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Human Kinetics
ORIGINAL RESEARCH

Effects of Different Vitamin C–Enriched Collagen Derivatives on Collagen Synthesis

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Nutritional strategies to improve connective tissue collagen synthesis have garnered significant interest, although the scientific validity of these interventions lags behind their hype. This study was designed to determine the effects of three forms of collagen on N-terminal peptide of procollagen and serum amino acid levels. A total of 10 recreationally active males completed a randomized double-blinded crossover design study consuming either placebo or 15 g of vitamin C–enriched gelatin or hydrolyzed collagen (HC), or gummy containing equal parts of gelatin and HC. Supplements were consumed 1 hr before 6 min of jump rope. Blood samples were collected immediately prior to supplement consumption and 4 hr after jump rope. A subset of blood samples ($n = 4$) was collected for amino acid analysis 1 hr after ingestion. Consumption of an equivalent dose of each supplement increased amino acids in the circulation similarly across all interventions. N-terminal peptide of procollagen levels tended to increase ~20% from baseline in the gelatin and HC interventions but not the placebo or gummy. These results suggest that vitamin C–enriched gelatin and HC supplementation may improve collagen synthesis when taken 1 hr prior to exercise. However, large variability was observed, which precluded significance for any treatment.

Keywords: gelatin, N-terminal peptide of procollagen, exercise, musculoskeletal, injury

Collagen is the primary structural protein in tendon, ligament, skin, and bone (Kjaer, 2004). In athletes, the integrity of these collagen-based tissues is instrumental to force transfer and underpins injury prevention and return to play. Therefore, interventions that improve the robustness of these tissues may have an effect on performance.

Dietary supplementation with gelatin or hydrolyzed collagen (HC) products has become popular among athletes aiming to improve connective tissue function and prevent or treat injury. However, the scientific validity of these nutritional interventions lags behind their hype.

Gelatin and HC have a similar amino acid profile and are particularly high in glycine, proline, hydroxyproline, hydroxylysine, and arginine (Eastoe, 1955). Gelatin is isolated from the collagen within the skin, tendons, ligaments, and bone of cattle, fish, or pigs through boiling. This large molecular weight protein can be further processed using acids or enzymes to produce the small peptides that are termed HC. Some variance exists in the peptide sequence and functional properties of the products, and this can be influenced both by the source and age of the animal as well as the processes used during extraction (Eastoe, 1955). The main functional difference between the two products is that HC is soluble in water and cannot form a gel.

Both gelatin and HC are both digested, absorbed, and incorporated into various connective tissues (Oesser et al., 1999; Shaw et al., 2017). Interestingly, dietary proline and HC are both incorporated into the skin of rats (Matsuda et al., 2006); however, HC is preferentially incorporated into musculoskeletal tissues including muscle, cartilage, and bone (Oesser et al., 1999). Furthermore, supplementing with HC has been shown to increase cartilage thickness (McAlindon et al., 2011) and decrease knee pain in athletes (Clark et al., 2008). However, little is known about how different collagen derivatives differ in digestibility, absorption, or their ability to stimulate collagen synthesis or how collagen synthesis is best measured in a healthy athletic human body.

Therefore, the current study aimed to determine the effect of different preparations of collagen supplements on collagen synthesis rates. Subjects were provided with 15 g of vitamin C–enriched collagen either as a drink containing gelatin or HC or as a gummy containing equal parts of gelatin and HC. One hour later, the subjects were asked to jump rope to stimulate bone collagen synthesis, and after a further 4 hr, blood was drawn to determine the circulating levels of the N-terminal peptide of procollagen (PINP). PINP in the blood primarily represents bone collagen synthesis (Pollmann et al., 2007). In the current work, it is assumed to be a marker of collagen synthesis in bone and not other collagenous tissues; however, with current methods, it is not possible to determine the source of this cleaved peptide measured. We hypothesized that collagen synthesis rates would be increased following the consumption of collagen, regardless of whether the collagen was provided as gelatin or HC or as a gummy.

