Metabolism and Toxicity of High Doses of Cyclo (his-pro) Plus Zinc in Healthy Human 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 diabetes [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...
Medicine Department of Biochemistry. Zinc and copper were analyzed using HPLC methods at the UCLA School of Medicine Department of Biochemistry. Plasma CHP panels, and lipid profiles were analyzed by the clinical chemists in 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 tubal ligation, bilateral oophorostomy or hysterectomy.

Test Table:

| Test (A) (Units) | 0 hours | 2 hours | 4 hours | 8 hours | 24 hours | P- Significance | Normal Range |
|-----------------|---------|---------|---------|---------|----------|----------------|--------------|
| 8 Placebos      | Mean    | SEM     | Mean    | SEM     | Mean     | SEM            |              |
| Sodium (mM/L)   | 139.9   | 0.4876  | 139.1   | 0.613   | 139.75   | 0.6296         | 140.5        |
| Potassium (mM/L)| 4.31    | 0.0738  | 4.456   | 0.1082  | 4.502    | 0.1278         | 4.4736       |
| Chloride (mM/L) | 105.1667| 0.7196  | 104.7   | 1.162   | 105.2    | 0.9153         | 104.2        |
| CO2 (mm/L)      | 29.72   | 0.6545  | 29.5167 | 0.3834  | 29.1667  | 0.4761         | 30.62        |
| Urea Nitrogen   | 32.14   | 0.6545  | 31.714  | 0.6191  | 31.612   | 0.6734         | 32.612       |
| Creatinine      | 1.0133  | 0.05058 | 1.027   | 0.04574 | 1.0171   | 0.05629        | 1.0171       |
| EGFR (mL/min)   | 91.6    | 5.293   | 91.07   | 6.119   | 91.73    | 5.571          | 92.43        |
| Glucose (mg/dL) | 139.9   | 0.4876  | 139.1   | 0.613   | 139.75   | 0.6296         | 140.5        |
| Chloride (mg/dL)| 105.1667| 0.7196  | 104.7   | 1.162   | 105.2    | 0.9153         | 104.2        |
| CO2 (mm/L)      | 29.72   | 0.6545  | 29.5167 | 0.3834  | 29.1667  | 0.4761         | 30.62        |
| Urea Nitrogen   | 32.14   | 0.6545  | 31.714  | 0.6191  | 31.612   | 0.6734         | 32.612       |
| Creatinine      | 1.0133  | 0.05058 | 1.027   | 0.04574 | 1.0171   | 0.05629        | 1.0171       |

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- Significance Normal
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. 3.5-5.5
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
CO₂ (mmol/L) 30.83 0.769 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 0.049 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 0.222 40.2 0.341 40.5 0.677 0.0078 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 29.252 0.4924 28.791 0.5132 0.9509 N.S. 27-34
MCHC (g/dL) 33.68 0.1438 33.918 0.1074 33.9556 0.1981 34.0444 0.1543 33.782 0.09856 0.4868 N.S. 33-36
RDW (%) 14.238 0.0738 14.1 0.2472 13.7556 0.1615 13.833* 0.1615 13.95 0.1355 0.0346 N.S. 12-15
PLT (k/uL) 232.889 28.186 258 29.163 265.13 37.2 269.56 28.062 254.55 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 113.82 1640 130.34 1844.4 128.01 1811.1 126.8 1700 83.559 0.003 N.S. 12-15
Monocyte % (%) 10.411 0.2039 9.1818 0.5774 5.574 0.8571 5.314 0.7213 5.8849 0.7933 0.7731 0.041 N.S. 1.5-10
Monocyte # (#/uL) 588.89 30.151 9.55 9.55 8.55 8.55 8.55 8.55 8.55 8.55 0.0001 ** 12-20
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 * 110-7700

