COVER PAGE

(KIWI) Speeding up a Slow Protein for Muscle Mass With Hay Kiwifruit

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DESIGN AND METHODS

*Our **specific hypothesis** is that the consumption of 2 Hayward green kiwifruit (containing actinidin protease) prior to 100g of ground beef will increase the rate of protein digestion from the beef in the elderly, leading to an increased uptake of the essential amino acids. Furthermore, this increased essential amino acid availability will produce a greater postprandial net anabolic protein response, as well as increased fractional synthetic rates of muscle and liver proteins.*

To test this hypothesis we will use a double-blinded crossover design involving two intervention arms; in both arms 100g of ground beef (cooked weight) will be consumed following either 2 Hayward green kiwifruit (containing actinidin protease) or 2 Hort16A Gold kiwifruit (devoid of actinidin protease) by elderly subjects.

In response to the interventions, our primary endpoints are:

- the rate of beef protein digestion in the stomach (7);
- plasma amino acids (AA) which will reflect dietary digestibility and availability of precursors for protein synthesis (1);
- the magnitude of the whole-body net anabolic response (NAR), defined as the difference between whole body protein synthesis and protein breakdown, to determine overall protein balance, accounting for all roles of protein in the body (4, 13);

and our secondary endpoints are:

- the muscle fractional synthetic rate (FSR) on muscle protein synthesis (4, 13);
- the fibrinogen FSR to determine the impact of actinidin-assisted protein digestion on liver protein synthesis (Wolfe, unpublished research);
- plasma glucose and insulin which will reflect the wider metabolic response (12).

These endpoints will enable achievement of the four objectives summarised in Table 1 on the second page of this proposal.

Experimental Overview and Rationale:
We will study a total of 15 healthy male and females between 60 and 85 years of age in a double-blinded crossover design composed of two intervention arms, as detailed in Figure 3. With a sample size of N=15 subjects, the crossover design will have 80% power to detect a significant effect, where statistical significance is set at $p<0.05$. The 60-85 year age group was chosen due to the significant loss of muscle mass apparent at this stage of aging, which will provide a backdrop for the most pronounced effects and thus most scientifically accurate measurements. The entire study will take place at University of Arkansas for Medical Sciences (UAMS), in collaboration with Professor Robert Wolfe. Professor Wolfe is an international expert in the use of stable isotope tracer technology for the measurement of protein metabolism and developed a majority of the methods considered to be gold-standard for this work. As this is a clinical study with complex methodology, completion of the experimental portion at UAMS under the supervision of Professor Wolfe and his highly experienced team, will ensure that all procedures are conducted in the manner required for maximum accuracy and sensitivity of the data.

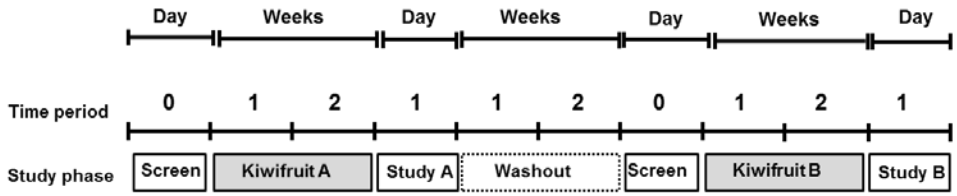

Figure 3. Complete outline of crossover design showing phases of study for a subject undergoing treatment A first and treatment B second.

Study Interventions. The interventions are as follows:

A. 100g of ground beef plus 2x Hort 16A Gold kiwifruit (Control)
B. 100g of ground beef plus 2x Hayward Green kiwifruit

The studies are referred to as Study A and Study B, respectively, in Figure 3. These amounts were chosen because 100g beef is the recommended serving size of beef for the elderly and we know from previous work that 100g of beef will induce a robust anabolic response (5, 6). Two Hayward Green, but not Hort16A Gold, kiwifruit had a significant effect on the rate of digestion of beef protein in pig trials (7). Ground beef will be produced to have an average protein composition of 25% (10); as 35g of protein is likely to saturate the muscle synthetic response, 25g will be within the range that permits detection of a difference in muscle protein synthetic rate between interventions. The use of ground beef will also limit any effects of masticatory differences between individuals. Subjects will also be selected to ensure masticatory function has not deteriorated.

Each subject will undergo both interventions, eating either Hort16A Gold fruit for the first arm and Hayward Green fruit for the second arm, or vice versa. Neither subjects nor meal distributors will be informed as to which kiwifruit variety contains actinidin; the study is therefore double-blinded.

Experimental Details:

Participant Selection and Screening: We will study a total of 15 healthy male and females between 60 and 85 years of age. Subjects will not participate in any organized exercise program, or perform any strenuous exercise 72 hours prior to the metabolic experiment.

Inclusion Criteria:

- Men and women, ages 60-85 years.

