NAVAL HEALTH RESEARCH CENTER

CONNECTIVE TISSUE BREAKDOWN AND BONE MORPHOLOGY CHANGE FOLLOWING INCREASED INTENSITY EXERCISE

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SUMMARY

Two plasma indicators of connective tissue (CT) degradation have been identified in previous work. One of these, hydroxyproline (OHP), is a widely accepted measure of CT turnover; the other, hydroxylysylpyridinoline (HP), has only recently been isolated in plasma. In this study, responses of OHP and HP are compared to bone morphology changes associated with a 9-week period of increased intensity aerobic exercise. Bone morphology was measured as tibial bone mineral density (BMD) from dual photon absorptiometry (DPA), and tibial cross-sectional area (XAREA) from quantitative computed tomography (QCT). Nine local club runners (TGRP: mean age 36.6 yrs, distance 22.9 km/wk, pace 3.30 m/sec) ran their normal program for a 3-week baseline period. Their pace was increased 4% (distance unchanged) for a 9-week period. Ten university staff personnel whose training remained stable for 9 weeks served as controls. OHP, HP, BMD, and XAREA were measured prior to (T1) and following (T2) the 9-week period. Repeated measures ANOVA revealed significant group by time interactions for OHP ($p=0.03$), HP ($p<0.001$), and BMD ($p=0.02$). OHP and HP increased and BMD decreased significantly for the TGRP at T2. XAREA was unchanged. A modest, but significant, Spearman Correlation (-0.44, $p=0.03$) was found between residualized gain scores for HP and BMD. The correlation between OHP and BMD residuals approached significance (-0.37, $p=0.06$). It was concluded that: 1) bone mineral density adjustments to increased intensity were not complete after 9 weeks, 2) increased intensity exercise at a 4% rate does not alter tibial XAREA, and 3) HP appears to be a better indicator of connective tissue degradation than is OHP.

INTRODUCTION

Exercise has been shown to lead to modification of bone density and bone mineral content (13). Cross-sectional studies indicate increasing levels of physical activity leads to increased bone density and bone mineral content, while decreased exercise levels lead to decreased bone density and bone mineral content. Within subject studies have been less clear. Margulies and coworkers (10) have shown increases of 8% to 14% in the bone mineral content of the distal third of the tibia after 14 weeks of strenuous military training. However, others have shown increases in bone mineral content with running training over longer periods of time (6,19). Simkin and colleagues (14) studied the effects of 5 months of loading exercise on distal forearm bone density. Results indicated localized increases in bone density with no change in bone mineral content. Beyer and colleagues (3) reported changes in bone density, mass, and mineral content of rats after training on a treadmill running 35 min/day, 5 days/wk for 16 weeks. Smith and coworkers (16) studied bone mineral density (bone mineral content divided by bone width) in middle aged women who participated in aerobic dance training 45 min/day, 3 days/wk for more than 3 years. These researchers found a 3.8% decrease in bone mineral density the first year, and an increase of 1.39% over the next two years. Clearly more work is needed to clarify the relationships between the nature of physical activity (mode, intensity, frequency, and duration) and its effects on bone morphology.

One adaptive response of bone to increased "stress" is to remodel. The remodelling process begins with resorption of the bone matrix and the concomitant breakdown of mature collagen. The resorption phase is followed by a constructive phase in which new bone matrix is laid down to better resist the imposed mechanical stress. During the resorption phase, the breakdown of collagen is reflected in the appearance of increased plasma concentrations of biochemical byproducts of that breakdown.

The amino acid hydroxyproline (OHP) has been widely accepted as one of the main biochemical indices of connective tissue turnover. OHP is formed by the intracellular hydroxylation of proline during procollagen synthesis. It is virtually unique to collagen. Plasma OHP can be derived from both intra- and extracellular

compartments. The appearance of OHP in plasma reflects the degradation of both procollagen (which has been extruded from the cell) and mature extracellular collagen.

When collagen synthesis is increased, the amount of procollagen formed which contains transcription errors is increased (2). This "error-containing" procollagen is catabolized and not incorporated into mature collagen. Thus, elevated levels of plasma OHP may reflect both increased procollagen synthesis rates and mature collagen breakdown.