Material and Methods

Participants, Blood Sampling, and Exercise Intervention

Recreationally active males provided written consent to participate in this repeated-measures designed study that was approved by the University of California Davis Institutional Review Board (1140867-1) and was written in accordance with standards set by the Declaration of Helsinki and submitted to the Clinical Trials Registry (ACTRN12616001092482). Prior to commencement of the study, subjects were familiarized with the study protocol. On the

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first day of the study, anthropometric data were collected using a bioelectrical impedance scale (Tanita™ BF-680 W, Arlington Heights, IL) and stadiometer. The subjects then returned to the lab after an overnight fast and provided a baseline blood sample ($t = -1$ hr) from the antecubital vein of the arm (Figure 1). Immediately following the blood draw, participants ingested 15 g of either gelatin or HC within a diluted cordial beverage (Ribena®; Suntory Limited, Uxbridge, United Kingdom) or as a gummy containing 7.5 g of both gelatin and HC. Over the course of the study, each subject performed each of the trials in a randomized order separated by at least a 48-hr washout period. The gummy was prepared by heating the water, adding the Ribena®, rapidly mixing in gelatin and then the HC into solution. The liquid mixture was then poured into silicon molds, refrigerated, and solidified for 24 hr. One hour after consuming the intervention supplement, participants completed 6 min of continuous jump rope to load the musculoskeletal system (Shaw et al., 2017). After completing the jump rope, participants were permitted to consume food ad libitum, which was recorded and simulated for each subsequent intervention. Participants were also instructed to remain in a rested state with only light activity such as walking or studying permitted. Four hours after jumping rope, a second blood draw was collected ($t = 4$ hr).

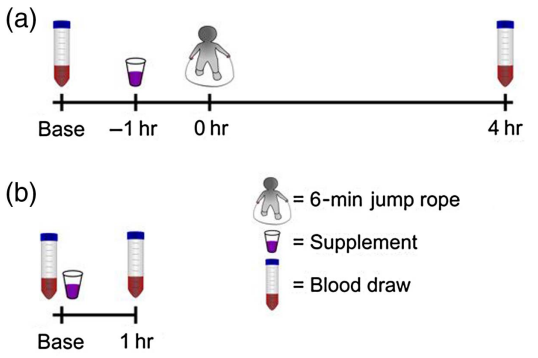

Figure 1 — Schematic of study design. (a) Primary study arm and (b) secondary study arm.

Nutritional Supplement

Four isocaloric nutritional supplements were provided to each subject in a random order: (a) 15-g maltodextrin + 80-ml blackcurrant cordial containing 48 mg of vitamin C at time of production (Ribena® Suntory Limited) + 250-ml water (PLA), (b) 15-g gelatin (GreatLakes™, Grayslake, IL) + 80-ml blackcurrant cordial + 250-ml water (GEL), (c) 15-g hydrolyzed collagen (Gelita™, Culumet City, IL) + 80-ml blackcurrant cordial + 250-ml water (HC), and (d) 120 g of a solid gummy with ~7.5 g each of gelatin and hydrolyzed collagen + blackcurrant cordial + water (GUM). Beverages were mixed well in an opaque drink bottle immediately prior to ingestion, and participants were instructed to consume the beverage or food as quickly as possible 1 hr prior to exercise. Treatments were separated by a minimum of 48 hr to avoid carryover effects from the previous treatment. Washout was successful as can be seen in the hydroxylysine levels at baseline in each trial (Figure 2). Nutritional intake in the 12 hr prior to and during each intervention was self-selected by participants under the instruction to manually record and simulate food and beverage consumption for each intervention and to avoid alcohol 24 hr prior to and during each intervention.

