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Metabolism and Toxicity of High Doses of Cyclo (his-pro) Plus Zinc in Healthy Human Subjects

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Abstract

In our previous studies, Cyclo-Z (Cyclo [His-Pro], [CHP] plus zinc) treatment in animals improved insulin sensitivity. However, the adverse effects of these ingredients in humans have not yet been established. his study was to evaluate the pharmacokinetics and possible toxicity of CHP and zinc in healthy human subjects. Forty-nine healthy subjects were randomly divided into 4 groups of 11-15 subjects. ll the subjects consumed 8 study capsules and then submit to a battery of blood draws. Group 1 consisted of subjects who received 8 placebo control capsules. Individuals in Group 2 received 2 capsules of Cyclo-Z containing 3 mg CHP plus 20 mg zinc, in addition to 6 capsules of placebo. Group 3 subjects received 4 capsules of Cyclo-Z and 4 capsules of placebo. Finally, Group 4 individuals received 8 capsules of Cyclo-Z. Blood samples were collected at 0, 2, 4, 8, and 24 hours for the analysis of liver, kidney and lipid panels. Biochemical analyses were also conducted to measure CHP, zinc, and cooper levels for pharmacokinetic analyses. No side effects were experienced in any of the subjects based on physical and blood chemistry examinations. Plasma CHP levels increased at 4 hours in each subject treated with CHP. However, all plasma CHP levels returned to normal levels by 24 hours. Plasma zinc levels were stable and did not increase beyond normal levels. However, a slight increase of plasma zinc at 4 hours was found for Group 4 who received160 mg zinc. There was no copper deficiency found in any of the test subjects. This clinical study demonstrated that acute intake of 24 mg CHP plus 160 mg zinc did not show any clinical side effects or copper deficiency in healthy subjects.

Keywords: Cyclo (his-pro); zinc, toxicity of zinc and CHP; zinc absorption; Cyclo (his-pro) absorption

Introduction

Zinc is involved in more than five physiochemical roles in the control of insulin sensitivity. First, zinc has an insulin-like activity in the absence of insulin. Without insulin binding to the insulin receptor, internalized zinc alone stimulates insulin receptor β-subunit autophosphorylation [1,2]. This activity is not only helpful to the improvement of insulin sensitivity in type 2 diabetics, but also extremely important for type 1 diabetes since zinc will help to utilize glucose in the absence of insulin. Through this zinc action, high blood glucose before insulin treatment can be prevented. Second, zinc is a cofactor for gene expression of Glut-4 [3]. Third, zinc inhibits glucose transport in the small intestine [4]. Fourth, zinc is an integral part of membrane- bound cellular proteases [5]. Lack of proteases is related to impaired insulin receptor-mediated signal transduction by inducing inadequate degradation of used proteins to rebuild the basement membrane [5]. Finally, zinc is also an integral part of insulin degrading enzyme (IDE) [6,7], which is necessary to maintain insulin sensitivity by removing internalized insulin molecules and other used protein fragments that interfere with propagation of insulin receptor mediated signal transduction mechanisms. IDE is located in the endosome, to where insulin bound insulin receptors are transported, insulin is released from its receptor, and IDE degrades insulin molecules to peptides. Finally, these peptides are completely degraded into amino acids in the lysosome, and Insulin receptors are recycled or completely degraded in the cytosol.

Cyclo (his-pro) (CHP) stimulates muscle zinc uptake [8], and increases muscle glucose uptake in Goto-Kakizaki (G-K) rats, a model of type 2 diabetics [9]. Histidyl-proline glycoprotein contains L-histidyl-proline in tandem (the precursor of CHP), and has a very strong zinc-binding activity in the plasma as well as copper binding activity which may transport zinc from small intestine to the tissue cells [10,11]. CHP plus zinc (Cyclo-Z) treatment enhanced IDE synthesis about 30 % more than control brain tissues in human amyloid beta transgenic mice [12]. Cyclo-Z treatment in diabetic animals ameliorated insulin resistance in rats and mice [9,13]. Treatment of young (1.5- month-old) G-K rats with Cyclo-Z for 4 weeks significantly decreased development of hyperglycemia for more than 2 months despite of the cessation of treatment [9]. These results suggest that Cyclo-Z may prevent and treat insulin resistant and diabetic patients as proven by troglitazone and metformin treatments [14-16]. Furthermore, Cyclo-Z treatment improved body weight control very significantly in obese and overweight rats [17]. Thus, it is hypothesized that Cyclo-Z intake may be effective in preventing and treating human diabetes and obesity with no or minimal side effects. However, no study has been performed to determine the clinical toxicity and pharmacokinetics of CHP and zinc. This study was designed to determine the effect of acute consumption from 3 to 24 mg CHP plus 20 to 160 mg zinc on the safety and pharmacokinetic profiles of CHP and zinc in humans.

