mg/kg). Homocysteine was checked at Day 0, Day 30 and Day 60. Data was analyzed using SPSS version 20.0 ($P \leq 0.05$). **Results:** All the therapeutic groups showed significant improvement, the best in Group 4 (pyridoxine 18mg/kg) in which HCY reduced from 10.635 µmol/L to 8.208 µmol/L. Lesser but significant improvement was evident in Group 9 (PIT 0.3mg/kg + PYR 18mg/kg + ACCE 200mg/kg) and Group 10 (PIT 0.15mg/kg + PYR 9mg/kg + ACCE 100mg/kg). **Conclusion:** Significant reduction in HCY by pyridoxine plus its combination with cinnamon and pitavastatin indicates usefulness in treatment of hyperhomocysteinemia and prevention of atherosclerosis.

Key words: Hyperhomocysteinemia, cinnamon, pyridoxine, pitavastatin

INTRODUCTION

Hyperhomocysteinemia is a condition of elevated levels of plasma homocysteine (HCY) i.e. >15 µmol/L (Table-1).[1] It is an important independent risk factor involved in the development of atherosclerosis, prematurely.[2] It has been reported that there is a significant association between raised homocysteine and coronary artery diseases (CADs) and young Pakistani population is at 3.5 times increased risk of suffering from CAD due to raised HCY.[3] Homocysteine (Fig-1), was discovered in 1932, and found chemically similar to amino acid "cysteine", hence the name. Its role in human cellular mechanisms was determined in 1935 by Vincent du Vigneaud.[4]

Classification of Hyperhomocysteinemia	Homocysteine Levels (Micromol/L) or (µmol/L)*
Moderate	16-30
Intermediate	31-100
Severe	>100
* Normal Levels of homocysteine in humans: <15	

Table-1: Reference ranges for serum homocysteine in humans[1]

Homocysteine is a sulfhydryl-containing amino acid and not a normal dietary constituent.[5,6] which is an intermediate in the biosynthesis and metabolism of amino acids, methionine and cysteine. It is only produced by demethylation of methionine.[4,7] In

plasma it exists in four forms: 1% as free thiol, 70-80% disulfide-bound to plasma proteins (mostly albumin), 20-30% with itself to form a dimer, rest with other thiol compounds.[4]

Homocysteine has several possible fates. It may enter the re-methylation cycle (Fig. 1) by *"folate, Vit-B_{12}-dependent methionine synthase"* enzyme or catalyzed by *"betaine homocysteine methyltransferase-2"* enzyme. Alternatively, HCY can insert itself into the pathway of "trans-sulfuration" when either excess methionine is there or synthesis of cysteine is required. The defects of the involved enzymes or deficiencies of certain vitamins (folate, pyridoxine, cobalamin, etc.) can shut these pathways. As a result, HCY aggregates and leads to raised HCY blood levels.[8]

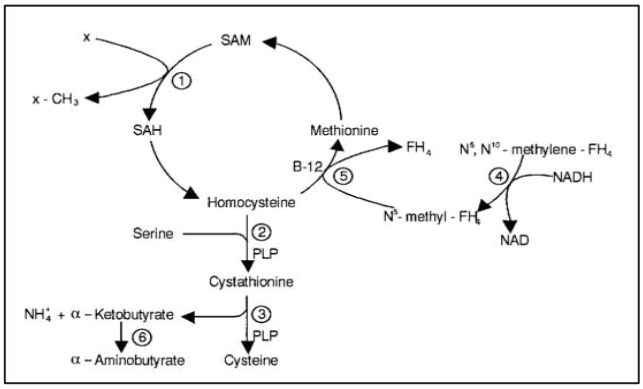

Fig-1: Methionine and homocysteine metabolism. Homocysteine is either remethylated (remethylation cycle) to methionine or metabolized by the transsulfuration pathway. Enzymes: 1: various methyltransferases; 2: CBS; 3: cystathionase; 4: MTHFR; 5:methyltetrahydrofolate homocysteinemethyltransferase; 6:transaminase(s). X: general methyl group acceptor; FH4: methyltetrahydrofolate.[9]

An increased load of various reactive oxygen species (ROS) and lipid peroxidation have been implicated in hyperhomocysteinemia. Moreover, this imbalance leads to oxidative modification of the structure of cellular membrane and intracellular molecules, oxidation of LDL (formation of ox-LDL) and a decrease in nitric oxide (NO), all of them have detrimental effects on vascular permeability, thus creating abnormalities of cardiac perfusion.[10]

