Naval Submarine Medical Research Laboratory

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Human Bone Matrix Changes During Deep Saturation Dives

by

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In 1999, 8 U.S Navy divers underwent a dry hyperbaric chamber dive to 305 meters of seawater (msw). Measurements of serum osteocalcin and urine crosslinked N-telopeptide were made at baseline, 305 msw, and immediately after surfacing. The bone marker levels for the divers and volunteers were analyzed by repeated-measures ANOVA, with planned comparisons made between markers and dive phase.The data for the 1999 dive revealed a significant decrease in urine Ntx, with no change in serum osteocalcin. Data from the 1999 dive were combined in a Meta-analysis with Ntx and osteocalcin measurements taken during two similar 305 msw dive profiles conducted in 1997 and 1998. The Meta-analysis revealed a reduction in serum osteocalcin following saturation at 305 msw but no significant change in Ntx at depth. Taken together these studies may have identified a response of bone metabolism to changes in ambient pressure, however questions still remain as to whether blood sample handling/decompression procedures during the 1998 dive affected the Meta-analysis results for osteocalcin.

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ABSTRACT

With the recent introduction of biochemical markers of bone resorption and formation it is now possible to explore potential bone metabolism changes that may occur with hyperbaric exposures. In 1999, eight U.S Navy divers underwent a dry hyperbaric chamber dive to 305 meters of seawater (msw). The bottom time for the dive was 79 hours with a 62 hour stay at 305 msw. Measurements of serum osteocalcin (a bone formation and turnover marker) and urine crosslinked N-telopeptide (Ntx, a bone resorption marker) were made at baseline, 305 msw, and immediately after surfacing. Additional Ntx and osteocalcin samples were taken from four control subjects at surface. Half of these control samples were put on ice and compressed to 305 msw to validate the medical lock decompression procedures for the samples drawn at depth. The bone marker levels for the divers and volunteers were analyzed by repeated-measures ANOVA, with planned comparisons made between markers and dive phase (compression or decompression). The validation study showed no effect of compression and decompression on Ntx or osteocalcin concentrations for the samples drawn at surface, compared to samples that were never pressurized. The data for the 1999 dive revealed a significant decrease in urine Ntx, with no change in serum osteocalcin. Data from the 1999 dive were combined in a Meta-analysis with Ntx and osteocalcin measurements taken during two similar 305 msw dive profiles conducted in 1997 and 1998. The Meta-analysis revealed a reduction in serum osteocalcin following saturation at 305 msw but no significant change in Ntx at depth. Taken together these studies may have identified a response of bone metabolism to changes in ambient pressure, however questions still remain as to whether blood sample handling/decompression procedures during the 1998 dive affected the Meta-analysis results for osteocalcin.

ACKNOWLEDGMENTS

As is often the case with research in the military, this study went through the hands of several principal investigators who were transferred before the study could be completed. The initial experimental work was done by Dr. John Sims, LT, MC, USN of the Naval Submarine Medical Research Lab (NSMRL), Groton, CT, with the help of Dr. Ronny Jackson, LT, MC, USN at the Naval Diving and Salvage Training Center, Panama City (NDSTC), FL. At the time I (LCDR Robert Perkins) arrived at NSMRL, Dr. Mike Waltz, LT, MC, USN had taken over the project along with David Fothergill, Ph.D. who was continuing his involvement as a co-investigator. Drs. Waltz and Sims have since left the Navy to pursue residency training. Dr. Fothergill and I took on the task of finishing data collection, analysis, and production of the final report. Our many thanks go to the divers and support personnel at NSMRL, NDSTC, and the Navy Experimental Dive Unit, without whom this work could not have been accomplished.

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INTRODUCTION

Bubble formation has been implicated in a number of diving related diseases, most notably decompression sickness (DCS) and arterial gas embolism. In bone, bubbles can also cause a mechanical blockage of blood flow that result in tissue damage and necrosis. Blood flow is centrifugal in bone; that is, nutrient arteries feed blood through a high-pressure system in the central marrow (medulla), which then drains to a low-pressure system of venous sinuses through the cortex to the periosteum.[1] It is thought that venous bubbles blocking outflow cause stasis and pressure in these sinusoids. This produces the characteristic bone and joint pain associated with DCS, from stretching of the sinusoids and the highly innervated periosteum.[2] Bubbles also cause inflammation and scarring as a result of the body's immune response to what is essentially a foreign object.[3] Either of these mechanisms can result in bone and joint damage, as well as serious deficits of both the central and peripheral nervous systems (weakness, sensory changes, paralysis, and sometimes death).

Chronic Effects: Dysbaric Osteonecrosi

Exposure to increased atmospheric pressure can sometimes be associated with necrosis of the hip and shoulder joints, as well as shaft lesions of the femur, tibia, and humerus. This condition, known as dysbaric osteonecrosis (DON), has been reported in compressed-air workers, saturation divers, non-saturation divers, and personnel rescued from disabled submarines.[4,5,6,7] The prevalence of DON has been reported to range from 1.7%-5% in military divers to 59.5% in Japanese fishing divers.[8,9] It is considered an important occupational disease and international consensus states that it is a long-term risk of hyperbaric exposure.[10] This agreement notwithstanding, much remains unknown about its pathogenesis, prevention, and treatment. DON is currently disqualifying for U.S. Navy divers, commercial divers, and tunnel workers based on a radiographic staging classification. Most radiographic lesions are asymptomatic, but whether such lesions progress and by what mechanism is unknown. There are a few studies that suggest that they do progress despite the absence of further hyperbaric exposures, but the longest follow-up to date is only 10 years.[11] If lesions progress and cause femoral fractures or osteoarthritis, the typical recourse for symptomatic treatment is surgical joint replacement.[7]

The principal mechanism of bone injury is generally accepted to be bubble formation during decompression that results in venous stasis and inadequate perfusion and oxygenation in the bone.[2] However, it is notable that switching to more conservative decompression tables has not entirely prevented the disease.[12] Also notable is that only 10% of DCS cases go on to develop necrosis and 25% of patients with diagnosed DON do not have a known history of decompression sickness.[13] Currently, there is no complete explanation for the particular susceptibility of the hip and shoulder joints, as well as for the almost universal sparing of the knees.[14]

The studies to date suggest that decompression related bubbles are sufficient, but not necessary, for the development of DON. The pattern of disease implicates another mechanism (or several), unrelated to gas bubbles, that either predisposes to bubble-induced necrosis or is in itself sufficient to cause the disease. A number of theories have been investigated to elucidate this

separate factor. They include fat embolism, decompression triggered intravascular coagulation, and oxygen toxicity secondary to raised partial pressures of oxygen.[15,16,17]