*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.
Randomization

When a subject was eligible to enroll into the study, he/she was asked to complete a Human Study Consent Form. Then the Clinical Research Coordinator notified the Research Pharmacist to assign a specific drug from 4 different gel capsule combinations (8 placebos, 2 Cyclo-Z plus 6 placebos, 4 Cyclo-Z plus 4 placebos, or 8 Cyclo-Z) in a randomized manner into one of 4 study groups. Forty-nine subjects finished the trial with 11 to 15 subjects in each of 4 groups. The pharmacist will keep records of drug distribution with an identifiable code of drug, and date for each study subject. After the initial baseline measurements, each subject took 8 capsules of a mixture of Cyclo-Z and placebo capsules with water over 5-minutes. Then, the subject consumed a high-carbohydrate breakfast with juice. Water was freely available but caffeinated or artificially sweetened beverages were not consumed. A high-carbohydrate lunch was served to be consumed after 2, 4 and 8 hours after taking Cyclo-Z, the symptom questionnaire over a period of < 15 minutes. Subjects could select the food items consumed. A high carbohydrate breakfast was served to be consumed the evening meal without alcohol as prescribed by the unit dietitian. The subjects returned before 8AM the next morning for the last of 24 hour testing. Some subjects participated in more clinical trials, but not participated in more than one test per week.

Physical examinations were performed at the VA Greater Los Angeles Healthcare System, Los Angeles, CA by a study physician. Chemical assays were performed by the Clinical Chemistry Laboratory at the VA Greater Los Angeles Healthcare System. Five blood samples (each containing about 10 mL) were drawn from each patient. One tube of blood was used for testing kidney, liver and lipid panels. The second tube of blood was used for hemogram, and the third sample for CHF, zinc and copper level measurements. The blood chemical analyses were as follows:

- Liver panel: alkaline phosphatase, alanine transaminase, lactic acid dehydrogenase, total blood protein, albumin, and total bilirubin.
- Kidney panel: blood urea nitrogen, sodium, potassium, chloride, carbon dioxide, creatinine, glucose, and pH.

| Test C (Units) | 0 hour | 2 hours | 4 hours | 8 hours | 24 hours | P-values | Significance | Normal |
|---------------|--------|---------|---------|---------|----------|----------|-------------|--------|
| 4placebo + 4 Cyclo-Z Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Range |
| Sodium (mmol/L) | 139.78 | 0.3964 | 139.6 | 0.4757 | 138.67 | 0.4019 | 140.22 | 0.8012 | 139.67 | 0.4496 | 0.3402 | N.S. | 136-146 |
| Potassium (mmol/L) | 4.603 | 0.0778 | 4.525 | 0.0877 | 4.3589 | 0.0746 | 4.2778 | 0.067 | 4.5133 | 0.13 | 0.0875 | N.S. | 3.5-5.5 |
| Chloride (mmol/L) | 90.62 | 9.138 | 107.4 | 10.526 | 105.3 | 4.942 | 104.3 | 10.136 | 106 | 0.4263 | 0.0537 | N.S. | 98-106 |
| CO2 (mmol/L) | 30.767 | 0.9025 | 30.6 | 0.3464 | 29.475 | 0.2496 | 29.133 | 0.7535 | 30.667 | 0.7965 | 0.0439 | N.S. | 22-31 |
| Urea Nitrogen (mg/dL) | 10.25 | 0.5789 | 12* | 0.4924 | 9.5 | 0.3371 | 11.0 | 0.3051 | 10 | 0.3051 | 0.01 | * | 4.0-15.0 |
| Creatinine (mg/dL) | 1.05 | 0.05436 | 2.04 | 0.4743 | 1.025 | 0.0446 | 1.142 | 0.0617 | 1.05 | 0.0544 | 0.98 | N.S. | 0.5-1-4 |
| EGFR (mL/min) | 94.25 | 3.745 | 90.5 | 7.441 | 96.25 | 0.4767 | 92.5 | 0.4767 | 90 | 0.4767 | 0.3402 | N.S. | 0.3-0.4 |
| Glucose (mg/dL) | 92.5 | 6.865 | 90 | 4.303 | 85.5 | 1.252 | 79.5** | 4.398 | 102.5 | 5.77 | 0.005 | ** | 70-110 |
| Chloride (mmol/L) | 90.62 | 0.03045 | 90.5 | 0.03045 | 90.475 | 0.03045 | 90.4 | 0.03045 | 90.4 | 0.03045 | 0.2831 | N.S. | 80-100 |
| Alkaline Phosp. (U/L) | 80.25 | 2.7 | 76.75 | 0.5126 | 75.3 | 0.4942 | 75.3 | 0.4942 | 75.3 | 0.4942 | 0.0537 | N.S. | 33-94 |
| ALT/WLA (U/L) | 21.5 | 1.098 | 22.25 | 0.9383 | 22.5 | 0.1508 | 22.75 | 0.9624 | 22.75 | 0.9624 | 0.4333 | N.S. | 7.0-45 |
| Bilirubin (mg/dL) | 0.7 | 0.04251 | 0.7 | 0.04251 | 0.65 | 0.04251 | 0.65 | 0.04251 | 0.65 | 0.04251 | 0.0537 | N.S. | 0.5-1-4 |
| RRBC (M/L) | 75.58 | 6.353 | 88.525 | 0.2358 | 86.25 | 0.8264 | 86.25 | 0.8264 | 86.25 | 0.8264 | 0.0537 | N.S. | 80-99 |
| MCH (pg) | 29.225 | 0.225 | 30 | 0.2093 | 29.075 | 0.2477 | 29.225 | 0.2477 | 29.225 | 0.2477 | 0.0537 | N.S. | 27-34 |
| MCHC (g/dL) | 33.95 | 0.1098 | 33.85 | 0.1177 | 33.725 | 0.05789 | 33.725 | 0.05789 | 33.725 | 0.05789 | 0.0537 | N.S. | 32-36 |
| RBC (%H) | 13.725 | 0.2093 | 13.925 | 0.1436 | 13.525 | 0.1303 | 13.825 | 0.1911 | 13.775 | 0.1009 | 0.4935 | N.S. | 12.0-15.0 |
| WBC (k/uL) | 5.325 | 0.4294 | 5.025 | 0.3582 | 5.9 | 0.4848 | 5.9 | 0.4848 | 5.9 | 0.4848 | 0.0537 | N.S. | 4.5-11 |
| LYM#-A (%) | 29.85 | 1.948 | 33.475 | 3.82 | 34.2 | 1.357 | 35.9 | 1.398 | 23.2*** | 1.452 | 0.001 | *** | 20-40 |
| Eosinophil # (#/uL) | 325 | 49.43 | 200 | 21.32 | 350 | 50 | 250 | 26.112 | 150*** | 15.076 | 0.0009 | *** | 0-500 |
| Basophil # (#/uL) | 25 | 13.056 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.999 | N.S. | 0-200 |

