Food Sci. Biotechnol. Vol. 17, No. 3, pp. 564 ~ 568 (2008)

Food Science and Biotechnology

Impact of Sodium Copper Chlorophyllin on Mercury Absorption Using an *in Vitro* Digestion with Human Intestinal Cell Model

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Abstract The effects of sodium copper chlorophyllin (SCC) on bioaccessibility and uptake of mercury from fish were investigated using an *in vitro* digestion coupled with a Caco-2 cell. Fish along with SCC was subjected to a simulated *in vitro* digestion, which simulates both the gastric and small intestinal phase *in vivo*. Mercury bioaccessibility, the amount of mercury released from fish to aqueous phase following a digestion, was measured. Various amounts of SCC (0.1-25 mg) significantly reduced mercury bioaccessibility in a dose dependent manner by 49-89% compared to the negative control (fish without SCC) ($p<0.05$). Mercury bioaccessibility in varying molar ratios of mercury to positive control, 2,3-dimercapto-1-propane sulfonate (DMPS) was between 24 and 52%. Mercury uptake by Caco-2 cells from test media containing aqueous phase following *in vitro* digestion was measured after 6 hr incubation at 37°C. Cellular mercury uptake with increasing amount of SCC ranged from 0.352 to 0.052 μg mercury/mg protein, while those in DMPS treatment were between 0.14 and 0.27 μg mercury/mg protein. Our study suggests that SCC can reduce mercury absorption following fish consumption and may be efficient as a synthetic chelating agent for long term chronic mercury exposure in fish eating populations.

Key words: mercury, sodium copper chlorophyllin, bioaccessibility, Caco-2 cell, uptake

Introduction

As the third most toxic compounds found in U.S., it is well known that mercury degenerates neurological development (1,2). Fish consumption has been identified as the main pathyway for the mercury exposure to human (3,4). U.S. Environmental Protection Agency and Food and Drug Administration have recently issued joint advisory of fish consumption to protect susceptible populations (i.e., women of child bearing age, infant, and young children) from mercury exposure through fish consumption (5). However, fish is a good source of high-quality protein, vitamin D, selenium, omega-3 fatty acids, and other nutrients (6,7). Therefore, studies are required to reduce the mercury absoprtion while maintaining the health benefits from fish consumption. As a therapeutic treatment, a synthetic chelating agent, such as 2,3-dimercapto-1-propane sulfonate (DMPS) has been used for reducing mercury absorption (4,8). Alternatively, many studies have suggested that dietary factors can affect mercury bioavailability (9-12). In addition to commercial chelating agent, co-consumption of wheat bran with mercury significantly decreased mercury accumulation in kidney and brain (12). Consumption of garlic showed an increase in excretion of mercury (4,13). It also has been suggested that biologically active components in plants may inhibit the adsorption of heavy metals (10).

Chlorophylls are rich in plant pigments and commonly found in the human diet (15,16). Sodium copper chlorophyllin (SCC), a commercial-grade water soluble mixture of copper-chlorophyll derivatives, exhibits potent antimutagenic and antioxidant activities (17-19). It was reported that the content of chlorophyll in vegetables had positive correlation with antimutagenic activity (20). SCC also exhibits potent carcinogenic activity against a variety of known dietary and environmental contaminants such as aflatoxins, benzo[a]pyrene, polycyclic aromatic hydrocarbons (PAHs), acrylamide, and heterocyclic amines (16,21,22). With increasing awareness of health benefits of SCC, it has been widely used for both a food supplement and colorant. (16,23).

In contrast to numerous reports about the apparent health promoting benefits of SCC, there is minimal information about its specific effects on bioaccessibility and human intestinal absorption of heavy metal such as mercury. Bioaccessibility is defined as the amount of a chemical that is released from food into gastrointestinal tract fluid following a simulated digestion and as a result, is available for absorption by the intestinal mucosa (24-27). Simulated gastrointestinal digestion coupled with human intestinal cell model has been well validated and utilized to assess bioaccessibility and absorption of nutrients or contaminants from foods (15,23,24,29,30). Therefore, the objective of this study was to determine the effects of SCC on mercury bioaccessibility and uptake from fish. As a model system, an *in vitro* digestion method coupled to a human intestinal cell absorption model was used and compared with DMPS, which is well used for reducing mercury poisoning.