Materials and Methods

Subjects recruitments

Tolerability of consuming Cyclo-Z capsules (each containing 3 mg CHP plus 20 mg zinc) in escalating doses was monitored to assess any adverse side effects and physical signs of intolerability. Zinc was in the form of zinc oxide fortified with gluconate. No difference in the

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intestinal zinc absorption between zinc oxide and zinc sulfate were reported although zinc oxide is less soluble than zinc sulfate in the water [18]. The eligibility of study subjects were screened with a full medical history and physical examination. Healthy volunteers were tested in a double-blind random order fashion Latin square design. In order to examine any physiochemical abnormalities due to Cyclo-Z intake, multiple blood measurements were performed over a 24-hour period after the intake of escalating doses of Cyclo-Z. For each test dose, at least 11 subjects were given the same dose. Subjects had to have baseline data and may have two Cyclo-Z dose data in different doses. Subjects entered the Phase 1 clinical trial unit before 8 AM after 12-hour fasting. Baseline measurements included: symptom checklist, vital signs (temperature, pulse, blood pressure, and respiration), complete blood count, chemistry panels, lipid profile, plasma CHP, zinc and copper levels, and electrocardiogram. Blood count, chemistry panels, and lipid profiles were analyzed by the clinical chemists in the VA Department of Clinical Chemistry Laboratory. Plasma CHP levels were evaluated using HPLC methods at the UCLA School of Medicine Department of Biochemistry. Zinc and copper were analyzed using ICP-MS methodology at the UCLA Department of Biochemistry. The symptom questionnaire was administered at baseline, and the questionnaire was repeated at 2, 4, 8 and 24 hours to check any patient complaining about the drug doses. This study is in accordance with the ethical standard of VA Greater Los Angeles Healthcare System Ethical Committee.

Subjects eligibility

Inclusion criteria: All subjects who met the following inclusion criteria:

1) Healthy subjects without any serious medical problems.

2) All ethnic groups

3) Both men and women.

4) Female subjects must not be lactating and must either be at least 12 month postmenopausal or surgically sterilized by bilateral tuba ligation, bilateral oophorostomy or hysterectomy.

Test (A) (Units)	0 hours		2 hours		4 hours		8 hours		24 hours		P-	Signi-	Normal
8 Placebos	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	values	ficance	Range
Sodium (mmol/L)	139.9	0.4867	139.1	0.613	139.75	0.6296	140.5	0.5337	139.133	0.4125	0.3352	N.S.	136-146
Potassium (mmol/L)	4.31	0.073	4.456	0.1082	4.502	0.1278	4.4736	0.1153	4.348	0.1057	0.658	N.S.	3.5-5.3
Chloride (mmol/L)	105.1667	0.7196	104.7	1.162	105.2	0.9153	104.2	1.23	104.83	0.958	0.9569	N.S.	98-106
CO_2 (mmol/L)	29.72	0.6545	29.51667	0.3834	29.1667	0.4761	30.62	0.2194	29.267	0.2827	0.1375	N.S.	22-31
Urea Nitrogen (mg/dL)	12	0.5887	11.714	0.6619	12.4	0.6734	12.6	0.8272	12.83	0.8234	0.8145	N.S.	4.0-15.0
Creatinine (mg/dL)	1.0133	0.05058	1.027	0.05474	1.02	0.05629	1.1071	0.07051	1.02	0.05089	0.7559	N.S.	0.5-1.4
EGFR (mL/min)	91.6	5.293	91.07	6.119	91.733	5.571	84.43	5.229	90	5.169	0.8697	N.S.	100-125
Glucose (mg/dL)	106.5	2.83	102.5	4.247	99.067	3.399	99.286	2.858	102.2	3.76	0.5542	N.S.	70-110
Alkaline Phosp. (U/L)	71.8	5	72.73	5.794	74.214	5.791	74.71	5.311	74.2	5.577	0.9956	N.S.	33-94
ALT/WLA (U/L)	23.063	2.22	23.73	2.576	24.231	2.727	24.5	2.851	24.6	2.675	0.9935	N.S	7.0-45
Bilirubin (mg/dL)	0.7	0.05422	0.7	0.04364	0.66	0.04338	0.543**	0.04389	0.74	0.0466	0.0061	**	0.2-1
RBC (M/uL)	4.729	0.1053	5.381	0.6775	4.717	0.102	4.7047	0.107	4.789	0.1159	0.5068	N.S.	4.4-5.9
Hemoglobin (g/dL)	13.91	0.2053	13.694	0.2014	13.727	0.2141	13.75	0.1952	13.933	0.1681	0.8683	N.S.	13.3-17.7
Hematocrit (%)	40.86	0.5364	40.456	0.5837	40.633	0.517	40.767	0.5444	41.407	0.5871	0.7937	N.S.	39-52
MCV (fL)	86.65	1.382	86.653	1.402	86.393	1.395	86.953	1.35	86.8	1.372	0.999	N.S.	80-99
MCH (pg)	29.573	0.6049	29.329	0.5988	29.29333	0.5992	29.393	0.5926	29.253	0.592	0.9962	N.S.	27-34
MCHC (g/dL)	33.95	0.2145	33.836	0.1869	33.76	0.2147	33.76	0.1802	33.6875	0.2404	0.921	N.S.	32-36
RDA (%)	14.114	0.2307	14.08571	0.2424	14.16	0.2638	14.12	0.2213	14.153	0.2286	0.9995	N.S.	12.0-15.0
PLT (k/uL)	227.3571	27.696	257.8	13.592	260.93	13.055	265.27	15.019	268.29	16.674	0.5081	N.S.	150-440
WBC (k/uL)	6.2067	0.403	6.4438	0.4899	6.42	0.3721	6.48	0.3478	6.5933	0.4366	0.9763	N.S.	4.5-11
LYMP%-A (%)	25.727	2.045	25.887	1.897	28.32	2.057	30.033	2.425	25.973	2.2211	0.5259	N.S	20-40
LYMP#-A (#/uL)	1513.3	87.759	1566.7	90.326	1753.3	102.76	1906.7	149.75	1636.7	105.77	0.0947	N.S.	600-4800
Monocyte % (%)	8.7667	0.463	8.12	0.4649	8.6733	0.5781	9.0933	0.2826	8.9667	0.3994	0.5927	N.S.	2.0-10.
Monocyte # (#/uL)	514.29	31.797	493.33	38.379	560	40	578.57	35.342	580	40.473	0.3588	N.S.	0-950
PMN% (%)	61.747	2.401	62.573	2.174	59.267	2.456	56.107	2.574	61.62	2.431	0.3231	N.S.	41-85
PMN# (#/uL)	3926.7	385.88	4086.7	450.27	3860	354.27	3692.9	303.38	4146.7	403.77	0.9235	N.S.	1100-7700
Eosinophil % (%)	3.22	0.6681	2.8933	0.6384	3.4769	0.6575	3.6923	0.6253	2.8733	0.4922	0.9117	N.S.	1.0-6.0
Eosinophil # (#/ul)	207.14	43.517	186.67	38.872	220	43.86	235.71	42.753	193.33	38.379	0.9212	N.S.	0-500
Basophil % (%)	0.54	0.03491	0.5267	0.07268	0.5714	0.05107	0.607***	0.07193	0.5667	0.05745	0.0001	***	0-1
Basophil # (#/ul)	13.33	9.085	20	10.69	21.429	10.995	35.714	12.839	33.333	12.599	0.5963	N.S.	0-200