Exclusion Criteria:

- Inability to chew meats or difficulty swallowing solid foods
- History of diabetes
- History of malignancy in the 6 months prior to enrolment
- History of gastrointestinal reduction or bypass surgery (Lapband, etc)
- History of a chronic inflammatory condition or disease (Lupus, HIV'AIDS, etc)
- History of chronic kidney disease or currently requiring dialysis.
- Allergy to beef or kiwifruit
- Subjects who do not or will not eat animal proteins
- Subjects who cannot refrain from consuming protein or amino acid supplements during their participation in this study
- Subjects who report regular resistance exercise (more than once per week)
- Hemoglobin less than 9.5mg/dL at the screening visit
- Platelets less than <150,000 at the screening visit

- Subjects who are not willing or able to suspend aspirin for several days prior to their muscle biopsies.
- Subjects who have been prescribed a blood-thinning medication (Coumadin, lovenox, heparin, Plavix, etc).

- Concomitant use of corticosteroids (ingestion, injection or transdermal)
- Any other disease or condition that would place the subject at increased risk of harm if they were to participate, at the discretion of the study physician

During the screening visit (Day 0), dual-energy X-ray absorptiometry (DEXA) will be performed to determine body composition. This will enable us to normalize data in terms of lean body mass as well as whole body weight. Subjects will be then randomly assigned by a study coordinator to group A or B. Subjects be provided with 2 kiwifruit per day and will be instructed to consume these immediately before their midday meal for two weeks (Figure 3, shaded box) prior to the study. Subjects will also be instructed to abstain from strenuous physical activity for >72 h before the initiation of the metabolic study.
After the first intervention arm, there will be a two-week washout period (Figure 3, dashed box) where subjects are instructed to consume no kiwifruit. After the washout period, the second arm of the study will begin, using the other Kiwifruit.

Experimental Details.
The experimental portion of the study involves a 4.5-hour basal fasted period and 5-hour post-meal period (total 9.5-h time period). This is shown in Figure 4. Samples will be taken in the fasted and fed states in order to determine basal and protein-related responses of the primary (plasma AAs, and NAR) and secondary (muscle and fibrinogen FSR, plasma glucose and insulin) endpoints.

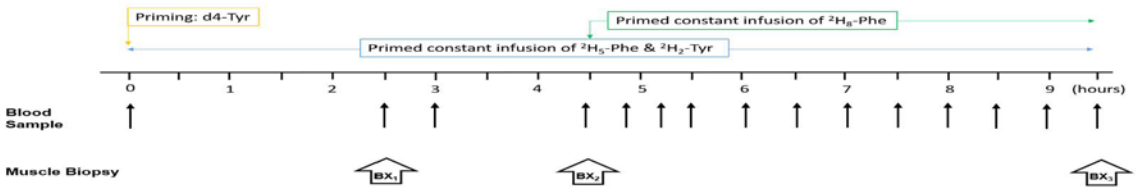

Figure 4. Tracer infusion and sampling protocol.

<u>*Stable isotope tracer infusion protocol.*</u> The 9.5-h tracer infusion protocol is an established method in Professor Wolfe's laboratory (5, 6, 13) and is presented in Figure 4. Subjects will report to UAMS after an overnight (after 2200) fast and will be catheterized with a venous catheter in the forearm of one arm for tracer infusion, and in a hand or wrist vein of the other arm for blood sampling using the heated hand technique. After obtaining a blood sample to determine background enrichments, infusion of L-[*ring*-$^2H_5$]-phenylalanine and L-[ring-$^2H_2$]-tyrosine will be started and maintained throughout the study period. Priming doses of L-[ring-$^2H_5$]-phenylalanine and L-[ring-$^2H_2$]-tyrosine as well as L-[ring-$^2H_4$]-tyrosine will be given IV at time 0. Blood samples (solid arrows) will be drawn at specific intervals between 2 – 4.5 hours elapsed total time to obtain values for determination of the basal rates of whole body protein synthesis and breakdown. Muscle biopsies will be taken using a Bergstrom needle at 2.5 (BX_1) and 4.5 (BX_2) hours (+/- 10 minutes) total time to determine the basal muscle protein FSR. A second primed isotope of phenylalanine (L-[ring-$^2H_8$]-

phenylalanine) will be started at 4.5 hours and used for the measurement of fibrinogen FSR due to the high turnover rate of fibrinogen Subjects will then eat 2 of either Hort 16A Gold (A) or Hayward Green (B) kiwifruit followed by 100g (cooked weight) ground beef immediately following the second muscle biopsy at T=4.5 hrs. The third muscle biopsy (BX_3) will be taken at the end of the study (i.e., at 9.5 hours). The last two muscle biopsies will be used to determine the post-meal muscle protein FSR response. Blood samples will be taken at 0,150, 180, and 270 min before consumption of a test food (the fasted blood samples) and at 290, 310, 330, 360, 390, 420, 450, 480, 510, 540, and 570 min (for fed blood samples) to measure tracer enrichment and plasma responses of amino acids, glucose, and insulin. A total of 15 blood samples will be taken during the study (approximately 90 ml).