Materials and Methods

Materials King mackerel (*Scomberomorus cavalla*) was obtained from the Florida Department of Environmental Protection (Tallahassee, FL, USA) and chemical analysis found it to contain 1 ppm of total mercury. 2,3-Dimercapto-1-

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Received August 23, 2007; Revised October 18, 2007;
Accepted October 24, 2007

propane sulfonic acid (DMPS, reagent grade) and sodium copper chlorophyllin (SCC, commercial grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Test meals preparation Fish tissue was homogenized in a food processor with stainless-steel blades (HC 3000; Proctor-Silex, Inc., Washington, NC, USA). A 5 g of fish tissue (5 μg of mercury) and 5 mL of saline (0.9% NaCl, Sigma-Aldrich) were twice homogenized using a cell disruptor (Cell Disruptor 185; Branson Sonic Power Co., Danbury, CT, USA) at 20 kHz at 150-500 W for 30 sec. To produce a finished test meal, homogenized fish tissue was mixed with SCC powder (0.1, 1, 5, 10, 17, and 25 mg) or DMPS (1.25, 2.5, 5, 10, 20, and 50 μg).

***In vitro* digestion** Representative aliquots of test meal; negative control (fish only), positive control (fish along with DMPS), treatment (fish along with SCC); were subjected to a simulated gastric and small intestinal digestion as described by Garrett *et al.* (24) and Ferruzzi *et al.* (23). For the gastric phase of digestion, porcine pepsin (3 mg/mL, Sigma-Aldrich) was added to samples, followed by acidification to pH 2 with 0.1 M HCl (analytical grade, Sigma-Aldrich). Samples were then incubated at 37°C for 1 hr in a shaking water bath at 150 rpm (VWR, Cornelius, OR, USA). To mimic digestion in the small intestinal, the gastric digest was neutralized to pH 5.3 by addition of 100 mM sodium bicarbonate solution (Sigma-Aldrich) then 9 mL bile extract/pancreatin/lipase mixture (0.4 mg/mL, Sigma-Aldrich), lipase (0.2 mg/mL, Sigma-Aldrich), and porcine bile extract (2.4 mg/mL, Sigma-Aldrich) was added. After the pH adjustment to 7.0±0.5 using 0.1 M NaOH (analytical grade, Sigma-Aldrich), the samples were incubated in a shaking water bath for 2 hr at 37°C, 150 rpm after flushing the top of the tubes with nitrogen gas (99.99%, air gas, Indianapolis, IN, USA). Aliquots of the final digest (30 mL) were centrifuged at 167,000×g for 35 min (Beckman L8-70M, Beckman Coulter, San Antonio, TX, USA) in order to isolate the aqueous phase from residual pellets. Decanted aqueous phase was filtered (0.2 μm pore size, Whatman Inc., Clifton, NJ, USA). Aliquots of raw material, digesta, aqueous fractions, and residual pellets were collected and stored at −80°C until analysis.

Uptake of mercury by Caco-2 intestinal cells The Caco-2 cellular model was utilized to investigate the uptake of mercury from test meals as described by Ferruzzi *et al.* (15,23) with slight modification. Conditions for the growth and differentiation of the TC7 clone of Caco-2 cells have been described previously (31). Caco-2 cell cultures used in this experiment were obtained from ATCC and passage # was between 70 and 80. For all experiments, cells were seeded in 6-well plastic dishes (35×10 mm, Costar Corning, New York, NY, USA) at a density of 0.064 $\times 10^6$ cells/mm^2, and incubated in a humidified atmosphere of air/CO_2 (95%/5%) at 37°C. Uptake experiments were performed when monolayers were 11-14 day post-confluent. High-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Introgen Corporation, Grand Island, NY, USA) containing L-glutamine, pyridoxine hydrochloride, and 110 mg/L sodium pyruvate were used for basal DMEM. The medium was completed with 3.7 g/L sodium bicarbonate (44 mM, J.T. Baker, Phillipsburg, NJ, USA), 10% of autoclaved HEPES (10 mM, Sigma-Aldrich), 10% of non-essential amino acids (0.1 mM, Sigma-Aldrich), 10% of penicillin/streptomycin (P/S, 100 U/L/100 U/L, Sigma-Aldrich), and 1% of gentamicin (50 μg/L, Sigma-Aldrich) at pH 7.2. A 20% of inactivated fetal bovine serum (Gibco, Introgen Corporation, Grand Island, NY, USA) in basal medium was used for the seeding of the cells. For the maintaining of the cells, a 10% of inactivated fetal bovine serum in basal medium was used every other day.