High HCY influences cholesterol to convert into lipoproteins, damaging the arteries by plaque formation. Some forms of HCY are reported to damage intima of vessels by a direct action[11] by enhancing the formation of products that can injure the endothelium by excessive sulfation of collagen.[4,7]

Pyridoxine (Vitamin B_6) is a water-soluble vitamin important for carbohydrate, lipid, and amino acid (methionine, cysteine, homocysteine, etc.) metabolism.[12] Its deficiency has been implicated in conditions of elevated HCY and risk of atherosclerosis. A study on type IV secondary hyperlipoproteinemia patients concluded that vitamins (pyridoxine, cyanocobalamin) and folate lower HCY and augment the lipid and lipoprotein-lowering[13]. Intravenous pyridoxine therapy is found to be helpful and appropriate in decreasing serum HCY in renal patients.[2]

Pyridoxal phosphate (PLP) is a coenzyme in reactions of transamination, deamination, decarboxylation and trans-sulfuration in the body (Fig. 1). Deficiencies of the involved enzymes may become better after pyridoxine administration. Pyridoxine is a cofactor in the breakdown of methionine to cysteine by the same enzyme[14] its deficiency leads to elevated plasma HCY as it may disturb the trans-sulfuration pathway and increase the risk of vascular and cardiovascular pathologies[15,16]. It also converts tryptophan to nicotinic acid which also has hypolipidemic activity.[12] It has also been suggested to have antioxidant potential because its deficiency increases lipid peroxidation in liver and blood[17], as it is involved in the synthesis of glutathione (powerful antioxidant).[18]

Several botanicals e.g. turmeric (*curcuma longa*)[11], ginger (*Zingiber officinale*)[19] and cinnamon (*Cinnamomum cassia*) have also been researched upon for the potential for treating hyperhomocysteinemia and preventing its complications. Cinnamon modulates homocysteine and overall oxidative stress[10, 19]. A higher activity of glutathione peroxidase has been attributed which is protective against oxidative damage[20]. Polyphenols present in cinnamon are also powerful antioxidants[21].

Statins, commonly used for the treatment of dyslipidemia, are strong inhibitors of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase. They have been reported to provide time-dependent protection against the lipid-related oxidative injury augmented by high cholesterol diet-induced hyperhomocysteinemia. It not only improved lipid parameters but also decreased hepatic lipid peroxidation whereas raised glutathione and antioxidant markers; hence, protective.[10]

Statins lower serum lipids especially plasma total cholesterol, low-density lipoprotein and triglycerides.[22] Pitavastatin (PIT) is a safer statin, which is also the newest. Its potency is comparable to atorvastatin and simvastatin.[23, 24] It is strongly

suggested that pitavastatin has a superior safety profile as per the clinical advantage and safety [24, 25].
In this study we employed pyridoxine, *Cinnamomum cassia* (aqueous bark extract) and pitavastatin (another statin) to compare their HCY-lowering effects: alone and in various combinations to search for the most efficient remedy.

MATERIAL AND METHODS

Pyridoxine (25mg) tablets and Pitavastatin (2mg) tablets were purchased from Clinix Pharmacy, Lahore. Cinnamon bark was purchased from Hamdard Dawakhana, Lahore.
Aqueous *Cinnamomum cassia* bark Extract (ACCE) was prepared at Pharmacology Laboratory, UHS, Lahore. 1kg *Cinnamomum cassia* bark was washed and dried in a shady place and later extracted with 4L of distilled water at 90°C for 16 hours. The process was repeated twice. Extract was filtered and then freeze-dried (lyophilized) for storage and preservation at room temperature till use. It is ready to be used after being diluted in normal saline (0.9%) and orally administered at 200mg/kg. Dry yield was 8% (w/w). [26]
60 healthy young (6 weeks of age) male albino rats weighing 150-170 grams, were purchased from UHS, Lahore and were kept in the Animal House of Experimental and Research Laboratories, UHS. They were left to acclimatize for 2 weeks and were kept under standard lighting conditions and room temperature of 25 ± 10°c, with relative humidity of 60-70% (maintained). They were fed laboratory prepared diet and tap water, and libitum. HFD was used for induction of hyperhomocysteniemia in experimental groups and it was purchased from University of Veterinary and Animal Sciences, Lahore.
Controls received normal diet throughout 60 days. Group 2 received High-Fat Diet for only 30 days and was later on reverted to normal laboratory diet till the end of the study period (Day 60) and were not given any treatment to equate the vital preventive role of only dietary modification. The rest of the groups 3-10 were continued on HFD throughout[27] and treatment with different drugs (Fig-2) was started at day 30 which continued till Day 60.
10 groups of 6 animals each were used in this study (Table-2).