Our study focused on theories that pressure differentials between the inside and outside of the bone could give rise to a number of possible scenarios that would contribute to disease. Of particular interest was that several of these mechanisms predicted pathologic consequences of rapid *compression*, where attention had heretofore been focused primarily on decompression-related effects. In a recent reassessment of DON theories, Hutter cites multiple studies between 1970 and 1993 in which rapid compression was identified as a risk factor for DON.[18] However, there are relatively few published studies that followed-up on these findings. One of the first theories implicating compression effects was that of bone ischemia secondary to intramedullary pressure differentials.[19,20] Gas-induced osmosis was also postulated, in which differential absorption of gases in the marrow caused osmotic flow of fluid into the intramedullary spaces during compression.[21] The result was the intramedullary stasis and ischemia that most feel is the final common pathway of DON. Most recently, Hutter has postulated that the "non-bubble" factor may be delayed transmission of pressure to the intramedullary space during compression (resulting in decreased intramedullary pressure, stasis and congestion), coupled with the spherical geometry of the hip and shoulder joints (large ratio of medullary volume to available surface area for venous drainage, as well as an avascular articular cartilage).[18]

A difficulty with all of these theories is the overall resiliency of bony tissue itself. An essential step in the process of aseptic necrosis is the death of the cells that mediate bone formation (osteoblasts) and resorption (osteoclasts). Most of the mechanisms described above occur over relatively short time frames. However it has been well documented that bone can survive 6-12 hours of impaired blood flow before sustaining irreversible damage, but both bone formation as measured by osteocalcin and bone resorption as measured by N-telopeptide (Ntx) are adversely altered under hypoxic condition.[22,23,24,25] For these mechanisms to account for aseptic necrosis (in the absence of or in conjunction with decompression related bubbles), they should either produce perfusion limiting effects that last beyond the immediate time frame of compression, have additive effects that do not resolve by the next exposure, or both.

Searching for Evidence of Bone Injury

Another difficulty with investigating DON is its long latency. Several months to years may pass after the initial insult before radiographic evidence of disease appears.[2] The time to diagnosis is even longer if there is no index of suspicion to prompt radiographic studies and joint pain becomes the first indication of a problem. Any clinical or radiographic diagnosis in an occupational setting could potentially be tied to hundreds of dives so establishing a cause is problematic at best. Clearly, some marker of bone injury would be of use to capture the effects of the mechanisms described above in the time frame they are felt to occur. To this end, we investigated a number of serum and urine markers of bone metabolism with the intent of using one or two to assess bone injury, as well as overall bone activity, during and after a saturation dive.

Bone is constantly undergoing remodeling in response to external physical forces and internal hormonal controls. Bone resorption (breakdown) and deposition is a constant process in which

osteoclasts dissolve old bone and osteoblasts lay down the matrix for new bone formation. Under normal conditions, this process is tightly controlled, or coupled. Diseases of bone can arise when one process becomes uncoupled and is no longer balanced by the other.[26] During the resorption process, osteoclasts digest and solubilize the collagen bone matrix. The biochemical products resulting from the breakdown of collagen are released into the blood and eventually excreted in the urine. These markers of bone resorption include urinary calcium, hydroxyproline, hydroxylysine glycosides, acid phosphatase, and N-telopeptide (Ntx).

Ntx is a relatively sensitive and specific bone marker. It is detected in the urine by an immunoassay that recognizes the non-helical amino-terminal fragment of collagen with the attached pyridinoline cross-links.[27] Increased urine concentrations of Ntx have been demonstrated in bone diseases such as osteoporosis, primary hyperthyroidism, and Paget's disease. Also, concentrations of Ntx correlate with the extent of these diseases and have been shown to be a useful measure of the effectiveness of treatment.[28] We selected it as our bone resorption marker for its diagnostic properties, as well as relative affordability and ease of collection.

Bone formation markers considered were alkaline phosphatase, procollagen I extension peptides, and osteocalcin. We chose osteocalcin as an established assay that is a good marker of overall bone turnover when formation and resorption are coupled, and a measure of formation when they are uncoupled.[26] Osteocalcin has a short half-life and undergoes rapid renal clearance.[29] We theorized that if formation and resorption were coupled, and if the osteoblast suffered an acute, but non-lethal, insult during the dive, the short half-life might produce a measurable decline in osteocalcin levels. Both markers could then be assessed later to see if there was an effect on overall bone metabolism.

Saturation Diving and Bone Injury: Prior Studies

As described above, saturation diving involves compression to a working or “storage” depth and remaining there for the entire duration of a project. The advantage here is that, after 12-24 hours, the required decompression time becomes a constant since the body has absorbed all the inert gas it can at that depth. It is also useful for investigating the questions discussed above because the periods of compression and decompression are longer than in non-saturation diving and, hopefully, make it easier to capture effects of interest.

In 1997 and 1998, the Naval Submarine Medical Research Laboratory (NSMRL) in Groton, CT conducted studies on 17 men who undertook saturation dives to 305 meters of seawater (msw), using the Ntx assay to assess the effect of saturation diving on this marker. The osteocalcin assay was added in 1998. In a poster presented at the 1999 Undersea and Hyperbaric Medical Society Annual Scientific Meeting, the investigators reported a statistically significant 26% increase in urine Ntx concentration during the initial compression phase, as well as similar statistically significant increases during decompression. The results were suggestive of changes in bone metabolism during the compression and decompression phases of a saturation dive, although these results must be interpreted with caution due to the small number of subjects involved. The presenters suggested that changes in pressure, rather than bubble formation, might be the underlying etiology for bone loss in divers, and that the differentials likely came from the gas-induced osmosis model.[30]

The same facility was used for both dives and, with the addition of the assay for osteocalcin, it was anticipated that the two markers together might provide a better picture of overall bone metabolism. Other research, however, seems to indicate that resorption and formation markers cannot be combined reliably in this fashion.[29] The results of the 1997 and 1998 dives will be included in the discussion to follow. Since a similar 1000 fsw saturation dive was again planned for 1999, we proposed to undertake a further study to measure bone markers. Our aim was to build on the past two studies by repeating the observations under nearly identical circumstances to include Ntx and osteocalcin assays, as well as pain indices that had been suggestive of compression injury in the earlier dives. Although this research was not specifically designed to refute the bubble theory of DON, preliminary evidence from the previous 1000 fsw dives implicated a compression related process for bone matrix changes. Additionally, since conditions during the three saturation dives were so similar, a later meta-analysis of the three studies could help offset the small subject numbers used in the separate studies. The small numbers were imposed by space limitations at the facility where the dives were carried out.