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

Table 3: Study Subjects who took 4 capsules of Cyclo-Z and 4 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

At 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- | Significance | Normal |
|----------------|---------|---------|---------|---------|---------|----|-------------|--------|
| Sodium         | 139.5   | 0.4713  | 139.5   | 0.3793  | 139.98  | 0.5568 | 140.17 | 0.4508  | 139.92  | 0.4681 | 0.5107 | N.S. | 136-146 |
| Potassium (mmol/L) | 4.146   | 0.0786  | 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  | 104.04 | 0.8124 | 0.688  | N.S. | 98-106 |
| CO₂ (mmol/L)    | 29.46   | 0.6954  | 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 |

*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 within the normal ranges. These findings showed that 8 capsules 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 75 pmol/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 366.5 pmol/mL from baseline. When the dose of Cyclo-Z was doubled to 4 capsules, plasma CHP levels increased to 506.0 ± 110.5 pmol/mL at 4 hours. The increase from baseline was 435.5 pmol/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 632.6 pmol/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 122.4 pmol/mL above basal levels. After taking 4 capsules, CHP concentration increased to 227.2 pmol/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 254.3 pmol/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 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 75 pmol/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 366.5 pmol/mL from baseline. When the dose of Cyclo-Z was doubled to 4 capsules, plasma CHP levels increased to 506.0 ± 110.5 pmol/mL at 4 hours. The increase from baseline was 435.5 pmol/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 632.6 pmol/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 122.4 pmol/mL above basal levels. After taking 4 capsules, CHP concentration increased to 227.2 pmol/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 254.3 pmol/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-Figure 2D), 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.