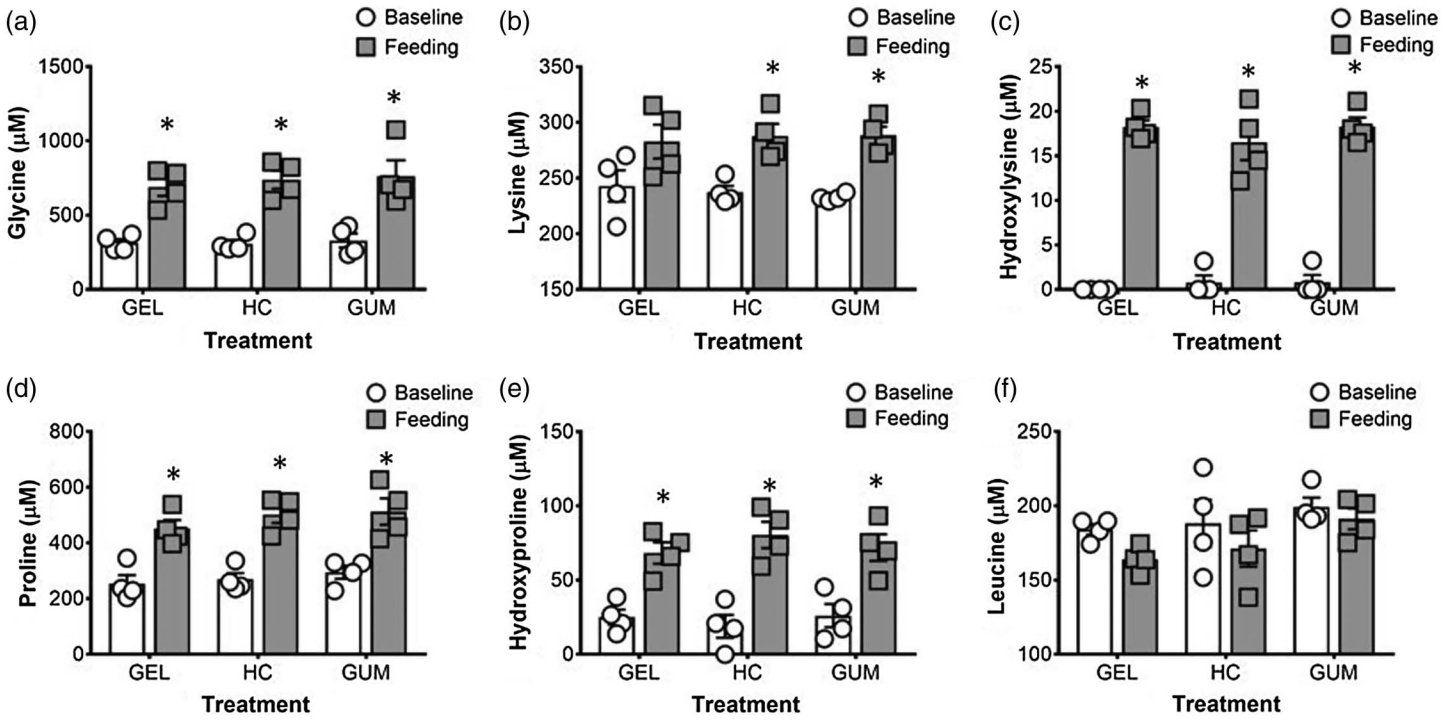

Figure 2 — Amino acid concentration in the serum following ingestion of 15 g of vitamin C–enriched GEL, HC, or GUM. The amount of (a) glycine, (b) lysine, (c) hydroxylysine, (d) proline, (e) hydroxyproline, and (f) leucine in the blood was determined by high performance liquid chromotography by the UC Davis Proteomics Core. The data are presented as mean ± *SD* for the four subjects who completed the crossover study. The two-way analysis of variance (treatment and time) indicated a significant time effect for all amino acids tested ($p < .05$) except for leucine. There were no significant differences in amino acid levels at baseline or 1 hr postingestion between any of the interventions. GEL = gelatin; HC = hydrolyzed collagen; GUM = gummy.

N-Terminal Peptide of Procollagen

Blood samples were collected in 3.5-ml clot-activated serum separating tubes (Vacutainer Serum Separating Tube [SST]; Becton Dickinson, Franklin Lakes, NJ), allowed to clot for 20 min before centrifugation at 4,150 rpm for 10 min. Serum samples were then aliquoted into 1.5-ml Eppendorf tubes and stored at −30°C until processing. PINP levels were analyzed by an enzyme-linked immunosorbent assay (ELISA) kit (Novatein Biosciences, Woburn, MA). Samples were measured in duplicate according to the manufacturer's instructions using an Epoch microplate spectrophotometer (BioTek, Winooski, VT). The intraassay %confidence variable was 0.76–1.37% and interassay was 1.12%, and the sensitivity of the assay was 1.0 pg·ml^{-1}.