Our original hypothesis stated that bone matrix would undergo a net resorption during a saturation dive; furthermore this change would be demonstrated to occur in both the compression and decompression phases. Due to the difficulty in assessing bone matrix changes with combined resorption and formation markers, the hypothesis was revised to simply look for an overall change in the bone markers during the dive. We would then perform planned comparisons based on the *a priori* expectation that compression and decompression are sufficiently distinct in their effects to justify evaluation as separate independent variables. Finally, any unexpectedly strong effects would be assessed with the appropriate post hoc analysis.

METHODS

The Committees for the Protection of Human Subjects at both the Naval Submarine Medical Research Laboratory and the U.S. Navy Bureau of Medicine and Surgery approved this study. Additionally, the Institutional Review Board of the School of Public Health at the University of North Carolina-Chapel Hill reviewed and approved the protocol retrospectively. Our study was carried out as an annex to the Navy Experimental Diving Unit's (NEDU) 1000fsw saturation dive (Deep Dive 99) in Panama City, FL on 16-30 November 1999. As in the past two Deep Dives, we recruited eight subjects from among male, military trained saturation divers at NEDU. Informed consent was obtained from each volunteer prior to participating in the study.

We were aware that exercise in this physically active population could potentially alter the metabolic markers of interest. Accordingly, the subjects were asked to monitor their physical activity over the week prior to the dive and record its frequency, duration and intensity. Those who reported exercising on an average of less than or equal to 3-4 times per week, for 16-30 minutes per session, were assigned to a "moderate" exercise category. Subjects reporting more than 3-4 sessions per week were assigned to the "hard" category. Other demographic data such as age, height, weight, and diving experience were also collected for later correlational analyses.

The dive took place in NEDU's Ocean Simulation Facility (OSF), a diagram of which appears in Figure 1. The facility consists of five dry hyperbaric chambers on the upper level and a 250 cubic

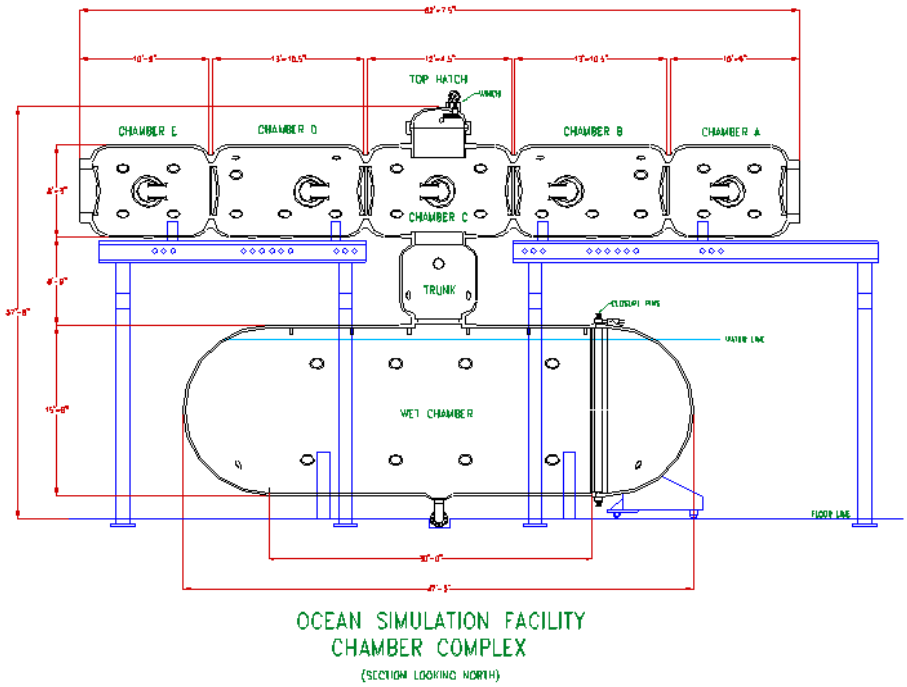

Figure 1: NEDU Ocean Simulation Facility

meter (55,000 gallon) “wet pot” on the lower level. The lower chamber can be filled with water and, along with the dry chambers above, the ambient pressure can be increased to a maximum equivalent depth of 685 meters of seawater (msw, 2,200 feet of seawater).

For this study, the divers were berthed in the dry chambers on the upper level. The dive profile is shown in Figure 2. The first phase was a simulated descent from sea level to 305 msw over 17 hours, an average descent rate of .298 meters/min. The second phase was a 62-hour stay at 305 msw. The final phase was a simulated ascent from 305 msw to the surface over approximately 11 days, representing an average ascent rate of 1.19 meters/hour.

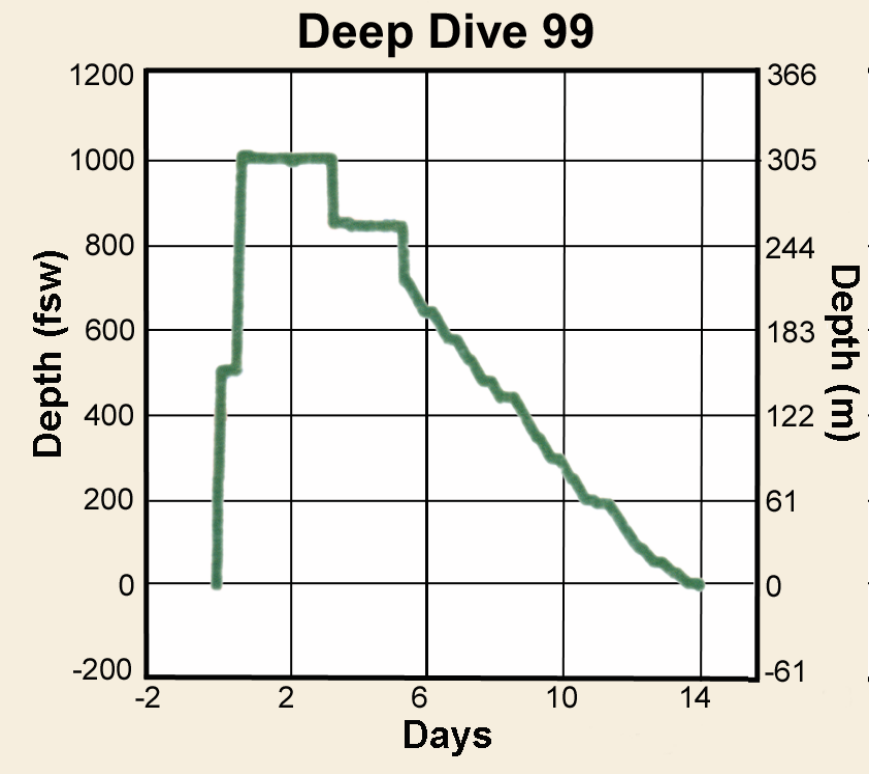

Figure 2: Deep Dive 99 Depth-Time Profile

Each participant provided second morning void urine samples for the Ntx assay on three different days. The baseline sample was collected on the first day of diving, just prior to compression. The second sample was collected at a depth of 305 msw, just prior to the beginning of the ascent to sea level. We had hoped to take samples immediately following the compression phase, however logistical and financial considerations required that we reduce the number of samples taken. The third sample was collected immediately after completion of decompression to sea level. Each urine sample (2 ml) was transferred to a screw-top vial for storage and shipping. Venous blood samples (3 ml) for the osteocalcin assay were obtained by venipuncture on the same days as the urine samples. All specimens were put on ice and immediately transferred to the surface via a medical lock at a decompression rate that prevented boiling.