**P<0.01; ***p<0.001 (at 0 vs. at 8 hours)

Table 1: Study Subjects took 8 capsules of Placebos.

5) Age 18 and above. Since this is the first toxicity study with Cyclo-Z, children younger than 18 years old were excluded from this study

Exclusion criteria: Subjects were excluded if they possessed any of the following criteria:

1) Taking any prescribed anti-diabetic medications which may affect study results

2) Any disease likely to limit risks of interventions:

3) Cancer requiring treatment in the past 5 years, with the exception of cancers which have been cured or in the opinion of the investigator carry a good prognosis.

4) Infectious disease: HIV positivity, and active Tuberculosis

5) Cardiovascular disease

6) Uncontrolled hypertension with blood pressure with average systolic blood pressure of > 160 mmHg and diastolic blood pressure > 95 mmHg on two screening visits. Pulse rate > 95 beats per minute on both screening visits

7) Gastrointestinal disease of any sort

8) Anemia: Hematocrit of < 36.0% in men or < 33% in women.

9) Exclusion for conditions or behaviors likely to affect the conduct of the study

10) Excessive alcohol intake

11) Exclusions related to medications such as Monoamine oxidase inhibitors (e.g. phenelzine, procarbazine, selegiline, furazolidone), and Antidepressive agents (lithium, prozac, zoloft, serzone, paxil, efflexor)

12) Any other medication that, in the opinion of the Investigator, may pose harm to the patient or affect the intervention.

Test B (Units)	0 hours		2 hours		4 hours		8 hours		24 hours		P-	Signi-	Normal
6placebo + 2 Cyclo-Z	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	values	ficance	Range
Sodium (mmol/L)	139.1	0.3877	139	0.6181	139.2	0.6159	139.3	0.6817	139.78	0.5646	0.3456	N.S	136-146
Potassium (mmol/L)	4.215	0.1143	4.092	0.104	4.235	0.1004	4.231	0.09353	4.2873	0.09184	0.5129	N.S	3.5-5.3
Chloride (mmol/L)	102.75	0.25	101.8	0.3498	101	0.7236	101.7	0.6186	103.2	0.9293	0.1615	N.S.	98-106
CO_2 (mmol/L)	30.83	0.679	29.96	0.3781	29.1667	0.4437	28.1**	0.5331	30.02	0.4505	0.0073	**	22-31
Urea Nitrogen (mg/dL)	14.25	1.388	13.2	0.3374	11.75	0.25	15.533	2.15	12.4	0.7797	0.0742	N.S.	4.0-15.0
Creatinine (mg/dL)	1.022	0.06742	0.991	0.05789	0.975	0.05789	0.9667	0.04942	0.9909	0.07384	0.825	N.S.	0.5-1.4
EGFR (mL/min)	96.78	9.175	98.73	7.966	100	7.967	97.44	5.144	101	12.449	0.6886	N.S.	100-125
Glucose (mg/dL)	97.56	9.175	96.45	7.966	95.2	7.967	95.778	5.144	94.818	12.449	0.7294	N.S.	70-110
Alkaline Phosp. (U/L)	72.25	5.10	80.9	6.033	91.111	6.308	91.78	7.447	82.82	10.441	0.0652	N.S.	33-94
ALT/WLA (U/L)	42.9	13.469	36.89	6.199	32.5	6.144	34.11	6.232	31.82	7.619	0.1214	N.S	7.0-45
Bilirubin (mg/dL)	0.756	0.07689	0.75	0.08925	0.578	0.0965	0.544*	0.0862	0.6545	0.0131	0.0411	*.	0.2-1
RBC (M/uL)	5.853	0.1202	5.627	0.1198	5.678	0.1102	5.626	0.1014	5.697	0.09829	0.0856	N.S.	4.4-5.9
Hemoglobin (g/dL)	13.78	0.2527	13.7	0.0528	13.722	0.1011	13.69	0.1372	13.691	0.266	0.0863	N.S.	13.3-17.7
Hematocrit (%)	40.45	0.4029	40.482	0.4193	40.422	0.2225	40.2	0.391	40.509	0.5677	0.0082	N.S.	39-52
MCV (fL)	84.93	1.195	85.445	1.219	87.1	1.25	86.567	1.163	85.109	1.303	0.9306	N.S.	80-99
MCH (pg)	28.733	0.5347	29.027	0.5172	29.533	0.5963	29.522	0.4924	28.791	0.5132	0.9509	N.S.	27-34
MCHC (g/dL)	33.68	0.1438	33.918	0.1074	33.95556	0.1981	34.0444	0.1543	33.782	0.09856	0.0468	*	32-36
RDW (%)	14.238	0.0738	14.1	0.2472	13.7556	0.1615	13.833*	0.1615	13.95	0.1355	0.0045	0.034	12.0-15.0
PLT (k/uL)	232.889	28.186	258	29.163	265.13	37.2	269.56	28.082	254.55	26.61	0.9998	N.S.	150-440
WBC (k/uL)	5.744	0.3258	5.8091	0.2614	5.822	0.3899	6.311	0.3247	5.8545	1.404	0.7898	N.S.	4.5-11
LYMP%-A (%)	29.422	0.5038	28.773	1.363	31.887	0.8981	30.533	1.571	29.745	0.9703	0.0936	N.S	20-40
LYMP#-A (#/uL)	1688.9	113.82	1640	130.34	1844.4	128.01	1811.1	126.81	1700	83.597	0.1563	N.S.	600-4800
Monocyte % (%)	10.411	0.2039	9.1818	0.5774	8.6778	0.7213	8.5889	0.7933	9.4455	0.7731	0.0401	N.S.	2.0-10.
Monocyte # (#/uL)	588.89	30.151	536.36	39.166	522.22	65.711	533.33	54.356	464.47	56.407	0.4749	N.S.	0-950
PMN% (%)	55.567	2.915	57.045	1.589	53.178	1.083	54.944	1.383	55.945	0.9473	0.0748	N.S.	41-85
PMN# (#/uL)	3062.5	145.19	3318.2	93.845	3100	151.51	3662.5*	164.28	3345.5	130.34	0.0114	*	1100-7700
Eosinophil % (%)	4.0778	0.7256	4.4545	0.6949	5.6889	0.6814	5.4222	0.7279	4.3909	0.6685	0.9905	N.S.	1.0-6.0
Eosinophil # (#/ul)	233.33	47.672	263.64	53.832	333.33	54.356	333.33	45.227	254.55	52.225	0.8746	N.S.	0-500
Basophil % (%)	0.522		0.5455	0.02611	0.578***	0.01306	0.5111	0.02132	0.4727	0.025	0.0002	***	0-1
Basophil # (#/ul)	11.111	0	18.182	0	11.111	0	11.111	0	0	0	0	N.S.	0-200