Amino Acid Content of the Blood

A random subgroup ($n = 4$) volunteered to provide a separate aliquot of blood for the purpose of determining the amino acid content of the serum after each treatment. This arm of the study involved a fasted baseline blood sample and ingestion of one of the three interventions GEL, HC, and GUM in a randomized order on three different occasions separated by 24 hr, then a second blood sample collection 1 hr after supplement ingestion (Figure 1). Whole blood was collected in 3.5-ml clot-activated serum separating tubes (BD SST) tubes, allowed to clot for 20 min, and then centrifuged at 4,150 rpm for 10 min. The resulting serum was separated into single 1.5-ml microtubes and stored at −30°C until subsequent analysis. Amino acids levels were quantified at the UC Davis Proteomics Core facility by high-performance liquid chromatography. Briefly, serum samples were hydrolyzed in 6N hydrochloric acid for 24 hr at 110°C. 50-µl of 10% sulfosalicylic acid was added to 200 µl of the hydrolyzed serum samples. This mixture was diluted 1:2.5 in aminoethylation-cysteine buffer, and a 50-µl injection volume was analyzed using a high-speed Amino Acid Analyzer (model L-8900; Hitachi High-Technologies, Pleasanton, CA).

Statistical Analysis

Sample size calculations for the study were based on the mean changes in PINP and *SD*s established in previous work in this area (Shaw et al., 2017). Given the mean changes and *SD* in PINP with gelatin supplementation ($SD = 12\%$), a sample size of 8 subjects per group was needed to yield a power of 80% to detect 20% differences with 95% confidence interval. A robust regression and outlier removal outlier test was performed for analysis of PINP levels to determine outlying data, and these outliers were removed from subsequent analysis. PINP levels were analyzed by two-way analysis of variance with one factor being time and the other being the supplement (version 5; GraphPad Prism, San Diego, CA). Data are presented as mean ± *SE* of the mean, and the significance level was set at $\alpha < .05$ for all comparisons. Paired *t* test was performed post hoc to compare each intervention individually to the PLA. Amino acid content and PINP levels were analyzed by two-way analysis of variance with one factor being time and the other being the supplement.

Results

The 10 recreationally active males who successfully completed the study were 22.7 ± 5.2 years old, 179.0 ± 8.0 cm tall, weighed 78.8 ± 7.74 kg, and had a body fat of 13.6 ± 3.11%.

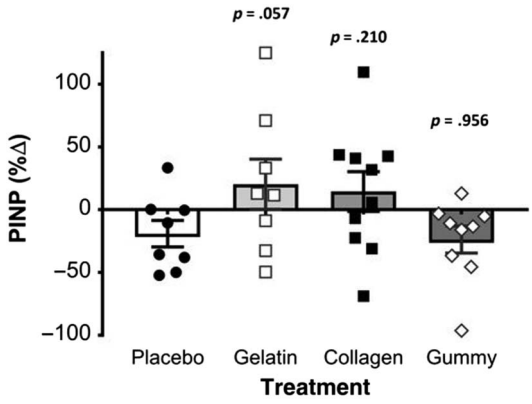

Figure 3 — Collagen synthesis following exercise and ingestion of placebo or equivalent dose vitamin C–enriched GEL, collagen of gummy food. Changes in serum levels of PINP at 4 hr postexercise from baseline (1 hr prior to exercise). Results from paired *t* tests between the placebo and each feeding treatment are shown over the appropriate bar. Note the strong trend for significance in the GEL group. No significance was found between interventions based on the two-way analysis of variance ($p < .05$). GEL = gelatin; HC = hydrolyzed collagen; GUM = gummy; PINP = N-terminal peptide of collagen.

Effects of Supplementation on PINP

Compared with PLA (−6.8 ± 43.7%), GEL and HC supplementation demonstrated a positive change from baseline PINP 4 hr after completing the jump rope (20.4 ± 52.9 and 19.6 ± 47.5%, respectively), whereas GUM demonstrated a negative change (−23.8 ± 30.5%; Figure 3). Mean changes in PINP from baseline were not statistically significant ($p > .05$) likely due to the high rate of variability between subjects. (A total of 5 outlying data points were removed.)