The medical locks are small decompression chambers used to transfer items in and out of the main chambers. Some concern had been raised during the 1997 and 1998 Deep Dives that the process of decompressing the samples might affect the results of the assay. To evaluate this concern, samples were also taken from volunteers in the control room day watch. Part of each sample was iced immediately and the other part was pressurized to 305 msw. These samples were then brought back to surface pressure using the same procedures as for test subject samples (i.e. decompression rate <3 msw/min). Each blood sample was immediately centrifuged and the serum supernatant was transferred to a screw top vial for freezing. The serum and urine specimens were stored in a biological freezer at -28 degrees Celsius until all samples were collected. At the end of the study they were all shipped frozen via courier for analysis by Quest Diagnostics. Urine was analyzed with an immunoassay for type-I collagen cross-linked N-telopeptide (NTx) by SmithKline Beecham laboratory, Rochester, Minnesota. NTx results were reported as the nanomolar concentration of bone collagen equivalents per millimolar concentration of creatinine (nM BCE/mM Cr) to adjust for urine volume and dilution.

In order to establish correlations, if any, between bone metabolism and pain symptoms, each diver was asked to document pain symptoms throughout the dive. Bone and joint pain was categorized by anatomical location and any occurrence at any time during or 1 week after the dive was recorded for both groups.

Statistical Analysis

The overall effect of the dive on mean osteocalcin and Ntx levels was compared using analysis of variance (ANOVA) for a repeated measures design. This analysis controls for individual variation and for biological measurement variation. The subjects serve as their own controls, so that variability owing to individual differences is eliminated from the error (residual) term, increasing the chances of observing significant differences between sampling intervals. If the ANOVA resulted in a significant F-statistic, planned comparisons were performed on compression and decompression effects. Pairwise comparisons for any unexpected (post hoc) strong effects were done using Tukey's WSD (Wholly Significant Difference: a moderately conservative post hoc analysis) with confidence levels. Pearson and Spearman correlational analyses were performed on associations among diver demographics and the dependent measures.

Since the experimental dive profiles for Deep Dives 1997, 1998, and 1999 were similar, a meta-analysis was performed on two aggregate data sets, one for Ntx and another for osteocalcin. Ntx measurements were made during all three studies, so data from 1997-99 were included in the Ntx analysis. There was more frequent sampling performed in 1997 and 1998, so any data that did not correspond with the three sampling periods of the 1999 study were dropped. Osteocalcin was not measured in 1997, so the data pool for this marker included only the 1998-99 data, and the non-corresponding data from 1998 were also dropped. Eight diver subjects participated in the 1997 Deep Dive and nine participated in the 1998 Deep Dive. None of the diver subjects participated in more than one Deep Dive experiment.

RESULTS

Validation of Decompression Procedure

Descriptive results are shown in Table 1 for the four control subjects we used to validate our method of decompressing samples through the medical locks. There were no smokers or former smokers in this group. No subject exercised less frequently than 3-4 times per week, so we did not include a category below "moderate".

Table 1: Descriptive statistics for method validation subjects (n=4)

Characteristic	Mean (s.d.) or percent	Range
Age (years)	38 (2.71)	36-42
Years Diving	17 (4.90)	11-21
Activity -% Moderate -% Hard	 75 25	
% Non-smoker	100	

The marker results are summarized in Table 2. The baseline samples were drawn on day 1 of the study. Another sample was taken 79 hours later (at the same time the test subjects were giving their 305 msw sample). This sample was divided in three parts. The first was frozen immediately. The second placed in the medical lock, pressurized to 305 msw, and returned to surface pressure. The third part was put on ice while the second sample was being pressurized and returned, after which they were both frozen. Table 2 also shows the results of the repeated-measures ANOVA on both groups of measurements. Since the F-statistic between treatment groups was not significant, no further analysis was done on these samples. The results are presented graphically in Figure 3.

Table 2: Bone marker results from the 1999 validation study

Marker	Mean (s.d.)	Range
Ntx† (nmol BCE/mmol Cr)		
-Baseline	30.3 (3.86)	28-36
-Immediately Frozen	35.3 (8.26)	25-45
-Compressed to 305	35.8 (8.30)	25-45
-Iced during compression	34.0 (8.41)	23-43
Osteocalcin‡ (ng/ml)		
-Baseline	15.2 (2.19)	12.8-18.1
-Immediately Frozen	17.0 (2.47)	14.1-20.1
-Compressed to 305	17.8 (1.40)	16.1-19.5
-Iced during compression	19.8 (4.25)	13.6-22.9

† Between groups F=1.36, df 3, 9 (p=0.3169)
‡ Between groups F=2.12, df 3, 9 (p= 0.1672)

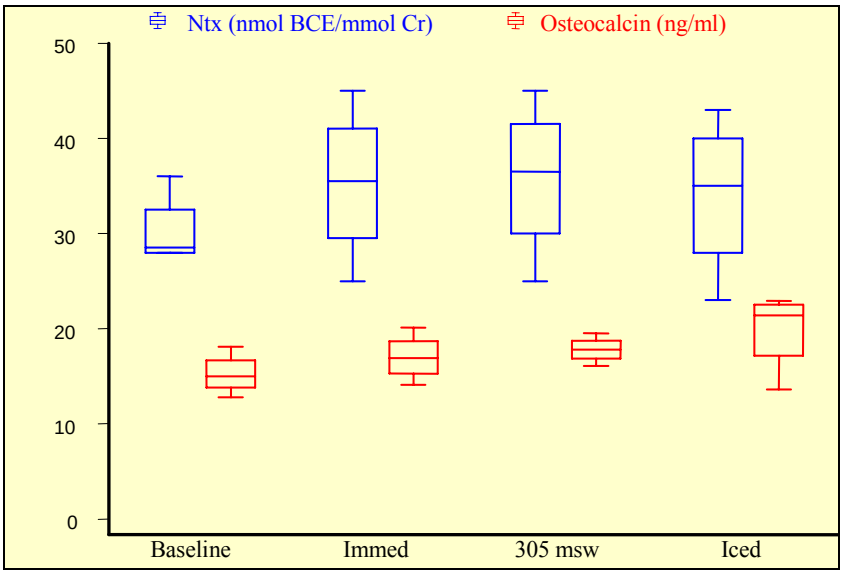

Figure 3: Ntx and Osteocalcin results from decompression method validation (see text). Horizontal line = median value, box = 25% to 75% confidence interval, whisker = 5% to 95% confidence interval.