Prior to uptake experiments, monolayers were washed twice with basal DMEM before adding 2 mL of the test medium containing filtered aqueous fraction from *in vitro* digestion and basal DMEM at a ratio of 1 : 3. At predetermined incubation time (6 hr), medium was removed by aspiration and cultures were washed twice with ice-cold phosphate buffered saline (PBS, Sigma-Aldrich). Cells were collected by scraping and then placing into 1.0 mL of ice-cold PBS and stored at −80°C until analysis. Each experimental treatment was performed in triplicate.

Assessment of toxicity and cellular viability Viability of Caco-2 cells was detected by Trypan blue staining (Sigma-Aldrich). Cells were counted using a hemocytometer and then % of cellular viability was calculated as followed;

Cellular viability (%)
= (No. of viable cell)/(No. of viable cell + No. of dead cell)*100

Cytotoxicity of test medium containing phytochemicals was determined using a methylthiazoletetrazolium (MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Sigma-Aldrich) assay. In brief, 2 mL of the medium supplemented with aqueous solutions of the above-mentioned foods (1 : 3, filtered aqueous solution to DMEM) were added to highly differentiated culture of Caco-2 cells which were 11-14 days post confluent. Medium was aspirated 6 hr after incubation at 37°C. Two mL of MTT in PBS (1.5 mg of MTT in 15 mL of PBS) was added, and cells were incubated for an additional 1 hr at 37°C. The MTT solution was removed by aspiration, 300 μL of DMSO was added to each well, and cells were dissolved for 5 min at 23°C in the dark. A 50 μL aliquot of the solution was transferred from each well to a 96-well plate (Costar Corning), and absorbance at 570 nm was measured in a 96-well plate reader (Bio-Tek Instruments Inc., Tustin, CA, USA). Each experiment was performed in duplicate.

Protein assay Cells were homogenized by sonic disruption and cell protein was measured by using a bicinchonic acid (BCA) protein assay kit according to manufacturer's protocols (Bio-Rad Laboratories, Rockford, IL, USA).

Determination of mercury Alliquots of raw material, digesta, aqueous fraction, and harvest cells was analyzed for total mercury using a thermal decomposition (gold) amalgamation/atomic absorption spectrophotometer (TDA/AAS) (DMA-80 Mercury Analyzer; Milestone Inc., Pittsburgh, PA, USA) as described by Shim *et al.* (32). Total mercury in aqueous fraction and residual pellet was determined. Certified reference materials (CRMs: Tort-2 and Dorm-2, Institute for Environmental Chemistry, National Research

Council Canada, Ottawa, ONT, Canada) were used to standardize the instrument. Total mercury levels obtained from each well of cells were normalized to the corresponding concentration of cellular protein. Total mercury concentrations in phytochemical rich foods/ingredients were below the limit of detection (0.01 ng total mercury).

Statistical analysis Results are presented as representative data from duplicate sets of experiments. Data are expressed as the mean±standard error of mean (SEM). Statistical analysis for each parameter assessed was performed by using analysis of variance (ANOVA) followed by Tukey's post-hoc test (SAS, Cary, NC, USA). Differences among means were considered statistically significant at $p<0.05$.

Results and Discussion

Mercury bioaccessibility Digested fish samples, 'digesta', were analyzed for total mercury or centrifuged to separate the residual pellet from the aqueous phase to measure bioaccessibility. Mercury recovery, which was determined by comparing the amount of mercury added in the fish tissue to the amount of mercury measured in the digesta, ranged from 94 to 108%. Bioaccessibility, which is the amount of methylmercury released from digested fish tissue into the aqueous phase, was measured for digested fish which did not have any food/food component added. The percentage of mercury measured in the aqueous phase, i.e., the bioaccessibility, from 2 (2 μg Hg), 5 (5 μg Hg), and 10 g (10 μg Hg) of fish tissue was 90, 80, and 75%, respectively (Fig. 1).