Recently Vailas and colleagues (18) have isolated a stable 3-hydroxylpyridinium collagen cross-link, hydroxylysylpyridinoline (HP) in plasma. HP is one of two characterized forms of non-reducible collagen cross-links (8). It contains a 3-hydroxypyridinium ring with three amino and three carboxyl groups; and can be identified by fluorescence of the ring structure. The HP cross-link is formed extracellularly during the linking of collagen strands to form structural, mature collagen. Increased plasma levels of HP then should indicate only the breakdown of mature collagen.

The extent to which the appearance of these markers of CT breakdown might reflect the observed changes in bone morphology has not been determined. As part of a program of studies to characterize marker response to exercise stress, the purpose of this study was to measure changes in bone morphology and plasma concentrations of OHP and HP in human subjects following 9 weeks of increased-intensity exercise training.

The hypotheses were: 1) changes in bone morphology would be associated with changes in levels of plasma markers of connective tissue degradation; and 2) that these associations would be stronger for the HP cross-link, which reflects extracellular breakdown of collagen, than for OHP, which is derived from both extra- and intracellular breakdown.

METHODS

Subjects. A total of 20 subjects participated in this study. They were divided into two groups, an experimental group and a control group of ten subjects each. The experimental group was recruited from a local running club. These runners were all following running programs which had been stable for several months prior to the

onset of the study. A group of 10 university staff members, similar in age and training to the running club members, participated in this study as a control group. All participants gave their written informed consent to participate.

Measures. This study used two measures of bone morphology: bone cross-sectional area and bone mineral density. Two plasma markers of connective tissue breakdown were also employed: hydroxyproline (OHP) and hydroxylysyl-pyridinoline (HP).

Bone cross-sectional area was determined at the mid-shaft of the tibia using Quantitative Computerized Tomography (QCT). Prior to x-ray imaging, the mid-shaft level was measured as 50% of the distance from the top of the tibial plateau to the medial malleolus. The position was marked and used to align the x-ray field. A glass rod of known diameter was placed between the legs during the x-ray imaging and used as the standard for calculating cross-sectional area from the image. Cross-sectional area was calculated as the total number of pixels in the image exceeding a defined gray scale level using image digitization software (Jandel Scientific, Corte Madera, CA). The measurement error for cross-sectional area determination, calculated as the mean absolute between-trial difference divided by the grand mean of two repeated trials, was 1.76%.

Bone mineral density was determined from dual-photon absorptiometry (DPA). DPA imaging (Norland Instruments) was also done at tibial mid-shaft. During imaging, the subject's leg was internally rotated 45°. This angle was chosen to provide the greatest separation between tibial and femoral images. Also during imaging, a water bag was placed on the medial side of the tibial shaft to provide similar phase transitions for the photon beam on all sides of the bone. The bone mineral content was determined from DPA and expressed in units of grams of minerals for a 1 cm wide path. This value was then divided by the bone cross-sectional area determined from QCT; to yield a bone mineral density in $g \cdot cm^{-3}$. Each participant was scanned twice during each session. Measurement error for bone mineral content, determined in a pilot study, was 1.89%. The correlation between repeated measurements for the participants in this study was 0.98.

OHP and HP were determined from a 10 ml blood sample. Blood was collected in heparinized tubes, centrifuged, and frozen at -70°C for analysis. At the time of analysis, the samples were thawed and the fats and steroids removed with ethyl acetate. The noncollagenous proteins, protein-bound OHP, and other interference substances were precipitated using 8% trichloroacetic acid (4,5,11). The samples were then hydrolyzed in an equal amount of 12N HCl for 24 hours at 110°C, cooled to room temperature, and dried under a vacuum with anhydrous pellets NaOH (11).

Hydroxyproline was determined using the method of Woessner (20). Dried plasma samples were resuspended in 1.0 ml of 0.065M NaCitrate buffer (pH=2.0), vortexed, and filtered (nylon 66 membrane, 0.45µm pore size; Gilman Sciences, Inc). A 0.6 ml aliquot was used to determine OHP concentrations colorimet-rically at a wave length of 557 nm. The remaining 0.4 ml filtered aliquot was dried for subsequent analysis of HP cross-links. The minimum detection limit for this assay is 1 pmol. The test-retest reliablility for duplicate samples in this assay was 0.96. The within subject reliability over a 1-7 day period was 0.71.