*p<0.05; **p<0.01; ***p<0.001 (At 0 hour vs. at 4 or 8 hours)

Table 2: Study Subjects who took 2 capsules of Cyclo-Z and 6 capsules of Placebos.

iii. Lipid panel: triglyceride, LDL, HDL, total cholesterol.

iv. Pharmacokinetic data: CHP, zinc, and copper

This study was performed with FDA-IND approval (IND #: 61, 897) and with approvals from the VA Greater Los Angeles Healthcare System IRB and R & D Committee.

Statistical analysis

Final analysis: An intent-to-treat paradigm was used for statistical analysis of all

Subject's data obtained at baseline compared to data obtained at 2, 4, 8, and 24 hours. Toxicity study was of particular importance, to see if there is increased incidence of adverse events with the consumption of 2-, 4- or 8-capsules of Cyclo-Z. In addition to repeated measure analysis of variance on laboratory measures, Poisson analysis was done to compare the relative rates of adverse events in each Cyclo-Z treatment group as compared to the control group.

Sample size consideration: A sample size of 10 per group would detect differences between placebo and Cyclo-Z treatment groups for any adverse effects. Using 0.5% as a minimum mean change with standard deviation estimated at 0.8%, the probability of detecting a clinically significant change among treatment groups is 81% with 10 patients per group (overall F-test with pair-wise contrasts). We planned to randomize 52 patients, 13 per each treatment group estimating that 2-3 subjects could drop out during the test period in each group. One subject can participate in 2-3 study groups after a 1-week washout period. Since this is an early phase study designed to evaluate safety, this sample size is generally considered adequate. Very low rates of adverse events or no side effects were expected. If the rate of reported adverse effects (AEs) among controls is 0.1, a sample size of 10 per treatment group would be adequate to detect a relative risk of 1.0 among treatment groups.

Results

A total 49 healthy volunteers completed all of the study procedures. The double blind study showed no adverse effects in subjects taking one time oral administration of 0, 2, 4, or 8 capsules of Cyclo-Z (Tables 1-4). All subjects had normal blood chemistry levels and cell numbers at the start of the trial (0 hours) and nearly all *p*-values of the