Pharmacokinetic data for cyclo (his-pro) plus zinc in healthy human subjects. J Drug Metab Toxicol 1:105. doi:10.4172/2157-7609.1000105

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 75 pmol/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 366.5 pmol/mL from baseline. When the dose of Cyclo-Z was doubled to 4 capsules, plasma CHP levels increased to 506.0 ± 110.5 pmol/mL at 4 hours. The increase from baseline was 435.5 pmol/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 632.6 pmol/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 122.4 pmol/mL above basal levels. After taking 4 capsules, CHP concentration increased to 227.2 pmol/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 254.3 pmol/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-Figure 2D), 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.

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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 an 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 dopaminergic 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 IC50 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

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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 (Figure 1B-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 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 attenuates 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/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 with 160 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 Responses. 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 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 excessive 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 further benefit for the treatment of diabetes or obesity.

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References
1. Ezaki O (1989) Iib group metal ions (Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat adipocytes. J Biol Chem 264: 16118-16122.
2. Tang X-H, Shay NF (2001) Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3 kinase and AKT in 3 T3-L1 fibroblasts and adipocytes. J Nutr 131: 1414-1420.
3. Keller SR (2003) The insulin-regulated aminoepitidase: A comparison and regulator of GLUT4. Front Biosci 8: e410-e420.
4. Watkins DW, Chenu C, Ripoche P (1989) Zinc inhibition of glucose uptake in brush border membrane vesicles from pig small intestine. Pflugers Arch 415: 165-171.
5. Zaqou P, Cantin JF, Alimardani –Bessette M, Monier F, Halimi S,et al (2000) Role of metalloproteases and inhibitors in the occurrence and progression of diabetic renal lesions. Diabetes Metab Suppl 4: 25-29.
6. Perlman RK, Rosner MR (1994) Identification of zinc ligands of the insulin-degrading enzyme. J Biol Chem 269: 33140-33145.
7. Valera Mora, ME, Searfone, A, Calvani M, Greco AV, Mingrone G. (2003) Insulin clearance in obesity. J Am Coll Nutr 22: 487-493.
8. Rosenthal MJ, Hwang IK, Song MK (2001) Effects of Arachidonic acid and cyclo (his-pro) on zinc transport across small intestine and muscle tissues. Life Sci 70: 337-348.
9. Song, MK, Hwang IK, Rosenthal MJ, Harris DM, Yamaguchi DT, Go, VLV (2003) Anti-hyperglycemic activities of cyclo (his-pro) in genetically diabetic Goto-Kakizaki rats. Exp Biol Med 228: 1338-1345.
10. Yip Y-Y, Hutchens TW (1991) Metal ion affinity absorption of a Zn (II)-transport protein present in maternal plasma during lactation: Structural characterization and identification as histidine-rich glycoprotein. Protein Expression Purification 2: 355-362 .
11. Borza DB, Morgan WT (1998) Histidine-proline-rich glycoprotein as plasma pH sensor. J Biol Chem 273: 5493-5499.
12. O’Barr SA, Song, MK, Mendoza K, Nguyen K, Shahidzadeh D, Schultz JJ (2009) Effects of zinc and cyclo (his-pro) on pathology, learning and memory in a transgenic mouse model of Alzheimer’s disease. 24th International Conferences of Alzheimer’s Disease International, Singapore.
13. Hwang IK, Go VLV, Harris DM, Yip I, Kang KW, Song MK (2003) Effects of cyclo (his-pro) plus zinc on glucose metabolism in genetically diabetic obese (ob/ob) mice. Diabet Obes Metabol 5: 317-324.
14. Sreenan S, Strius J, Pugh W, Burant CF, Polonsky KS (1996) Prevention of hyperglycemia in the Zucker diabetic fatty rat by treatment with metformin or troglitazone. Am J Physiol 271: E742-E747.
15. Simpson RW, Shaw JE, Zimet PZ (2003) The prevention of type 2 diabetes—lifestyle change or pharmacotherapy? A challenge for the 21st century. Diabetes Res Clin Pract 59: 165-180.

16. Kim YB, Ciaraldi TP, Kong A, Kim D, Chu N, Mohideen P, et al. (2002) Troglitazone but not metformin restores insulin-stimulated phosphoinositide 3-kinase activity and increases p110beta protein levels in skeletal muscle of type 2 diabetic subjects. Diabetes 51: 443-448.