Mercury uptake by Caco-2 cell Caco-2 cellular uptake of mercury from test media was studied for as long as 1 hr. Uptake at 37°C was found to increase linearly (R^2=0.981) up to 0.08 μg mercury per mg cellular protein at over 1 hr. However, mercury uptake by Caco-2 cells was significantly decreased in cultures incubated at 4°C over the same 1 hr period with final cellular mercury content of 0.02 μg mercury per mg cellular protein at 1 hr (Fig. 2A). Previous reports have indicated a temperature dependent uptake/ accumulation of inorganic mercury by renal cells (33,34). Aduayom *et al.* (8), however, reported that for inorganic mercury (i.e., mercuric chloride) accumulation by Caco-2 cells was not significantly different at 4°C and room temperature. In contrast, our results show a significant increase in methylmercury uptake by Caco-2 cells from fish tissue at 37°C compared to cells incubated at 4°C (Fig. 2A). Therefore, these data support the possibility that methylmercury as opposed to elemental mercury may be transported into enterocytes by a facilitated process (8). In addition, transfer of mercury to the aqueous fraction was not affected by exclusion of bile extracts from the standard digestion. More information is needed to determine the nature of this temperature dependence.

In the experiment of determination of the maximum mercury uptake for 24 hr, the greatest mercury uptake was shown at 6 hr incubation and it showed plateau after that time point (data not shown). From these observations, 6 hr incubations were used for further studies to assess the effects of the treatments (DMPS or SCC) on cellular mercury uptake. Caco-2 cellular mercury accumulation was proportional to increasing amounts of mercury from test medium containing aqueous fraction of *in vitro* digestion (0-10 g fish tissue). Test media mercury content ranged from 0-0.25±0.11 μg and resulted in intracellular content ranging from 0.0062 to 0.4869 μg of mercury per mg protein after incubation at 37°C for 6 hr (Fig. 2B).

Effect of SCC on mercury bioaccessibility The effects of DMPS on the concentration of mercury in the aqueous phase and the digesta (aqueous phase plus suspended

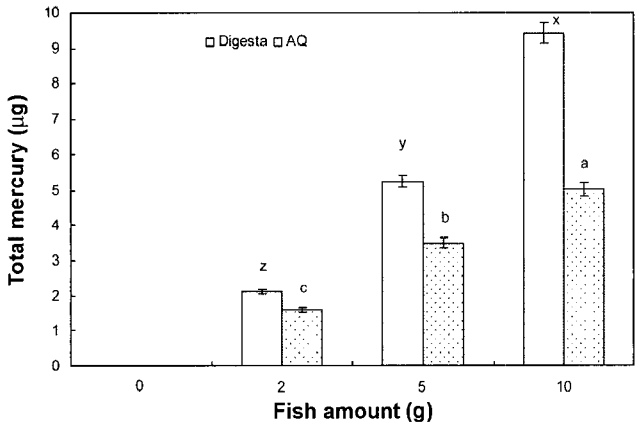

Fig. 1. Total mercury in digesta and aqueous phase (AQ) following *in vitro* digestion of fish tissue. Digesta includes both the AQ and suspended solids. Values represent means±SEM for 3 replications. Different letters (xyz or abc) indicates significance at the α=0.05 level.