Amino Acid Concentration in the Blood

We hypothesized that the lower PINP in the GUM group was the result of a decrease in the rate of digestion and absorption of the GUM compared with the GEL and HC groups. To test this hypothesis, subjects were invited back, and the circulating levels of amino acids were determined in the blood 1 hr after consuming the supplement. Levels of glycine, lysine, proline, hydroxyproline, and hydroxylysine, but not leucine were all elevated at the 1-hr time point. However, in contrast to our hypothesis, the increase in each amino acid was consistent across all three interventions (Figure 2).

Discussion

Nutritional interventions, particularly the use of collagen derivatives, have become popular as a means to increase collagen synthesis in the body (Katarzyna & Walczak, 2009). However, there is limited understanding as to how the delivery of these collagen products affect amino acid appearance in the blood and subsequent collagen synthesis. The current study has demonstrated that a dose of 15 g of vitamin C–enriched gelatin or HC similarly increases PINP, an indirect marker of collagen synthesis. However, likely due to the large variability seen in the control and experimental groups, these changes were not statistically significant. Interestingly, a composite of gelatin and HC in a vitamin C–enriched gummy actually decreased PINP. We hypothesized that the poor response to the

gummy was the result of slowed digestion and absorption; however, circulating amino acid concentrations were similar in all three interventions. Overall, the results from this study suggest that vitamin C–enriched gelatin and HC ingested prior to 6 min of jump rope result in a comparable, albeit modest, increase in collagen synthesis.

The major shortcoming of this study was the extreme variability in the PINP measures in all but the gummy group. This is in stark contrast with our previous work using the exact same ELISA plate (Shaw et al., 2017). A great deal of time was spent troubleshooting the variability of the PINP assay, and we discovered that vitamin C can inhibit ELISA reactions (Meng et al., 2005). The ELISA plate manufacturer has since confirmed that adding vitamin C to the samples decreases the colorimetric reaction used for this assay. Several factors may affect vitamin-C concentration in the samples and subsequently the ELISA results. Individual variability in subject dietary vitamin-C intake and the concentration of vitamin C in the cordial drink at time of ingestion are the primary factors that could affect vitamin-C content of the samples. Vitamin C is known to degrade with storage time, heat, and the type of storage container (Igwemmarm et al., 2013; Peleg, 2017). Each bottle or batch of cordial may therefore have very different levels of active vitamin C at the time of ingestion. A second important factor that may have contributed to the variation was the duration that the serum was left to clot before centrifugation, pipetting, and freezing. The blood processing time may influence vitamin-C concentration within the sample. Previously, blood samples were allowed to clot for 2 hr before processing (Shaw et al., 2017), whereas in the current study, the blood was centrifuged after 20 min of clotting. The longer time may result in the degradation of vitamin C, and thus, decreased the variability of the resulting ELISA reaction. In support of the hypothesis that difference in vitamin-C levels contributed to the variability in the ELISA, the GUM group, where the juice was heated and the vitamin C likely inactivated, showed the least PINP variability of the four groups. To further investigate this, we have since measured PINP levels in serum allowed to clot for precisely 20 min, 1 hr, and 2 hr and have shown that variability is least after the 1-hr serum clotting time. Therefore, for groups looking to replicate this work, it would be prudent to allow blood samples to clot for exactly 1 hr before processing, regardless of established phlebotomy handling guidelines.

Aside from this potential technical issue, this study produced several interesting results. First, the recognized precursors for collagen synthesis, glycine, lysine and proline, and indicators of collagen digestion/absorption (hydroxyproline and hydroxylysine) increased to the same extent in all of the interventions (Figure 2), whereas the key muscle signaling amino acid, leucine, did not. The change in serum amino acids reflects the composition of collagen: glycine (about 33% amino acid residues), proline (12–14%), hydroxyproline (<14%), and hydroxylysine (1.5%) (Parry, 1988). However, since the levels of hydroxylysine and hydroxyproline were lowest at baseline, the relative increase in these nonproteinogenic amino acids with feeding was largest. Others have also measured significant increases in hydroxyproline that peak 0.5–2 hr after ingestion (Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2011). The changes in glycine, proline, leucine, and lysine following all three feedings were also comparable to those reported by Shaw et al. (2017), indicating that the consumption of either GEL, HC, or a gummy results in similar, reliable, and repeatable change in serum amino acid levels.