Test D (Units)	0 hours		2 hours		4 hours		8 hours		24 hours		P-	Signifi-	Normal
8 Cyclo-Z	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	values	cance	Range
Sodium	139.5	0.4713	139.5	0.3793	139.08	0.5568	140.17	0.4508	139.92	0.4681	0.5107	N.S.	136-146
Potassium (mmol/L)	4.148	0.078	4.314	0.1209	4.3	0.1004	4.1758	0.0719	4.1091	0.0757	0.4322	N.S.	3.5-5.3
Chloride (mmol/L)	105.2	0.8001	103.4	0.9275	104.3	1.022	103.5	1.19	104.4	0.8124	0.688	N.S.	98-106
$C0_2$ (mmol/L)	29.46	0.6547	29.4	0.5874	27.96	1.142	28.8	1.523	29.36	0.7243	0.7977	N.S.	22-31
Urea Nitrogen (mg/dL)	13.4	1.208	12.6	1.632	13.8	1.157	14.6	1.208	12.2	0.9696	0.6807	N.S.	4.0-15.0
Creatinine (mg/dL)	0.925	0.06411	0.942	0.06207	0.917	0.07679	1.0333	0.08195	0.9333	0.0666	0.7451	N.S.	0.5-1.4
EGFR (mL/min)	103.3	6.051	101	6.279	107.83	9.036	92.17	5.875	102.7	6.634	0.5963	N.S.	100-125
Glucose (mg/dL)	107.6	5.912	91.67	6.261	92.583	2.832	88.333*	2.435	103.92	7.13	0.0495	*	70-110
Alkaline Phosp (U/L)	76	8.842	75.46	8.395	78.333	8.804	78.75	7.705	75.75	8.865	0.9978	N.S.	33-94
ALT/WLA (U/L)	34.667	4.978	33.54	4.848	34.417	5.129	35.17	5.11	35.58	5.479	0.9989	N.S.	7.0-45
Bilirubin (mg.dL)	0.842	0.0829	0.807	0.0759	0.718	0.0645	0.667	0.0753	0.8417	0.0583	0.342	N.S.	0.2-1
RBC (M/uL)	4.709	0.1155	4.688	0.1236	4.689	0.1184	4.6633	0.1156	4.665	0.1221	0.9987	N.S.	4.4-5.9
Hemoglobin (g/dL)	14.08	0.2826	14.1	0.2753	14.042	0.3103	13.93	0.2944	13.883	0.3005	0.9798	N.S.	13.3-17.7
Hematocrit (%)	41.442	0.7754	41.667	0.7188	41.383	0.7507	41.275	0.753	40.983	0.7868	0.9788	N.S.	39-52
Mean C Volume (fL)	83.56	5.17	88	1.354	88.285	1.318	89.009	1.616	88.317	1.435	0.5916	N.S.	80-99
MCH (pg)	30.127	0.7212	30.033	0.6379	30.09167	0.6578	29.975	0.656	29.942	0.6759	0.9996	N.S.	27-34
MCHC (g.dL)	34.045	0.281	33.858	0.2475	33.94167	0.2379	33.7417	0.1975	33.85	0.2512	0.9312	N.S.	32-36
Red Dist.Width (%)	13.885	0.3013	13.73333	0.329	13.8	0.3136	13.7167	0.2966	13.736	0.3304	0.9945	N.S.	12.0-15.0
Platelet (k/uL)	225.6923	13.542	229.3333	15.742	222.25	14.748	228.5	14.892	226.67	12.891	0.9974	N.S.	150-440
WBC (k/uL)	6.5583	0.6437	6.6333	0.7451	6.8545	0.6131	6.7545	0.59	6.4333	0.5699	0.9917	N.S.	4.5-11
Lymphocyte-A (%)	25.7	2.801	24.925	2.498	27.1	2.335	29.192	2.162	25.775	1.971	0.7356	N.S.	20-40
Lymphocyte-A (#/uL)	1553.8	160.77	1591.7	181.52	1783.3	194.95	2009.1	218.03	1625	167.03	0.4167	N.S.	600-4800
Monocyte (%)	8.8385	0.8329	8.0333	0.6242	8.5833	0.6911	8.5273	0.556	8.325	0.6901	0.9408	N.S.	2.0-10.
Monocyte (#/uL)	546.15	58.415	525	59.193	558.33	55.677	600	72.613	541.67	54.297	0.932	N.S.	0-950
PMN (%)	61.592	3.17	63.123	2.97	59.842	2.84	57.018	2.675	61.355	2.796	0.6598	N.S.	41-85
PMN (#/uL)	4084.6	605.93	4258.7	676.28	3984.6	469.3	3910	461.55	4025	493.45	0.9941	N.S.	1100-7700
Eosinophil (%)	3.6154	0.7501	3.5583	0.8083	3.9417	0.7941	4.2727	0.8117	3.7	0.8476	0.9715	N.S.	1.0-6.0
Eosinophil (#/uL)	215.4	55.287	209.09	63.896	250.04	64.536	281.82	61.523	233.33	56.855	0.9223	N.S.	0-500
Basophil (%)	0.4308	0.04585	0.4333	0.05195	0.5333	0.05271	0.4636	0.06778	0.55	0.0646	0.418	N.S.	0-1
Basophil (#/uL)	0	0	0	0	8.3333	8.333	9.0909	9.091	8.333	8.333	0.9999	N.S.	0-200

*p<0.05 (At O vs. at 8 hours)

Table 4: Study Subjects who took 8 capsules of Cyclo-Z.

means indicated that there were no significant changes among these values after Cyclo-Z intake. Less than 0.05 *p*-values indicate statistical significance and only the bilirubin values in the placebo group (Table 1) and glucose values in the 8 capsules of Cyclo-Z group (Table 4) were significantly different from the baseline ($p < 0.05$), but the levels were still all within the normal ranges. Comparisons among different study groups were not performed since all the biochemical data were within the normal ranges. These findings showed that 8 capsules of Cyclo-Z effectively reduced blood glucose levels from 107.6 ± 5.9 to 88.3 ± 2.4 mg/dL from baseline at 8 hours after a single dose of 8 capsules of Cyclo-Z. Twenty-four hours later, the blood glucose levels returned to approximately the initial levels from 108 to 102 mg/dL.