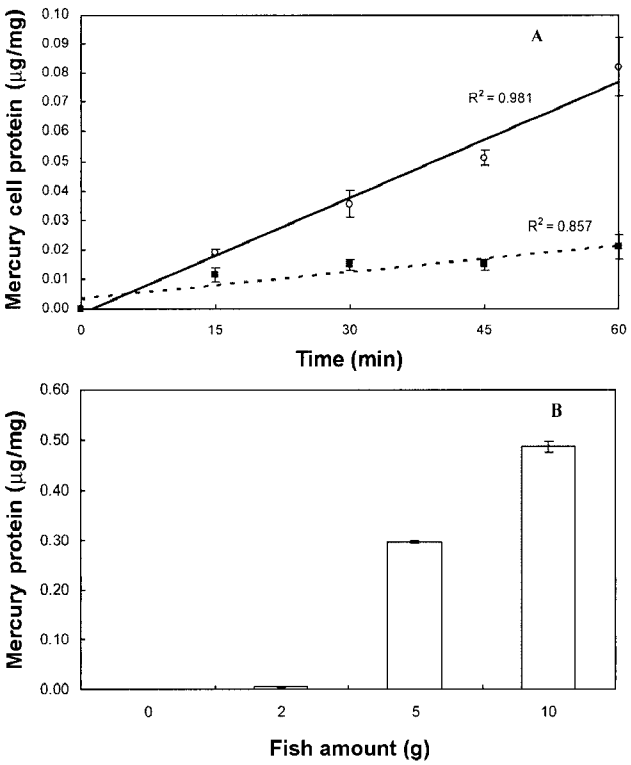

Fig. 2. Uptake of mercury by TC7 clone of Caco-2 human intestinal cells (incubated at ○, 37°C; ■, 4°C) (A) and from different fish tissue amounts when incubated at 37°C for 6 hr (B). Means±SEM for 3-4 independent measurements are reported.

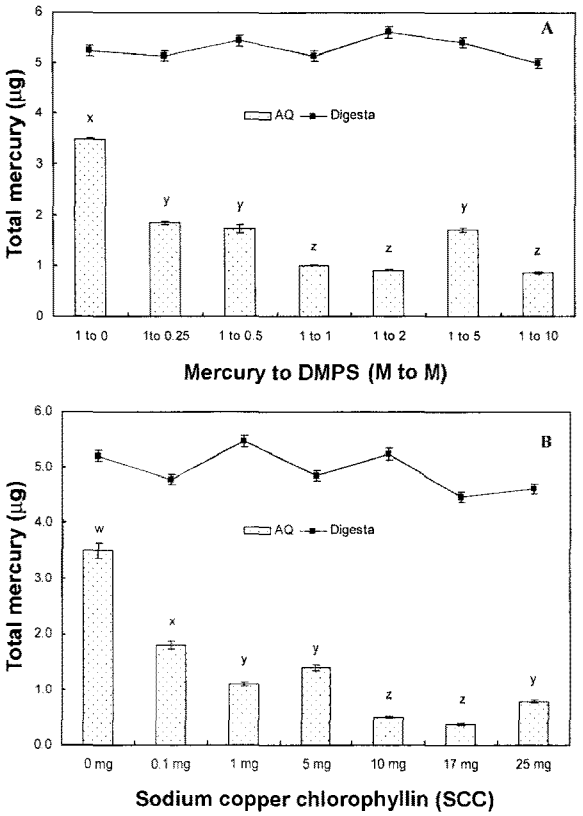

Fig. 3. Total mercury in digesta and aqueous fraction (AQ) from *in vitro* digestion of 5 g fish tissue (5 μg mercury) in the presence of increasing amounts of DMPS (A) and SCC (B). AQ includes liquid that was decanted from pellet. Values represent means± SEM for 3 replications. Different letters (xyz) indicates significance at the α=0.05 level.