Deep Dive 99 Result

Table 3 includes the descriptive information on the subject group for Deep Dive 99. Once again, there were no subjects reporting less than 3-4 times per week for exercise, so no category less than "moderate" was employed. Although no subject reported being a current smoker, two reported quitting in the past 6 months with an average 3 pack-year history prior to quitting.

Table 3: Subjects' descriptive statistics for Deep Dive 99 (n=8)

Characteristic	**Mean (s.d.) or percent**	**Range**
Age (years)	34 (5.06)	26-41
Years Diving	12.1 (5.49)	2-17
Activity -% Moderate -% Hard	 62.5 37.5	
% Non-smoker	100	

The marker results for the Deep Dive 99 subjects are presented in Table 4. Baseline levels were drawn just prior to the beginning of pressurization. The 305 msw sample was taken 79 hours later, just prior to the beginning of ascent. The post dive surface sample was taken upon completion of decompression to 1 atmosphere. The F-statistic for the repeated-measures ANOVA was not significant for osteocalcin, so no further testing was done on this marker. There was a significant

overall effect on Ntx. The results are presented in Figure 4. Repeat analysis without the outliers shown did not significantly change the results.

Table 4: Bone marker results from Deep Dive 99

Marker	Mean (s.d.)	Range
Ntx†(nmol BCE/mmol Cr)		
-Baseline	43.1 (15.4)	24-75
-At 305 msw	33.1 (8.69)	21-46
-Surface	46.9 (19.5)	26-89
Osteocalcin‡(ng/ml)		
-Baseline	18.3 (4.38)	12.2-24.7
-At 305 msw	19.2 (3.27)	14.9-25.1
-Surface	15.3 (3.03)	11.8-18.9

† Between depths F= 5.41, df 2, 14 (p=0.0182)
‡ Between depths F=3.41, df 2, 14 (p= 0.0622)

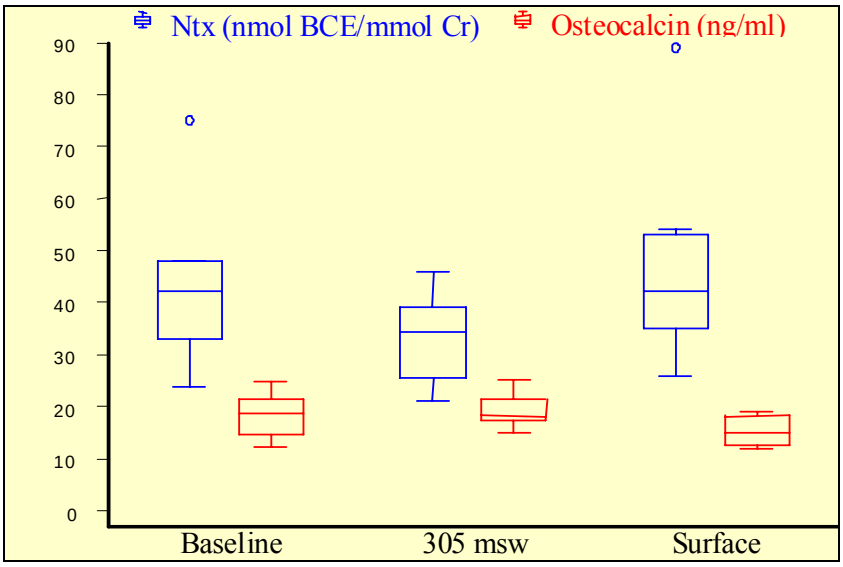

Figure 4: Box and whisker plot of Osteocalcin and Ntx changes during Deep Dive 99. Horizontal line = median, box = 25% to 75% confidence interval, whisker = 5% to 95% confidence interval. Several outliers for Ntx are also shown.

Since there was an expectation based on *a priori* theory that dive phase (compression vs. decompression) should exert independent effects on bone markers, a paired t-test was performed on Ntx levels before and after compression and decompression. "Compression" compared the mean baseline measurement and the mean 305 msw measurement. "Decompression" compared the 305 msw measurement and the post dive surface measurement. The results are summarized in Table 5.

Table 5: Paired t-test results for 1999 Deep Dive Ntx data.

Marker	Mean difference (s.d.) (nmol BCE/mmol Cr)	95% CI	p	Mean % change
Ntx				
-Compression	-10 (9.90)	-18.3, -1.72	0.0244	-20
-Decompression	13.8 (15.1)	1.11, 26.3	0.0369	+50

Meta-analysis: 1997-99

Figure 5 shows a composite graph of each of the depth-time profiles for the 1997-99 dives. The average compression rate (measured from initiation of compression to arrival at 305 msw) for the 1999 dive was .298 m/min. For the 97-98 dives it was .052 m/min and .042 m/min respectively. Decompression for 1999 (averaged from beginning of ascent to arrival at surface) was done at 1.19 m/hour. Decompression rates for 1997-98 were .866 m/hr and .975 m/hr respectively. The mean age of the diver subject for the aggregate data was 34.3 years (s.d. 4.94) with a range of 26-48 years. The results of the Meta analysis for Ntx and osteocalcin bone markers are given in Table 6.