Analytical methods. The preparation and subsequent analysis of samples for determination of isotopic tracer enrichment will be carried out by means of gas chromatography-mass spectrometry (GCMS: Models 7890A/5975; Agilent Technologies, Santa Clara CA) as described previously (5, 6). Briefly, 125 ul of 10% sulfosalicylic acid will be added to plasma samples to precipitate protein. Plasma free amino acids will be then extracted from 300 ul supernatant fluid by cation exchange chromatography (Strata-X-C; Phenomenex, Torrance, CA) and dried under Speed Vac (Savant Instruments, Farmingdale, NY). Enrichments of phenylalanine and tyrosine will be measured on the *tert*-butyldimethylsilyl derivative with the use of GCMS (5, 6). Ions of mass to charge ratios of 234, 235, and 239 for phenylalanine and of 466, 467, 468, and 470 for tyrosine will be monitored with electron impact ionization and selected ion monitoring. Plasma glucose concentrations will be measured spectrophotometrically on a Cobas c 111 analyser (Roche, F. Hoffman-La Roche, Basel, Switzerland). Plasma insulin concentrations will be measured by using commercially available human insulin ELISA kit (Alpco Diagnostics, Salem, MA). Plasma AA concentrations will be determined by using liquid chromatography-mass spectrometry (LCMS: QTrap 5500 MS; AB Sciex) using the internal standard method (6). Preparations of muscle tissue samples obtained from the vastus lateralis muscles will be performed as previously described (5, 6). Phenylalanine tracer enrichment from muscle bound protein will be determined as in plasma analyses.

Calculations of protein kinetics at whole body and muscle levels. Whole body protein kinetics will be calculated based on the determinations of the rate of appearance (*R*a) into the plasma of phenylalanine and tyrosine and the fractional *R*a of endogenous tyrosine converted from phenylalanine as we have previously described (5, 6, 13). Briefly, the area under the curve (AUC) of plasma enrichments of phenylalanine and tyrosine tracers will be calculated using Graphpad Prism 6 for Mac (Graphpad Software, La Jolla, CA) (5, 6). Whole body protein kinetics will be calculated by dividing kinetic values of phenylalanine by its fractional contribution to protein (4%) (13). For the calculations for whole body protein breakdown rate, contribution of phenylalanine from exogenous meal and tracers infused will subtracted from total R_a. The following equations will be used for the calculations of whole body protein kinetics:

- Total rate of appearance of tracer into plasma (R_a) = F / E
- Fractional Ra of Tyrosine from Phenylalanine (Fractional Ra of Tyr from Phe) = $E_{Tyr\ M+4}$ / $E_{Phe\ M+5}$
- Rate of phenylalanine hydroxylation to tyrosine (Phe hydroxylation rate) = Fractional R_a of Tyr from Phe x R_a Tyr
- Protein synthesis rate = [(R_a Phe – Phe hydroxylation rate) x 25]
- Protein breakdown rate = [(R_a Phe – F Phe) x 25 - PRO]
- Net protein balance = Protein synthesis rate – Protein breakdown rate
- Muscle protein fractional synthesis rate (FSR, %/h) = [(E_{BP2} – E_{BP1})/ (E_m x t)] x 60 x 100

In these equations, enrichment (E) is expressed as tracer-to-tracee ratio (TTR) or mole percent excess (MPE), calculated as TTR/(TTR + 1). TTR will be used for calculations of rates of protein breakdown whereas MPE will be used for calculations of rates of protein synthesis. E is enrichment of respective tracers. *F* is the tracer infusion rate into a venous site: *F* Phe for phenylalanine tracer. E Tyr M+4 and E Phe M+5 are plasma enrichments of tyrosine tracer at M+4 and of phenylalanine tracer at M+5 relative to M+0, respectively. The correction factor of 25 is for conversion of kinetic

value for phenylalanine to protein based on the assumption that contribution of phenylalanine to protein is 4% (100/4 = 25) (2). PRO is the amount of exogenous protein (g) that is the amount of amino acids appearing in the circulation as a result of the exogenous protein digestion, accounting for splanchnic extraction (29%) of amino acids in young adults (11). Phe hydroxylation rate is the rate of appearance of tyrosine derived from phenylalanine through process of hydroxylation. Calculation of muscle protein FSR will be performed as previously described (5, 6). E_{BP1} and E_{BP2} are the enrichments of protein bound L-[*ring*-$^2H_5$]-phenylalanine in the first and second muscle biopsies, respectively, and E_m is the mean plasma enrichment of the L-[*ring*-$^2H_5$]-phenylalanine. *t* is the duration of time in minutes elapsed between two muscle biopsies; 60 and 100 are factors used to express muscle protein synthesis in percent per hour.

Statistical analysis and power calculations. Where effects of both the intervention and time require analysis, a two-way analysis of variance (ANOVA) model with repeated measures will be used. When significant differences between the interventions were identified by ANOVA, post-hoc Student's t-tests will be performed. When analysis involves the comparison of the total response to each intervention only, paired student's t-tests will be used. For all comparisons, statistical significance will be set at $p<0.05$.

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