17. Song MK, Rosenthal MJ, Song AM, Uyemura K, Yang H, et al. (2009) Body weight reduction by oral treatment with zinc plus Cyclo-(his-pro). Br J Pharmacol 158: 442-450.

18. Holz C, DeHaene J, Woodhouse LR, Villalpando S, Rivera JA, et al. (2005) Zinc absorption from zinc oxide, zinc sulfate, zinc oxide + EDTA, or sodium-zinc EDTA does not differ when added as fortificants to maize tortillas. J Nutr 135: 1102-1105.

19. Tran CD, Miller LV, Krebs NF, Lei S, Hambidge KM (2004) Zinc absorption as a function of the dose of zinc in aqueous solution. Am J Clin Nutr 80: 1570-1573.

20. Hilton CW, Prasad RC, Vo P, Mouton C (1992) Food contains the bioactive peptide, cyclo-(his-pro). J Clin Endocrinol Metab 75: 375-378.

21. Prasad C, Hilton CW, Svec F, Onai SI, Vo P (1991) Could dietary proteins serve as cyclo-(his-pro) precursors? Neuropeptides 19: 17-21.

22. Hilton CW, Prasad C, Svec F, Vo P, Reddy S (1990) Cyclo-(His-Pro) in nutritional supplements. Lancet 336: 1455.

23. Hilton CW, Prasad RC, Wilber JF (1990) Change in circulating cyclo(his-pro) concentrations in rats after ingestion of oral glucose compared to intravenous glucose and control. Endocr Res 16:139-150.

24. Hilton CW, Prasad C, Wilber JF (1990) Acute alterations of cyclo(his-pro) levels after oral ingestion of glucose. Neuropeptides 5: 55-59.

25. Prasad C (1988) Cyclo(his-pro): Its distribution, origin and function in the human. Neurosci Biobehav Rev 12: 19-22.

26. Pekary AE, Reeve JR, Smith VP, Friedman S, Hershman JM (1985) In vitro production of a TRH-homologous peptide and his-pro diketopiperazine by human semen. J Androl 6: 379-385.

27. Taubert D, Grinberg G, Stenzel W, Schonig E (2007) Identification of the endogenous key substrates of the human organic cation transporter OCT2 and their implication in function of dopaminergic neurons. PloS One 2: e635.

28. Jung N, Lehmann C, Rubbert A, Knispel M, Hartmann P, et al. (2008) Relevance of the organic cation transporters 1 and 2 for antiretroviral drug therapy in human immunodeficiency virus infection. Am Soc Pharmacol Exp Ther 36: 1616-1623.

29. Faden, AI, Fox GB, Knoblauch SM, Cernak I, Mullins P, et al. (2003) Neuroprotective and Nootropic actions of a novel cyclopeptide after controlled cortical impact injury in mice. J Cereb Flow Metab 23: 355-363.

30. Faden AI, Knoblauch SM, Movsesyan SM, Cernak I (2004) Novel small peptides with neuroprotective and nootropic properties. J Alzheimer's Dis 6: S93-S97.

31. Prasad C, Balasubramaniam P (1988) Cyclo(His-Pro) and the development of tolerance to the hypotensive effect of ethanola. Neuropeptides 12: 75-79.

32. Faden AI, Movsesyan SM, Knoblauch SM, Ahmed F, Cernak I (2005) Neuroprotective effects of novel small peptides in vitro and after brain injury. Neuropharmacology 49: 410-424.

33. Minelli A, Conte C, Grottelli S, Bellezza M, Cacciatorre I, Bolanos JP (2009). Cyclo (His-Pro) promotes cytoprotection by activating NFkbeta-mediated up-regulation of Antioxidant defense. J Cell Mol Med 13: 1149-1161.

34. Minelli A, Conte C, Grottelli S, Bellezza M, Emiliani C, Bolanos JP (2009) cyclo (His-Pro) up-regulates heme oxygenase 1 via activation of NFkbeta signaling. J Neurochem 111: 956-966.

35. Fragner P, Presset O, Bernard N, Martinez J, Roze C, Aratan-Spire S (1997) A new biological contribution of cyclo (His-Pro) to the peripheral inhibition of pancreatic secretion. Am J Physiol 273: E1127-E1132.