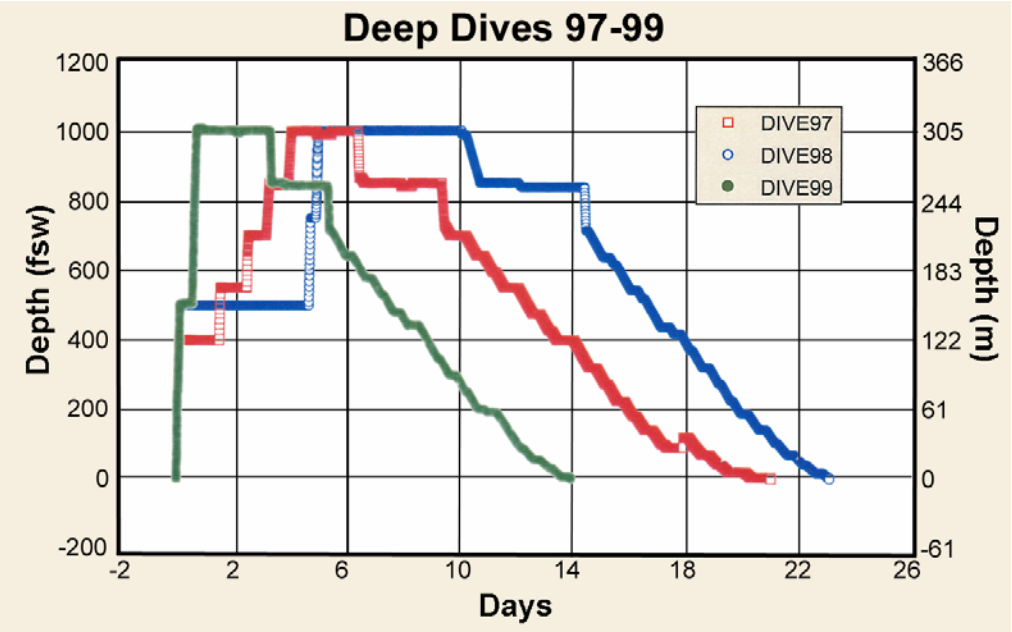

Figure 5: Depth-Time profile for Deep Dives 1997-99.

Table 6: Bone marker results from Deep Dives 1997-99

Marker	Mean (s.d.)	Range
Ntx† (nmol BCE/mmol Cr)		
-Baseline	40.5 (18.9)	20-107
-At 305 msw	41.8 (19.9)	21-102
-Surface	44.2 (23.4)	15-122
Osteocalcin‡ (ng/ml)		
-Baseline	19.6 (5.79)	10.5-32.5
-At 305 msw	12.1 (7.61)	2.0-25.1
-Surface	14.9 (5.52)	5.0-31

† Combined data 97-99 (n=25), Between depths F= 0.63, df 2,48 (p=0.5381)

‡ Combined data 98-99 (n=17), Between depths F=6.62, df 2,32 (p= 0.0039)

Although sampling was done more frequently for both the 1997 and 1998 dives, only the data that corresponded to the collection points in the 1999 study were included. No osteocalcin data was collected for 1997, so that pool reflects only samples taken during 1998 and 99. Since the F-statistic for the Ntx data was not significant, no further analysis was done on this data. The results of the Ntx data are presented graphically in Figure 6.

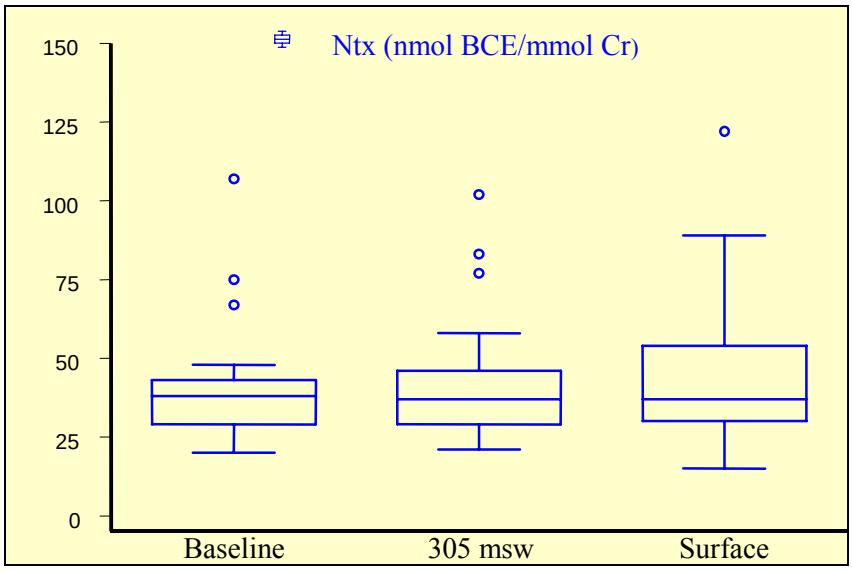

Figure 6: Ntx results from Deep Dives 1997-99 (n=25). Horizontal line = median, box = 25% to 75% confidence interval, whisker = 5% to 95% confidence interval. Several outliers are also shown.

The F-statistic for the osteocalcin data was significant, indicating an overall effect of depth group on osteocalcin levels. Figure 7 shows the graph of these measurements. A paired t-test was performed on osteocalcin levels before and after compression (baseline to 305 m) and decompression (305 m to Surface). The results are summarized in Table 7. As no other unexpected significant effects were identified in the ANOVA, no further post hoc testing was done.

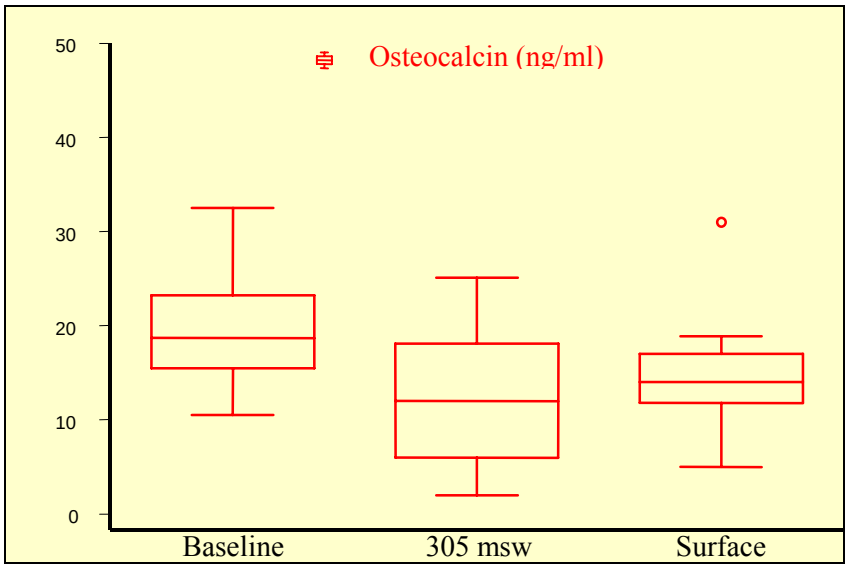

Figure 7: Osteocalcin results from Deep Dives 1998-99 (n=17). Horizontal line = median, box = 25% to 75% confidence interval, whisker = 5% to 95% confidence interval. One outlier is also shown.

Table 7: Paired t-test results for 98-99 osteocalcin data.