Groups
Control Group:
Group 1
Preventive Group:
Group 2 (HFD + Normal Diet)
Experimental Groups:
Group 3 (HFD + 0.3mg/kg Pitavastatin)
Group 4 (HFD + 18mg/kg Pyridoxine)
Group 5 (HFD + 200mg/kg Aqueous Cinnamon Extract - ACCE)
Group 6 (HFD + 0.3mg/kg Pitvastatin + 18mg/kg Pyridoxine)
Group 7 (HFD + 18mg/kg Pyridoxine+200mg/kg ACCE)
Group 8 (HFD + 0.3mg/kg Pitavastatin+200mg/kg ACCE)
Group 9 (HFD + 0.3mg/kg Pitavastatin + 18mg/kg Pyridoxine + 200mg/kg ACCE)
Group 10 (HFD + 0.15mg/kg Pitavastatin + 9mg/kg Pyridoxine + 100mg/kg ACCE)
Groups 2-10 were given HFD for induction which was composed of: Casein - 120g, Corn starch - 549.6g; Soybean oil - 250g; Cholesterol - 10g, Choline - 0.4g; Salt mixture - 50; Vitamin mixture – 10g; Cellulose – 10g; Total calories (Kcal)/1000g of diet = 5018.4 Kcals[35]

Table-2: Groups Distribution and composition of High-Fat Diet for experimental groups

Serum homocysteine levels were checked at Day 0, Day 30 and Day 60. Samples were collected via Cardiac puncture technique (Fig-3) into serum vials containing anticoagulant, centrifuged at 3000 RPM for 15 minutes for plasma to separate and stored in a freezer at -20 ± 2ºC in Eppendorf tubes. Biochemical tests were performed on Beckmann's Auto analyzer by biochemical kits at Biochemistry Laboratory of Shaikh Zayed Hospital, Lahore.

Fig-2: Dose administration

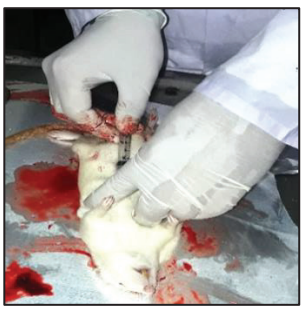

Fig-3: Cardiac puncture technique

Statistical analysis:
The data was analyzed using SPSS version 20.0. Mean ± SD was given for quantitative variable i.e. homocysteine. At each given time, comparison among groups was made by using One-way ANOVA. A p-value of < 0.05 was considered statistically significant.

RESULTS

Group	n	Serum Homocysteine in µmol/L *			
		Day 0	Day 30 (After Induction)	Day 60 (After Treatment)	
		Mean ± SD	Mean ± SD	Mean ± SD	ANOVA (Among Groups) - Day 60
1	6	7.087 ± 0.226	6.782 ± 0.364	6.952 ± 0.269	0.000
2	6	7.102 ± 0.323	10.220 ± 0.383	10.067 ± 0.674	
3	6	6.647 ± 0.382	10.082 ± 0.695	9.008 ± 0.440	
4	6	6.822 ± 0.308	10.635 ± 0.252	8.208 ± 0.503	
5	6	7.082 ± 0.348	9.988 ± 0.293	9.282 ± 0.489	
6	6	6.920 ± 0.290	10.113 ± 0.240	8.955 ± 0.516	
7	6	6.747 ± 0.454	9.983 ± 0.451	9.128 ± 0.231	
8	6	6.618 ± 0.128	10.008 ± 0.288	9.077 ± 0.246	
9	6	6.767 ± 0.324	10.032 ± 0.332	8.358 ± 0.297	
10	6	6.473 ± 0.619	10.393 ± 0.305	8.745 ± 0.376	
*In rats, serum homocysteine<10 µmol/L is considered normal					