36. Aguilar MV, Laborda JM, Martinez-Para MC, Gonzalez MJ, Mesequer I, et al. (1998) Effects of diabetes on the tissue Zn/Cu ratio. J Trace Elem Med Biol 12: 155-158.

37. Terres-Martos C, Navarro-Alarcon, M, Martin-Lagos F, Lopez-G (de la Serrana H, Perez-Vatero V, et al. (1998) Serum zinc and copper concentrations and Cu/Zn ratios in patients with hepatopathies or diabetes. J Trace Elem Med Biol 12 : 44-49.

38. Song MK, Mooradian AD (1988) Intestinal zinc transport: Influence of streptozotocin-induced diabetes, insulin and arachidonic acid. Life Sci 42: 687-694.

39. Singh RB, Niaz MA, Rastogi SS, Rastogi S, Bajaj S, et al. (1998) Current zinc intake and risk of diabetes and coronary artery disease and factors associated with insulin resistance in rural and urban population of North India. Am J Coll Nutr 17: 564-570.

40. Apostolova MD, Choo KH, Michalka AE, Tohyama CM (1997) Analysis of the possible protective role of metallothionein in streptozotocin-induced diabetes using metallothionein-null mice. J Trace Elem Med Biol 11: 1-7.

41. Tobia MH, Zdanowicz MM, Wingertzahn MA, McHelfey-Atkinson B, Stolmin AE, et al. (1998) The role of dietary zinc in modifying the onset and severity of spontaneous diabetes in the BB Wistar rat. Mol Genet Metab 63: 205-213.

42. Marchesini G, Bugiansi E, Ronchi M, Fiamma R, Thomaseth K, et al. (1998) Zinc supplementation improves glucose disposal in patients with cirrhosis. Metabolism 47: 792-798.

43. Brando-Net J, da Silva CA, Figueiredo NB, Shuahama T, da Cunha NF et al.(1999) Lack of acute zinc effect in glucose metabolism in health and insulin-dependent diabetes mellitus. Biometals 12: 161-165.

44. Blostein-Fuji A, DiSilvestro RA, Frid D, Katz C, Malarkey W (1997) Short-term zinc supplementation in women with non-insulin-dependent diabetes mellitus: effects on plasma S-nucleotide activities, insulin-like growth factor I concentrations, and lipoprotein oxidation rates in vitro. Am J Clin Nutr 68: 639-642.

45. Leonard A, Gerber GB, Leonard F (1986) Mutagenicity, carcinogenicity and teratogenicity of zinc. Mutat Res 168: 343-353.

46. Roney N, Osier M, Paikoff SJ, Smith CV, Williams M, et al. (2006) ATS/IDSA evaluation of the health effects of zinc and relevance to public health. Toxicol Ind Health 22: 423-493.

47. Wallraven PA, Hambidge KM (1978) Growth of infants fed a zinc supplement formula. Am J Clin Nutr 29: 1114-1121.

48. Lewis MR, Kokan L (1998) Zinc gluconate: acute ingestion. J Toxicol Clinical Toxicol 36: 99-101.

49. Yadrick MK, Kenney MA, Winterfeld EA (1989) Iron, copper, and zinc status: response to supplementation with zinc or zinc and iron in adult females. Am J Clin Nutr 49: 145-150.

50. Davis CD, Milne DB, Nielsen FH (2000) Changes in dietary zinc and copper affect zinc –status indicators of postmenopausal women, notably, extracellular superoxide dismutase and amyloid precursor proteins. Am J Clin Nutr 71: 781-788.

51. Prasad AS, Brewer GJ, Schoomaker EB, Rabbani P (1978) Hypocalcemia induced by zinc therapy in adults. JAMA 240: 2166-2168.

52. Gyorffy EJ, Chan H(1992) Copper deficiency and microcytic anemia resulting from prolonged ingestion of over-the-counter zinc. JAMA 267: 44-49.

53. Banham M, O'Connor JM, Aldender HD, Couiler J, Walsh PM, et al. (2003) Zinc supplementation has no effect on circulating levels of peripheral blood leucocytes and lymphocytes subset in health adult men. Br J Nutr 89: 695-703.

54. Maret W, Sandstead HH (2006) Zinc requirement and the risks and benefits of zinc supplementation. J Trace Elem Med Biol 20: 3-18.