Hydroxylysyl-pyridinoline samples were resuspended in 1.0 ml 0.065M NaCitrate buffer (pH=2.0), vortexed, and filtered. The supernatant was applied to a low pressure cation exchange column (Benson amino acid resin, BH-4, 7-20 µ) and eluted with 0.065M NaCitrate buffer (pH=4.6) at 0.6 ml/min. The fluorescence of the eluent was detected using an in-line detector (Waters, model 420) with excitation at 395 nm, and emission at 420 nm and integrated to yield total sample cross-link content. The system was calibrated using a pyridoxamine standard (Sigma Chemical). The minimum detection limit for this assay was 5 pmol. The test-retest reliability for duplicate samples was also 0.96, and the within subject reliablility over 1-7 days was 0.82.

Procedures. Subjects reported to the laboratory and provided their informed consent to participate. The subjects had the maximal rate of oxygen consumption (VO_2 max) determined from open-circuit spirometry measurements during a progressive treadmill running test in which both speed and grade were increased. Their percent body fat was determined from hydrodensitometry using the equation of Siri (15), and their 1.5 mile run time was measured.

Prior to the onset of increased exercise intensity, the exercise logs of the experimental subjects were reviewed and the mean running speed and distance for the month prior to the study was determined. The subjects were required to run at this speed and distance under supervision 5 days per week, for 3 weeks prior to the onset of increased intensity exercise. This was done to stabilize their exercise patterns prior to study onset.

Following the three weeks of exercise stabilization, the subjects were taken to the Radiology Laboratory at the University of California at Los Angeles, where bone cross-sectional areas and bone densities were determined. Blood samples for OHP and HP determination were also taken.

After baseline measurement, the running pace of the experimental group subjects was increased on alternate days for 3 of the 5 daily exercise sessions of each week. The distance remained the same. The net effect was, the average pace increased by 3.9% (range = -4.2 to +9.8). Only one individual did not show a net increase in pace. This increased intensity exercise program was followed for 9 weeks. The measures taken at baseline were then repeated within 3 days of the last exercise session. A training period of 9 weeks was picked because the cardiovascular and metabolic adjustments to training should be virtually complete after this period (1).

The control group had only the bone and blood measures made prior to and following a 9-week period in which they were instructed not to change their training program from what it had been over the last month. Physical characteristics and fitness were not determined.

Analysis. Changes in bone and blood parameters following the 9-week training period were assessed using a mixed design analysis of variance (ANOVA) with time as the within-subject factor and training program (experimental or control) as the between-subjects factor. These analyses were carried out using the MANOVA procedure of SPSSX (17). When significant interactions were found, post-hoc analysis was carried out using the appropriate t-test.

Associations between the blood markers and changes in bone morphology were determined from individual responses to the training program. The regressions to predict post-training values of bone cross-section, bone mineral density, plasma OHP,

and plasma HP from their pre-training values were calculated (including values from both groups), using the SPSSX REGRESSION procedure. The post-training values were then expressed as standardized residuals about the pre-post regression. These residualized gain scores for the blood measures were then correlated with the residualized gain scores for the bone measures, using Spearman rank-order correlation coefficients, to determine the associations between variations from the average response on these variables.

RESULTS

Responses to training. The experimental subjects attended a mean of 86.3% of the sessions. Table 1 shows the pre-training and post-training characteristics of the experimental subjects. It can be seen from this table that weight, percent fat, and running pace were with training, but VO_2max and weekly distance did not.

Table 1.
SUBJECT CHARACTERISITICS
(Experimental Group, N=10)

	Pre-Training	Post-Training
Age (yrs):	**39.4 ± 4.7**	**-**
Height (cm):	**177.4 ± 7.8**	**-**
Weight (kp):	**81.6 ± 11.5**	**79.6 ± 10.1***
% Fat:	**18.9 ± 7.8**	**17.5 ± 8.2***
VO_2max (l/min):	**4.5 ± 0.3**	**4.4 ± 0.2**
Running Dist. (km/wk):	**22.9 ± 6.0**	**24.0 ± 9.8**
Running Pace (m/sec):	**3.4 ± 0.5**	**3.5 ± 0.5***

***Differs significantly ($p<0.05$) from pre-training value.**

Figure 1 shows the mean tibial cross-section values with standard deviations for each group prior to and following the 9-week training period. The changes in tibial cross-sectional area were not significant for either group.