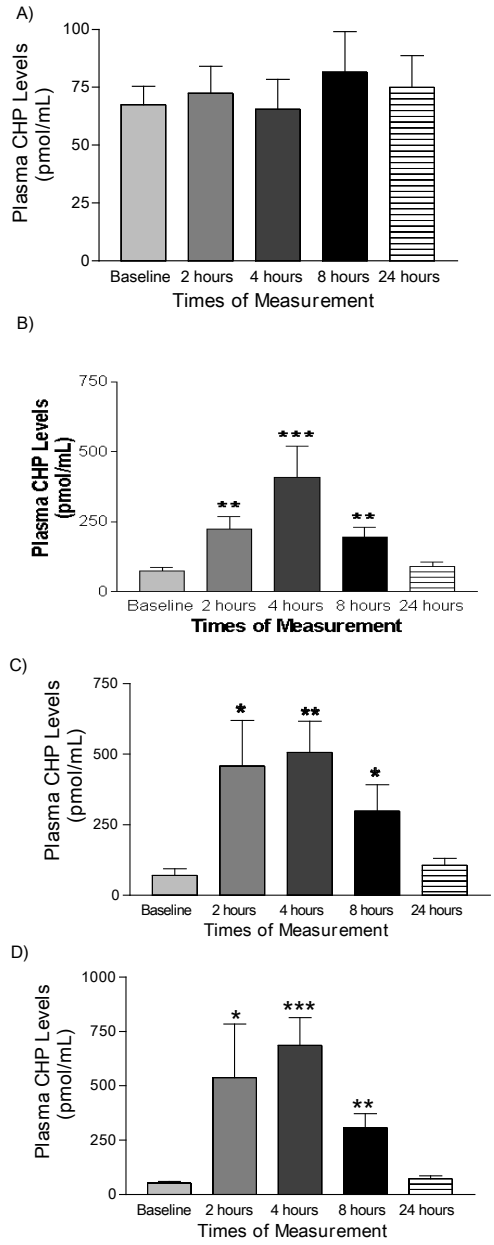

Figure 1: Mean (± SEM) plasma CHP levels measured at 0, 2, 4, 8, and 24 hours in all the healthy study subjects who consumed 8 study pills before breakfast. A: 8 capsules of placebo (n=15). B: 2 Cyclo-Z and 6 Placebo capsules (n=11). C:4 Cyclo-Z and 4 Placebo capsules (n=11). D: 8 capsules of Cyclo-Z (n=12).

Pharmacokinetic data for CHP: CHP concentrations in blood samples collected at 0, 2, 4, 8, and 24 hours after oral ingestion of Cyclo-Z were measured by HPLC method at the UCLA Department of Chemistry. The average plasma CHP concentration for subjects taking placebo was unchanged over a 24 hour period (Figure 1A). Although the normal plasma CHP concentration is approximately 75pmol/mL, the highest CHP levels in all of the study groups were at 4 hours after oral ingestion of Cyclo-Z capsules. After ingestion of two capsules of Cyclo-Z, plasma CHP levels increased to 409 ± 110.8 pmol/mL at 4 hours (Figure 1B), which is an increase of 366.5pmol/mL from baseline. When the dose of Cyclo-Z was doubled to 4 capsules, plasma CHP levels increased to 506.0 ± 110.5pmol/mL at 4 hours. The increase from baseline was 435.5pmol/mL, which is only 18.8 % higher than the level after taking 2 capsules (Figure 1C). When the dose of Cyclo-Z was increased to 8 capsules, plasma CHP concentration increased to 632.6pmol/mL (Figure 1D), which is an increase of 72.6 % compared to levels after taking 2 capsules. At 8 hours after taking 2 capsules of Cyclo-Z, the level of CHP was only 122.4pmol/mL above basal levels. After taking 4 capsules, CHP concentration increased to 227.2pmol/mL at 8 hours. This increase was about double level of 2 Cyclo-Z doses. When 8 capsules were taken, plasma CHP concentration increased to 254.3pmol/mL from the baseline, which is about the same increase as that of consuming 4 capsules of Cyclo-Z. Essentially, all of the orally ingested CHP had been completely metabolized within 24 hours for all three Cyclo-Z doses.

Pharmacokinetic data for zinc: As shown (Figure 2B-Figure2D), high dose of zinc did not increase plasma zinc levels after the intake of 160 mg zinc with 24 mg CHP. Zinc is absorbed linearly up to 10 mg zinc intake [19]. When zinc intake is increased from 10 mg to 30 mg, zinc was absorbed parabolic manner between 8 to 12 mg. Then, very little additional zinc absorption occurs from more than 30 mg zinc intake. However, when 8 capsules of Cyclo-Z were given to the study subjects, plasma zinc levels showed a tendency to increase slightly at 4 hours without a significant difference from the baseline zinc levels.

Plasma Copper level changes: The most important zinc toxicity is copper deficiency. As shown (Figure 3B-figure 3D), high dose of zinc did not decrease plasma copper levels, indicating that there is no copper deficiency when consumed acutely 160 mg of zinc with 24 mg CHP.

Discussion

High levels of CHP are present in many food sources [20-22], and readily absorbed in the gut without chemical or enzymatic destruction [23,24]. Naturally occurring CHP is distributed in most of human tissues in high concentrations [25,26] and in several common foods [20-22] and nutritional supplements such as *Ensure* and *Glucerna* [22] In the human semen, there are approximately 5-13 μg/mL CHP [26]. CHP is a endogenous key substrate of the organic cation transporter (OCT2), which is crucial for nigral cell integrity and its deficiency perhaps be a risk for Parkinson's disease [27]. OCT 2 requires CHP as a substrate for its activity. Incubation of human dopaminogenic neuroblastoma cells in medium containing 23. 4 mg/L CHP was studied without killing cells. This study supported our findings that acute human consumption of 24 mg CHP does not pose any toxicity in humans weighing 70 kg (Table 4). Carrier mediated transport across cell membranes is a very important determinant of activity, resistance, and toxicity of chemotherapeutic agents [28]. In the presence of OCT, the IC_{50} was consistently lowered in human immunodeficiency virus infections. CHP treatment also shows a considerable neuroprotective activity in vitro and in vivo [29,30]. CHP is very effective in the neuroprotective activity in traumatic brain

injury in rats and mice. When animals were treated with 0.1 to 10 mg/kg CHP, neuroprotection was observed between 0.5 to 8 hours, but not at 24 hours [29]. This is probably due to the disappearance of CHP after 8 hours as shown in our studies (Figure1B-Figure 1D). As shown in Figs 1A-1D, high CHP level after the intake of Cyclo-Z remained until 8 hours but CHP was completely metabolized or excreted by 24 hours. CHP is also protective against glutamate and β-amyloid neurotoxicity [30]. This effect shows a potential treatment value for Alzheimer's diseases as we have observed in our preliminary data [12].