Marker	Mean difference (s.d.) (ng/ml)	95% CI	p	Mean % change
Osteocalcin				
-Compression	-7.49 (10.3)	-2.21, -12.8	0.0083	-32
-Decompression	2.79 (8.83)	-1.75, 7.33	0.2105	+15

Pearson's correlation coefficients were computed between diver demographics (age, dive experience and physical activity level) and the dependent measures for both the 1999 data, as well as the aggregate data. There was a correlation between age and experience ($r=0.9316$, $p=0.0008$), but no significant correlations were demonstrated between the demographic measures and the Ntx and Osteocalcin results.

DISCUSSION

Validation of Decompression Procedure

The different methods for handling the samples did not result in any significant variation of measured levels for either marker. By comparing the pressurized samples with samples that underwent both immediate and delayed freezing, the effects of compression/decompression on the sample results should have been isolated. None of the handling methods differed significantly from baseline or from each other. We therefore concluded that the method of decompressing samples through the medical locks did not significantly affect the results in 1999.

Deep Dive 99

The results for the 1997-98 dives reported an increase of Ntx levels during the early compression phase.[30] Data from the 1999 dive showed a significant decrease in Ntx at the end of the stay at 305 msw followed by a return to baseline immediately after decompression. A reason for this discrepancy might be the difference in descent rates between the dives. Figure 5 shows that the descent rate for Deep Dive 1999 was significantly faster than for either of the previous two dives. Since resorption is related to overall bone turnover (and presumably to the overall metabolic activity of the osteoclast), the faster compression rate may have resulted in osteoclast injury (or decreased osteoclast metabolism by some other mechanism) from which the cells later recover. Since the Ntx samples taken at depth during the 1999 Dive were taken after the divers had saturated at the final 305 msw storage depth, it is possible that these samples simply missed the earlier effect seen in previous dives. The issue of whether an increase or decrease in Ntx is observed following hyperbaric stress may then be a question of where in the cycle of bone injury and recovery a measurement was taken.

Alternatively, the significant decrease in Ntx at depth may have been the result of a Type I error resulting from the impact of outliers in this small sample size (see Fig 4). It is of note that the pre-dive and post-dive Ntx standard deviations (SD) given in Table 4 are much larger than the SD for the Ntx levels at depth. These large SDs are largely attributed to the two outliers shown in Fig 4. Although the magnitude of the decrease may be significant, the clinical significance of that decrease is uncertain.

The lack of effect seen in osteocalcin measurements during Deep Dive 1999 may have also been the result of differences in compression rate. In this case, the short time to arrival at 305 msw could result in taking the bottom measurement before any effects are seen in OC levels. Additionally, both Ntx and OC are subject to diurnal variation of as much as 30%, analytical variation of up to 15%, and some seasonal variation.[31] While the 1997-98 dives were conducted in the summer, the 1999 dive was done in late fall. None of the analyses accounted for time of day or season, although the repeated measures design should address some of this variation. Since the 1999 osteocalcin results approached significance, the trend seen toward a decrease in OC levels may be real, but underpowered.

Meta-analysis: 1997-99

The similarity of the dive profiles made aggregation and analysis of all three deep dives a reasonable approach to reducing the uncertainty introduced by small sample size in the individual dives. Any data from 1997-98 that did not match the baseline, 305 msw, and surface collection times of the 1999 study were dropped. The intent was to balance the relative contribution of each dive to any effect seen. With the addition of the 1997-98 data, the Ntx measurements no longer showed any significant change over the course of the dives. However, there was a significant effect on osteocalcin levels and the planned comparison revealed a decrease in OC immediately after compression that persisted throughout the dive.

We conclude that compression to storage depth and/or bottom time during a saturation dive appears to induce a decrease in serum osteocalcin levels, which persists after decompression to the surface. While no such effect was noted in Ntx levels for the combined 1997-1999 data, it is possible that this is an effect of sample scheduling and earlier or more frequent sampling on this dive might have produced different results. Since bone formation and resorption are most likely coupled in these generally healthy subjects, OC can be interpreted as a marker of overall bone turnover or metabolism during these dives. Over long time frames, changes in OC could be attributed to a number of physiologic regulatory mechanisms, as well as to seasonal variations. However, since the effect we observed occurred over just a few days it is possible that the decrease in OC levels represents an acute change in osteoblast activity or metabolism. Another factor to consider is the discovery of hemolysis in many of the 1998 OC samples taken at depth. Enzymes released from the red blood cells are known to degrade OC, and could thus have contributed to lowering the recorded values. We were unable to determine whether any of the 1999 samples had hemolyzed, however the validation study results (no significant change in OC levels after cycling to 305 msw and back) speak against it.

The magnitude of the negative OC change is much greater in the 1998 data alone than in the combined 1998-99 data, and likely accounts for a disproportionate fraction of the observed effect. Although studied under very similar conditions, it is obvious from Figure 5 that these two dives had the biggest difference in dive profile. The 1998 dive had a slower rate of compression and the total duration under pressure was over a week longer. If compression rate or time at depth were significantly associated with osteocalcin production, the significance of the combined results would be more questionable. Again, it may simply be that hemolytic lowering of OC levels was a significant factor in the 1998 dives (and not in the 1999 dives) and accounts for most of the effect seen in the meta-analysis.

Finally, a possible explanation for the results of our study is that serum osteocalcin levels vary with acute, sub-lethal osteoblast injury, perhaps as a result of transient ischemia or some other insult. As previously mentioned, OC has a short half-life (5 minutes) and serum levels would thus be expected to decrease temporarily in the face of such an acute injury.[29] If this is the case, saturation diving may not be the best practical model to investigate these effects. Among Navy divers, it is not clear whether there is a relationship between saturation diving and DON that is independent of overt DCS.[32] While done regularly, saturation dives are also not representative of the majority of commercial dives. Most commercial dives are done at shallower depths, with more frequent ascents and descents (a known risk factor for DON). Accordingly, we have undertaken a

follow-up study to measure osteocalcin and Ntx levels in a series of single and repetitive dives to 40 fsw. The results of Perkins & Fothergill (2002) suggest that shallow, no-decompression diving also decreases serum OC levels.[33] However, some concern still remains that hemolysis in some of their samples taken at depth may have artificially lowered OC values in their study also. It will thus be useful to further investigate blood draw procedures at depth and handling/decompression of blood samples to better document the occurrence and impact of hemolysis on osteocalcin levels. Careful attention to the timing of blood draws within a profile may also help explain why effects differed in direction across these dives.