The second interesting finding was that even though all three nutritional interventions increased amino acids similarly, only the GEL and HC treatments tended to increase PINP in the serum. Collagen synthesis increased approximately 20% from baseline in the GEL and HC groups; however, in the GUM or PLA groups, there was no increase in PINP 4 hr after completing the 6 min of jump rope. The finding that GEL and HC increase PINP ~20%, although not significant, aligns with our previous work demonstrating a 30% increase in serum PINP when consuming 15 g of vitamin C–enriched gelatin. In this context, it is interesting that the GUM group did not show an increase in PINP even though serum amino acid levels rose similar to the GEL and HC groups (Figure 2). One possible explanation for this disparity is that in the preparation of the gummies, Ribena® was exposed to heat to dissolve the gelatin. Since heating is known to degrade vitamin C (Peleg, 2017), it is possible that following an overnight fast there is not sufficient vitamin C in the serum to support measurable levels of collagen synthesis. Further work is needed to understand the role of vitamin C of collagen synthesis and whether coingestion of vitamin C is integral to this process. However, as discussed previously, these studies need to employ methods to reduce vitamin C in the serum samples prior to PINP measures to minimize the inhibitory effect of vitamin C on the ELISA reaction.

A second discrepancy between the current work and our previous work in this area (Shaw et al., 2017) is that the PLA group did not increase PINP 4 hr after jump rope. We had previously measured an increase in PINP in the placebo (exercise alone) group (Shaw et al., 2017). PINP is the amino (N) terminal peptide that is cleaved from the procollagen molecule in the process of collagen synthesis, and its presence in the serum is a validated marker of bone collagen synthesis (Orum et al., 1996). While the decrease in PINP in the current study might be the result of the vitamin-C issue described previously, it is also possible that the subjects in the current study were more acclimated to this type of mechanical loading (being boxers and runners), and therefore, the loading stimulus would need to be greater to stimulate adaptations within the bone (Kjaer, 2004; Shaw et al., 2017).

Another related limitation of the current work is that we do not provide any evidence that the increase in PINP in the blood corresponds with an increase in collagen within tendons, ligaments, or other tissues. In fact, there are cases where changes in PINP within the tendon is opposite the change in collagen incorporation into the same tissue (Hansen et al., 2009) This indicates that collagen synthesis does not always reflect collagen accumulation in tissue. As a result, further work is needed to determine whether the changes in PINP measured here reflect increased collagen accumulation in tissue.

A third interesting finding in this study was that there was large variability in specific amino acid appearance between subjects and interventions. This biological variability is likely influenced by differences in habitual dietary intake and possibly body weight (Daniel, 2004; Gómez-Guillén et al., 2011; West et al., 2015). Habitual meat intake, or polymorphisms, may directly affect the level or activity of peptide transporters, endopeptidases, and/or exopeptidases and influence the absorption of amino acids from the various collagen derivatives (Daniel, 2004). As the use of collagen supplementation for prophylactic and treatment purposes in sport grows, these are important factors to consider for future investigation of the efficacy of nutritional modulation of collagen synthesis rates (Gómez-Guillén et al., 2011). Another important factor to consider is whether there is anything specifically important about collagen-based proteins for the synthesis of collagen within the body. In other words, would 15 grams of a different type of protein (e.g., whey) have the same effect as gelatin or HC?

Conclusion

This study demonstrated that ingestion of equivalent doses of gelatin and HC in conjunction with a 6 min exercise stimulus increased PINP levels comparatively. Large variability in PINP levels was evident, which obviated proper statistical analysis between the interventions. However, the fact that the increase in key amino acids was similar for all interventions strengthens the notion that gelatin and HC had similar effects on collagen synthesis. One further finding, based on the results from the gummy intervention is that it is possible that co-ingestion of vitamin C may be required to drive collagen synthesis following an overnight fast. Finally, vitamin C can impair ELISA results, and this, alongside a 1-hr blood clotting time, should be considered when replicating the current work.

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