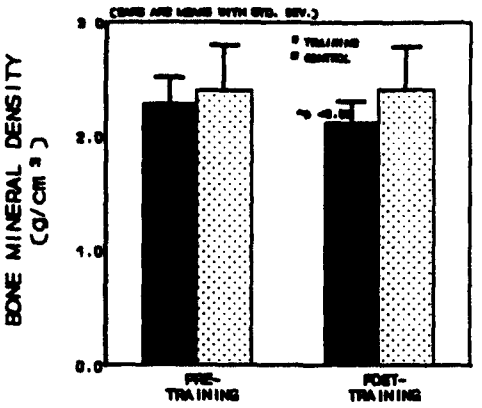

Figure 1. TIBIAL CROSS-SECTION PRIOR TO AND FOLLOWING 9 WEEKS TRAINING

Similarly, Figure 2 shows the mean bone mineral density values prior to and following the 9-week training period. The ANOVA revealed a significant Group x Time interaction ($F(1,18)=6.97$, $p=0.017$), which post-hoc analysis indicated was due to a significant decrease in density for the experimental group ($t=2.84$, $df=9$, $p=0.019$), but no change for the control group ($t=-0.32$, $df=9$, $p=0.757$).

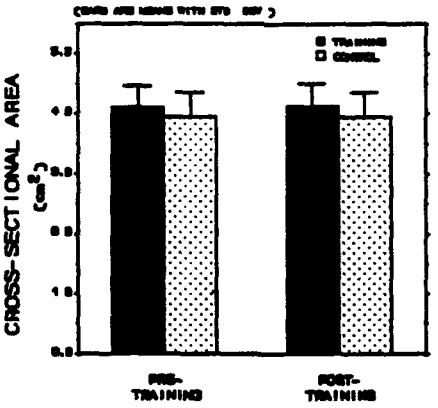

Figure 2. BONE MINERAL DENSITY PRIOR TO AND FOLLOWING 9 WEEKS TRAINING

Figure 3 depicts the mean plasma OHP values for each group prior to and following the 9 weeks of training. Again, the ANOVA revealed a significant Group x Time interaction ($F(1,18)=4.93$, $p=0.039$). Post-hoc analysis showed a significant elevation in plasma OHP following training in the experimental group ($t=3.69$, $df=9$, $p=0.005$) but no significant change for the control group ($t=0.40$, $df=9$, $p=0.689$).

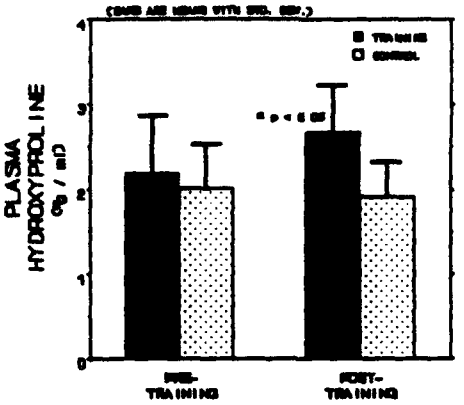

Figure 3. PLASMA HYDROXYPROLINE PRIOR TO AND FOLLOWING 9 WEEKS TRAINING

A similar pattern in HP values is seen in Figure 4. There is a significant Group x Time interaction ($F(1,18)=17.56$, $p=0.001$) due to a significant ($t=4.10$, $df=9$, $p=0.003$) elevation in plasma cross-link post-training in the experimental group with no change in cross-link values for the control group ($t=1.12$, $df=9$, $p=0.291$).

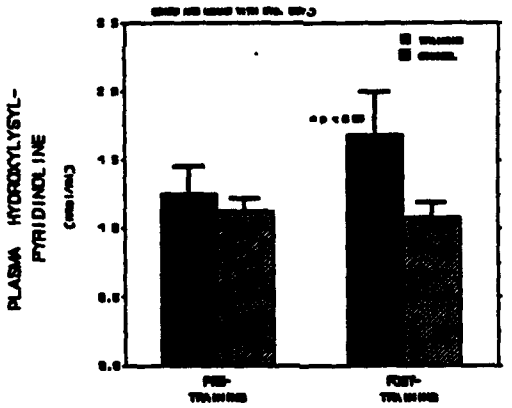

Figure 4. PLASMA HYDROXYLYSYLPYRIDINOLINE PRIOR TO AND FOLLOWING 9 WEEKS TRAINING

Associations among the variables. Table 2 shows the Spearman rank order correlation coefficients and associated probability values among the regression residuals for the blood markers and bone morphology measures. The two plasma markers were significantly correlated, but the two morphology measures were not. Neither of the plasma marker residuals were significantly correlated with the bone

cross-section residuals. The HP residuals were significantly correlated with the bone mineral density residuals, and the correlation between OHP residuals and bone mineral density approached significance (p=0.06).