REFERENCES

[1] Brookes M. The blood supply of bone. In Hutter CDD. Dysbaric osteonecrosis: a reassessment and hypothesis. *Med Hypotheses.* 2000; 54(4): 585-590.
[2] Walder DN. Aseptic necrosis of bone. In Bove AA, ed. *Bove and Davis' Diving Medicine 3rd ed.* Philadelphia, PA: W.B. Saunders Company; 1997: 227-234.
[3] Moon RE and Sheffield PJ. Guidelines for treatment of decompression illness. *Aviat Space Environ Med*. 1997; 68: 234-43.
[4] Lam TH and Yau KP. Dysbaric osteonecrosis in a compressed air tunneling project in Hong Kong. *Occup Med.* 1992; 42(1): 23-9.
[5] McCallum RI. Aseptic bone necrosis in commercial divers. *Lancet.* 1981; 2(8243): 384-8.
[6] Harrison J. Aseptic bone necrosis in divers. *Br J Radiol.* 1972; 45(533): 393.
[7] Schrantz WF, Bosse M, Goral A, Nowicki SD. Dysbaric osteonecrosis of the femoral diametaphysis. *Mil Med.* 1993; 158(5): 352-5.
[8] Amako T, Kawashima M, Torisu T. Bone and joint lesions in decompression sickness. *Semin Arthritis Rheum.* 1974; 4(2): 151-90.
[9] Harrison J. Aseptic bone necrosis in divers. *Br J Radiol.* 1972; 45(533): 393.
[10] Hope A, Lund T, Elliot D, Halsey M, Wiig H. Long term health effects of diving. Bergen, Norway: Norwegian Underwater Technology Center, 1994.
[11] Van Blarcom ST, Czarnecki DJ, Fuerdi GA, Wenzel MS. Does dysbaric osteonecrosis progress in the absence of further hyperbaric exposure? *Am J Roentgen.* 1990; 155(1): 95-7.
[12] Kindwall EP. Compressed air tunneling and caisson work decompression procedures: development, problems, and solutions. *Undersea Hyperb Med.* 1997; 24(4): 337-345.
[13] Walder DN. Bone Necrosis. In Hutter CDD. Dysbaric osteonecrosis: a reassessment and hypothesis. *Med Hypotheses.* 2000; 54(4): 585-590.
[14] McCallum RI and Harrison JAB. Dysbaric osteonecrosis: aseptic necrosis of bone. In Bennett PB and Elliott DH, eds. *The Physiology and Medicine of Diving, 4th ed*. Philadelphia, PA: W.B. Saunders Company; 1993: 563-584.
[15] Jones JP, Ramirez S, Doty SB. The pathophysiological role of fat in dysbaric osteonecrosis. *Clin Orthop (United States)*. 1993; 296: 256-64.
[16] Slichter SJ, Stegall P, Smith K, Huang TW, Harker LA. Dysbaric osteonecrosis: A consequence of intravascular bubble formation, endothelial damage, and platelet thrombosis. *J Lab Clin Med.* 1981; 98(4): 568-90.
[17] Jones GR. Dysbaric osteonecrosis (caisson disease of bone): Are active oxygen species and the endocrine system responsible, and can control of the production of free radicals and their reaction products confer protection. *Free Radic Res Commun.* 1987; 4(3): 139-47.
[18] Hutter CDD. Dysbaric osteonecrosis: a reassessment and hypothesis. *Med Hypotheses.* 2000; 54(4): 585-590.
[19] Harrelson JM, Hills BA. Changes in bone marrow pressure in response to hyperbaric exposure. *Aerosp Med.* 1970; 41: 1018-21.
[20] Hungerford DS, Lennox DW. The importance of increased intraosseous pressure in the development of osteonecrosis of the femoral head: Implications for treatment. *Orthop Clin North Am.* 1985; 16(4): 635-54.
[21] Hills BA. Gas induced osmosis as an aetiological agent for inert gas narcosis, gouty arthritis, and aseptic bone necrosis induced by exposure to compressed air. In Bove AA, ed. *Bove and Davis' Diving Medicine. 3rd ed.* Philadelphia, PA: W.B. Saunders Company; 1997:227-234.
[22] Woodhouse CF. Dynamic influences of vascular occlusion affecting the development of avascular necrosis of the femoral head. In Bove AA, ed. *Bove and Davis' Diving Medicine, 3rd ed.* Philadelphia, PA: W.B. Saunders Company; 1997: 227-234.

[23] Park JH, Park BH, Kim HK, Park TS and Baek HS. Hypoxia decreases Runx2/Cbfa1 expression in human osteoblast-like cells, *Molecular and Cellular Endocrinology.* 2002; 192(1-2): 197-203.
[24] Irwin R, LaPres JJ, Kinser S, McCabe LR. Prolyl-hydroxylase inhibition and HIF activation in osteoblasts promotes an adipocytic phenotype. Journal of Cellular Biochemistry. 2007; 100(3): 762-772.
[25] Arnett TR, Gibbons DC[1], Utting JC, Orriss IR, Hoebertz A, Rosendaal M, Meghji S. Hypoxia is a major stimulator of osteoclast formation and bone resorption. Journal of Cellular Biochemistry. 2003; 196(1): 2-8.
[26] Christenson, RH. Biochemical markers of bone metabolism: an overview. *Clin Biochem.* 1997; 30(8): 573-593.
[27] Hanson DA, Weis MA, Bollen A, Maslan SL, Singer FR, Eyre DR. A specific immunoassay for monitoring human bone resorption: Quantitation of type I collagen cross-linked N-telopeptides in urine. J Bone Miner Res. 1992; 7: 1251-8.
[28] Uebelhart D, Schlemmer A, Johansen JS, Gineyts E, Christiansen C, Delmas PD. Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium cross-links. *J Endocrinol Metab.* 1991; 72: 367-73.
[29] Garnero P and Delmas PD. Biochemical markers of bone turnover: applications for osteoporosis. *Endocrinol Metab Clin North Am,* 1998; 27(2): 303-323.
[30] Sims JR, Fothergill DM, Jackson R. Compression arthralgias and bone resorption. Abstract: UHMS Annual Scientific Meeting June 26-29, 1999. Undersea Hyperb Med. 1999; 26(Supplement): 21-22.
[31] Woitge HW, Scheidt-Nave C, Kissling C, Leidig-Bruckner G, Meyer K, Grauer A, et al. Seasonal variation of biochemical indexes of bone turnover: results of a population-based study. J Clin Endocrinol Metab. 1998; 83(1): 68-75.
[32] Jones, JP Jr., and Neuman TS Dysbaric Osteonecrosis. In Bennet PB and Elliott DH, eds. *The Physiology and Medicine of Diving, 5th ed.* Philadelphia, PA, Saunders; 2004: 662.
[33] Perkins, RW and Fothergill, DM. Effect of repetitive, no-decompression dives on human bone matrix. Abstract: UHMS Annual Scientific Meeting June 2002. http://archive.rubicon-foundation.org/1079