Acute pretreatment of rats with CHP decreases ethanol induced hypothermia, suggesting that CHP treatment plays an

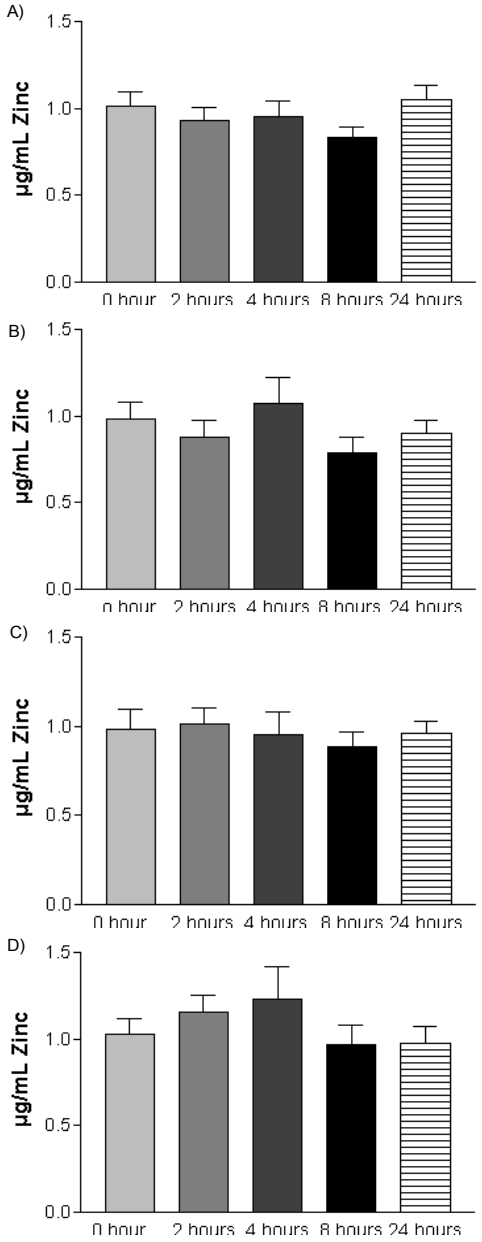

Figure 2: Mean (± SEM) plasma zinc levels measured at 0, 2, 4, 8, and 24 hours in all the healthy study subjects who consumed 8 study pills before breakfast. A: 8 capsules of placebo (n=15). B: 2 Cyclo-Z and 6 Placebo capsules (n=11). C:4 Cyclo-Z and 4 Placebo capsules (n=11). D: 8 capsules of Cyclo-Z (n=12).

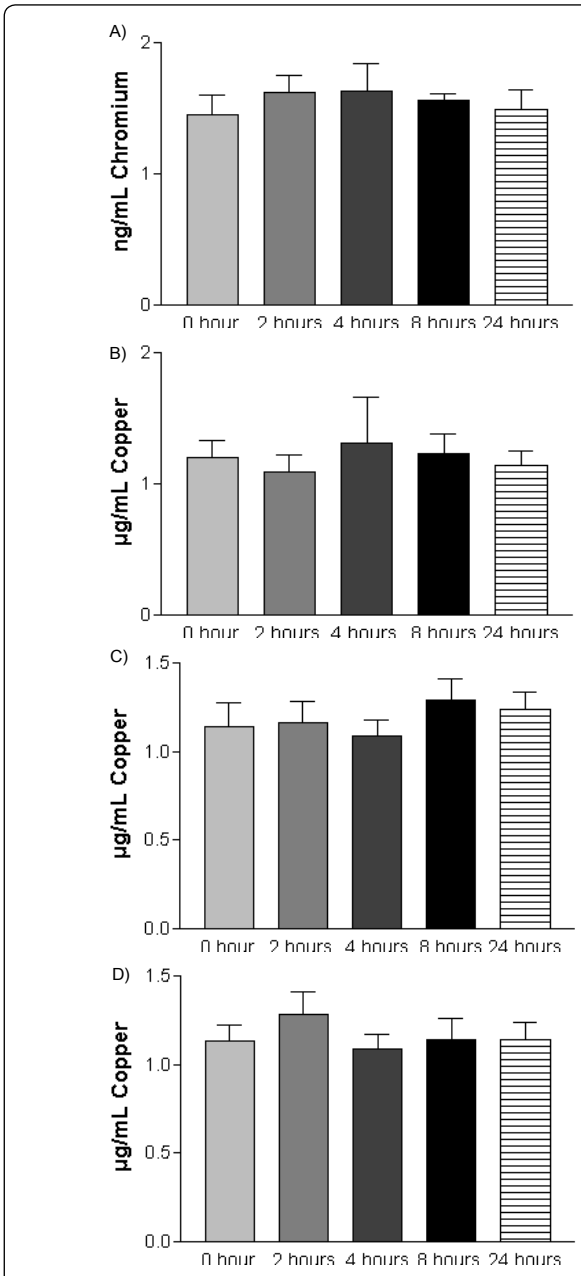

Figure 3: Mean (± SEM) plasma copper levels measured at 0, 2, 4, 8, and 24 hours in all the healthy study subjects who consumed 8 study pills before breakfast. A: 8 capsules of placebo (n=15). B: 2 Cyclo-Z and 6 Placebo capsules (n=11). C:4 Cyclo-Z and 4 Placebo capsules (n=11). D: 8 capsules of Cyclo-Z (n=12). .