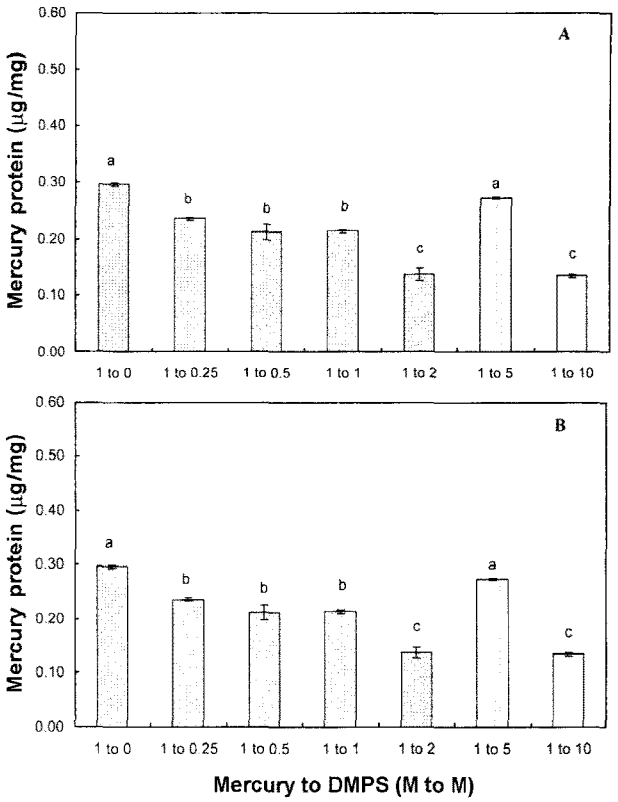

Fig. 4. Effects of DMPS (A) or SCC (B) on uptake of mercury by TC7 clone of Caco-2 human intestinal cells. Means±SEM for 3-4 independent measurements are reported.

solids) are shown in Fig. 3A. DMPS significantly ($p<$ 0.05) reduced mercury bioaccessibility from fish tissue in a dose-dependent fashion by between 24 and 52%. SCC was found to have a significant effect on mercury bioaccessibility using an *in vitro* digestion model (Fig. 3B). SCC decreased mercury bioaccessibility by 49-89% compared to the control.

Effects of SCC on mercury uptake by Caco-2 cells The effects of SCC on the cellular uptake of mercury from the aqueous phase were investigated over 6 hr at 37°C. Cellular mercury accumulation with added DMPS ranged from 0.14 to 0.27 μg of mercury per mg of cell protein at varying molar ratios of mercury to DMPS (Fig. 4A), indicating that intracellular mercury levels were reduced in a dose-dependent fashion relative to concentration of DMPS in test meal, with the exception of 1 : 5 mercury to DMPS ratio. Increasing amounts of SCC reduced cellular mercury from 0.352 to 0.052 μg mercury/mg protein (Fig. 4B). Mercury found in the aqueous phase was predicted to be most bioavailable by absorption across the intestinal epithelium. This study found a strong correlation between mercury bioaccessibility and cellular mercury uptake, particularly when combined with SCC. It indicates that the assay of mercury bioaccessibility after *in vitro* digestion can be a good indicator for estimating mercury bioavailability without requiring further cellular work. The effect of SCC on accumulation of mercury by Caco-2 cells was investigated by co-digestion of fish tissue with SCC. The digestive environment allows for physiologically significant interaction between mercury from the fish tissue and phytochemicals occurring in a manner similar to co-consumption *in vivo*. SCC showed significant trends of decreased bioaccessibility of mercury from fish tissue following *in vitro* digestion with these ingredients in a combined meal. It is plausible that these phytochemicals may form insoluble complexes with mercury and reduce bioaccessibility. Our data revealed that SCC, which is a water soluble derivative of chlorophyll, had significant potential for reducing mercury absorption in human intestine cells. These data support several reports that chlorophyllin reduced the absorption of compounds, such as cadmium, benzo(a)pyrene and aflatoxin B_1, as a result of formation of insoluble complexes (15,28). Therefore, these results suggest that food which is rich in SCC may be efficient as synthetic chelating agents (e.g., DMPS) for long-term chronic methylmercury exposure in fish-eating populations by reducing mercury bioavailability. Although the specific mechanisms by which SCC attenuate mercury uptake/accumulation have not been completely elucidated, several theoretical mechanisms may be proposed: 1) SCC can reduce the possibility of mercury absorption from the gut by binding mercury making it insoluble thereafter unavailable to intestinal mucosa; and 2) SCC may induce demethylation of rapidly absorbed methylmercury to more poorly-absorbed inorganic mercury resulting in decreased intestinal solubility and absorption.

In conclusion, this study supports the positive effects of

SCC on the inhibition of mercury absorption when evaluated using an *in vitro* gastric digestion model coupled with a model that uses human intestinal cells. We therefore suggest that modification of dietary factors can help to reduce mercury exposure to the human body via foods such as fish. In order to evaluate the toxicological significance of methylmercury exposure by fish consumption, further studies are required to determine whether the effects observed in this study are also found when using an *in vivo* system. The benefits of the techniques described in this manuscript relate to a system that provides rapid and cost-effective alternative for evaluating bioavailability of mercury.

Acknowledgments

The authors wish to thank Drs. Charles R. Santerre, Mario G. Ferruzzi, Elsa M. Janle in the Dept. of Food Science at Purdue University for their advice.

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