Table 2.
CORRELATIONS AMONG RESIDUALS*

	Hydroxy-proline	Cross-Section	Mineral Density
Hydroxylysylpyridinoline:	**0.529 (p=0.01)**	0.010 (p=0.34)	**-0.457 (p=0.02)**
Hydroxyproline		0.032 (=-0.45)	**-0.350 (p=0.06)**
Tibial Cross-sectional area:			-0.141 (p=0.27)

*Coefficients are Spearman ρ values.

DISCUSSION

Table 1 indicates the increased intensity training resulted in a loss of body mass and a decrease in percent fat. On average, experimental subjects lost 2 kg mass, of which 1.5 kg was fat loss and 0.5 kg was fat-free mass loss. There was, however, no change in VO_2max. This may be explained by the fact that increasing the exercise intensity by increasing the speed with no change in distance requires that the duration of exercise decrease. Hence, the volume of training; i.e., the product of intensity, duration, and frequency, was unchanged.

Bone mineral density was the only bone morphology parameter to change following 9 weeks of increased intensity training. It was decreased by 7.5% in the experimental group, and unchanged in the control group. Bone cross-section was not different on the average at 9 weeks in either group.

The lack of change in bone cross section may indicate that the mid-shaft is not the ideal point of measurement in the tibia. Changes may have been manifest, but not measured, at the proximal and/or distal ends of the bone. Additionally, bone may remodel such that shaft thickness may vary fore and aft and side to side without net

change in cross-section. Vailas (personnel communication) has evidence to suggest that such remodelling takes place in chickens subjected to a running program.

Following 9 weeks of exercise, bone mineral density was decreased in the increased intensity training group and unchanged in the control group. Numerous studies have shown that bone mineral content and bone density are increased when the volume of running training is increased. The decrease in bone mineral density at 9 weeks in the experimental group suggests that bone remodelling in response to the intensity increase is not yet complete. Certainly the finding of elevated OHP and HP is consistent with this interpretation. These findings are consistent with the results of Smith and coworkers (16) who found that bone mineral density decreased for one year in response to a training program. However, they are not well reconciled with the findings of Margulies and colleagues (10), who found increases in bone mineral content following 14 weeks of military training. These authors did not report bone cross-section values, so it is not certain that the increases in bone mineral content also indicate increases in bone mineral density.

Plasma levels of the markers of collagenolysis were only moderately correlated with the final states of bone mineral density change. This finding suggests that collagen sources other than bone may have contributed substantially to the plasma marker levels. HP is more highly concentrated in cartilage than in bone, and changes in HP would then reflect degradation of cartilage more than bone. HP may also be released from the connective tissue associated with other tissues, such as skeletal muscle (9,12). In this study, blood was sampled at only two times, and in addition, we did not measure collagen synthesis. Therefore, the net balance between synthesis and degradation is unknown. If the observed level of degradative processes (indicated by the elevated degradation markers), is taking place at a time of greatly increased collagen synthesis, the associations between plasma concentrations of HP and OHP and bone mineralization would be decreased.

The degree of bone mineralization is also dependent on the weight of the individual. Drinkwater and Chesnut (7) in their study of women during pregnancy found that as body weight increased, reflecting growth of the fetus, bone mineral density also increased. Following delivery, both maternal body weight and bone

density decreased. In that the subjects in our experimental group lost body mass over the course of the 9-week increased intensity training period, this may have provided a stimulus for bone mineral loss, and may be a contributing factor in the decreased bone mineral content seen after 9 weeks.

Regression residuals for HP and OHP were found to be more strongly associated with the residuals for bone mineralization. There are several factors which limit the use of OHP as a marker of bone resorption. (1) OHP can be released from non-bone collagen sources. These sources are numerous and include mature collagen of cartilage, tendon, ligament, muscle fascia, intra and extracellular procollagen, and soluble collagen which is degraded and not incorporated into collagen fibrils; (2) in addition, free OHP can be converted by the liver to pyroline-3-hydroxy-carboxylic acid and thereby removed from the circulation; (3) and finally, dietary sources can contribute to the total measurable pool of OHP. Given these factors and the fact that these limitations do not apply to HP, the findings of this study are not unexpected.

CONCLUSIONS

We conclude that bone mineral content adjustments to increased intensity exercise are not complete at 9 weeks. Increased intensity training does not appear to alter tibial cross section, at least near mid-shaft. Based upon its relationship to bone mineral density changes, HP appears to be a better indicator of connective tissue degradation than OHP.

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