important role in ethanol intoxication, tolerance, and/or addiction [31]. Furthermore, CHP reduces neuronal cell death in vitro and in vivo [32]. At a mechanistic level, CHP attenuate both apoptotic and necrotic cell death in primary neuronal cell cultures [30]. CHP protects cells against hydrogen peroxide-mediated apoptotic death [33] and it causes cellular protective responses against paraquat–mediated cell death [34]. The infusion of CHP 2.25 -5.5 nmol/kg/hr for 3 hours, decreased 2-D glucose induced stimulation of pancreatic secretion, which did not cause cell death [35]. When converted this value to mg/kg and calculated for 70 kg weighing humans, it will be 37.1-90.3 mg/70 kg CHP intake/hr. These previous findings [20-35] support the observation that one time consumption of 24 mg CHP is

not toxic (Table 1-Table 4). These data clearly demonstrated that CHP intake is rather protective against genotoxicity and neurotoxicity and yet poses no adverse side effects. Therefore, we do not expect any CHP toxicity in humans during the upcoming phases 2 and 3 clinical trials using Cyclo-Z.

Since 160 mg zinc intake with 24 mg CHP intake did not increase plasma zinc levels (Figure 2D), it is apparent that CHP may act as a buffering agent for zinc metabolism. CHP clearly stimulates intestinal zinc absorption in the everted gut sac experiments and zinc uptake in muscle tissues of rats [8]. However, (Figure 2B-Figure 2D) showed that there are no signs of increased plasma zinc levels when less than 80 mg zinc are consumed, but just showed a tendency of a slight increase within normal ranges in the plasma with160 mg of zinc consumption. Thus, CHP may act as a zinc transport regulating agent to control zinc uptake and excretion from the cells and probably from the small intestine. Under these conditions, no copper deficiency was exhibited with CHP plus zinc treatment (Figure 3B-Figure 3D). These data suggest that Cyclo-Z intake is very safe for human consumption and it rather plays very important biological roles in detoxication from many cell damaging agents or cellular injury. Epidemiological studies have indicated that the prevalence of diabetes and/or glucose intolerance is significantly higher among subjects consuming lower dietary zinc [36,37]. However, intestinal zinc absorption in diabetic animals and humans is decreased [38,39]. Zinc has a protective role in the pathogenesis of Type 1 DM [40,41], and administration of 200 mg zinc sulfate 3 times a day for 60 days improved glucose tolerance in type 2 diabetic patients [42]. However, treatment of diabetic animals and human subjects with high physiological doses of zinc was minimally effective in controlling blood glucose metabolism [43,44]. Toxicity of zinc is low but zinc deficiency is hazardous for human health [45]. In support of this finding, the Agency for Toxic Substances and Disease Registry (ATSDR) prepared toxicological profiles on hazardous chemical for the Comprehensive Environmental Reponses. Compensation and Liability Act (CERCLA). [46], ATSDR and US Department of Human Health Service, published on the toxicological profiles of zinc metabolism including absorption, distribution, metabolism and excretion (ADME).

Infants fed a milk formula supplemented with 4 mg/L zinc in addition of 1.8 mg/L zinc in the existing formula grew significantly more than non treated infants at 6 months [47]. During this treatment period, no sign of zinc toxicity was shown in the zinc supplemented milk fed infants. There are no acute minimum risk level (MRL) data currently available showing at least more than 570 mg zinc is toxic [48]. Long term zinc exposure has been shown to cause copper deficiency. At low doses of about 0.7 to 0.9 mg zinc/kg per day administration for 6-13 weeks showed subclinical changes in copper enzymes such as superoxide dismutase [49,50]. At about 2 mg zinc/kg//day chronic zinc intake induced symptoms of copper deficiency and anemia [51,52]. However, other hematological and immunological studies were performed to show that 40 mg/day zinc supplementation is not detrimental to health in healthy men [53]. The estimated chronic oral MRL for zinc is 0.3mg/kg/day. Thus, 60-90 kg weight subjects should be able to consume 18-27 mg/day of zinc safely. The MRL means that the 18-27 mg zinc/day over a long period of time induces neither nutritional deficiency in healthy, nonpregnant, adult humans, nor results in adverse effects from excess consumption. These findings suggest that CHP plus zinc (Cyclo-Z) may be one of the most effective anti-diabetic agents for the normalization of zinc metabolism in zinc deficient human subjects including type 2 diabetic and/or obese subjects.

In conclusion, conservative estimate suggests that about 25% of world's population is zinc deficient [54]. A large number of populations may benefit from Cyclo- Z intake. Our data (Figure1D) shows that of the 24 mg CHP dose taken in not more than 6-9 mg CHP is absorbed, which is about the optimal dose of it for the treatment of diabetes and obesity [9,12,16]. Thus, a dose of CHP higher than 9 mg will not be more effective in reducing blood glucose or body weight. Furthermore, an excess dose of zinc will not be absorbed more than 12 mg zinc when given zinc more than 30 mg at a time [19]. More importantly, 160 mg zinc with 24 mg CHP does not show any significant increase in plasma zinc levels (Figure 2D). This data shows that CHP may have a significant impact on the physiological and cellular zinc metabolism by acting as a zinc buffering agent. Thus, dose of CHP of more than 9 mg /day with 20 mg zinc may not pose any toxicity or any further benefit for the treatment of diabetes or obesity.

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