Table-3: Results for serum homocysteine at Day 0, Day 30 and Day 60

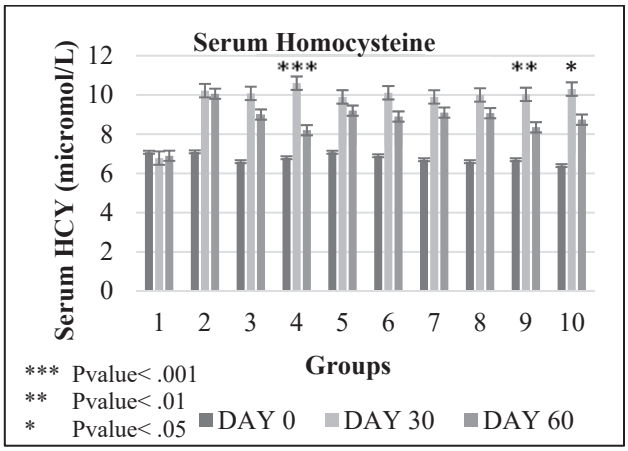

Fig-4: Comparison of serum homocysteine values at Day 0, Day 30 and Day 60

DISCUSSION

Homocysteine (HCY) is one of the several risk factor which culminate in the development, progression and complications of atherosclerosis.[10,11,13] HCY can exert direct pathological actions on the vessel wall leading to angiopathies. However, HCY can indirectly complicate dyslipidemia and its consequences by increasing the chances of lipid incorporation into the vessel wall.[10] Therefore, in this study, HCY levels were assessed to determine the effects of plant products, a vitamin and a standard anti-hyperlipidemic drug on high-fat diet-induced hyperhomocysteinemia.

At baseline, all the rats showed normal levels of serum HCY. In group 1 (control) no fluctuation in HCY occurred for the whole duration of study. After induction of dyslipidemia at Day 30, homocysteine showed a significant rising trend in all the experimental groups, when compared to baseline results. The difference among groups was significant (P: 0.000). However, HCY didn't increase above the upper normal range for rats except for a few readings in a few groups.

At the end of the study, in the preventive Group 2, decline in HCY following the alteration in diet was only a meager 1.6% (from 10.22µmol/L to 10.067µmol/L) when compared to Day 30 results.

In therapeutic groups, treatment presented with declining HCY levels. The most appreciable results were shown by Groups 4, 9 and 10 (Table-3). In Group 4 (PYR 18mg/kg/d) 23% reduction (from 10.635 µmol/L to 8.208µmol/L); in Group 9 (PIT 0.3mg/kg + PYR 18mg/kg + ACCE 200mg/kg) 16.7%reduction (from 10.032µmol/L to 8.358µmol/L) and in Group 10 (PIT 0.15mg/kg + PYR 9mg/kg + ACCE 100mg/kg) a 16% reduction (from 10.393µmol/Lto 8.745µmol/L) was noticed. These results were significantly (P: 0.000) different from Group 2 highlighting the beneficial effects of treatment with drugs in lowering HCY. It is interesting to see half the doses of these agents (Group 10) producing the same effects on hyperhomocysteinemia as full doses (Group 9).

Pyridoxine's HCY-lowering effect has already been established in previous animals and human trials.[9,15,28] Martinez et al. has reported that inadequate intake of vitamin B_6 has substantial effects on one-carbon metabolism and overall protein turnover kinetics, thus affecting homocysteine metabolism.[29] Cystathionine is a substrate for the pyridoxine dependent "cystathionase" enzyme which metabolizes cystathionine to cysteine and α-ketobutyrate in trans-sulfuration pathway, a pathway known to be involved in the metabolism of homocysteine.[9] Statins and cinnamon have also been reported to lower HCY, probably due to their anti-oxidative mechanisms.[10] So, our results have indicated that combination regimens involving all of these agents, have additive or even synergistic effects in lowering

serum HCY and associated risk of atherosclerosis, possibly due to anti-oxidant effects and prevention of lipid inclusion into vessel walls.[7,10]

CONCLUSION

It can be concluded from the results of this study that pyridoxine alone, as well as its combinations with pitavastatin and cinnamon can reduce serum homocysteine levels significantly, and can thus be considered for correcting hyperhomocysteinemia and related disorders, making it a valuable option as an adjunct in reducing the risk of atherosclerosis and subsequently coronary artery diseases in humans.

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