The effects of hyperbaric exposure on bone cell activity in the rat: implications for the pathogenesis of dysbaric osteonecrosis

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THE EFFECTS OF HYPERBARIC EXPOSURE ON BONE CELL ACTIVITY IN THE RAT: IMPLICATIONS FOR THE PATHOGENESIS OF DYSBARIC OSTEONECROSIS

Stephanie Anne Kapfer

1995
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Signature of Author
5/4/95
Date
The Effects of Hyperbaric Exposure on Bone Cell Activity in the Rat: Implications for the Pathogenesis of Dysbaric Osteonecrosis

Abstract of
A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Stephanie Anne Kapfer
1995
THE EFFECTS OF HYPERBARIC EXPOSURE ON BONE CELL ACTIVITY IN THE RAT: IMPLICATIONS FOR THE PATHOGENESIS OF DYSBARIC OSTEONECROSIS. Stephanie A. Kapfer (Sponsored by Roland Baron, Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, CT).

Prior research on the pathogenesis of dysbaric osteonecrosis has focused on the mechanisms by which bone cell death might occur following a hyperbaric exposure. While it has been known that not all hyperbaric exposures result in bone cell death, no study has examined whether or not such exposures might cause any other effects. The present study was designed to observe changes in bone cell activity during and immediately following hyperbaric exposure. In this study, experimental animals (220 g male rats) were exposed to a hyperbaric environment consisting of a twelve-hour dive to 120 ft (~ 5 atmospheres). Histomorphometric analysis was completed on undecalcified femur sections, triple-labeled with fluorescent markers. The results demonstrate a significant increase in absolute and relative osteoclast numbers in the dived animals (2.4 ± 1.4 and 2.1 ± 1.0) as compared to the non-dived animals (0.8 ± 0.3). Over the time period observed, none of the other parameters, including osteoblast number, osteoid volume, and mineralization rates, showed significant differences between the dived and non-dived animals. The results suggest an activation of bone resorption both during and immediately following hyperbaric exposure which may or may not be followed by an activation of bone formation. This study has demonstrated, therefore, that hyperbaric exposure affects bone cell activity. The significance of such changes remains unclear. Further exploration of these observations is warranted and may lead to a better understanding of the pathogenesis of dysbaric osteonecrosis.
The Effects of Hyperbaric Exposure on Bone Cell Activity in the Rat: Implications for the Pathogenesis of Dysbaric Osteonecrosis

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Stephanie A. Kapfer
13 March 1995
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I. INTRODUCTION

Definition
Dysbaric osteonecrosis is a form of aseptic osteonecrosis which results from exposure to compressed air. The disease occurs in a young population of manual laborers working in compressed air environments or as professional divers. It is characterized by necrotic lesions, seen radiographically as areas of increased density in the long bones, particularly the femur and humerus. The lesions are generally asymptomatic unless they are located directly beneath an articular surface. These so-called juxta-articular lesions can progress to structural collapse and failure. The end result is a painful, stiff joint with limited movement and use in an otherwise healthy young individual.

History

Compressed air and caissons. Dysbaric osteonecrosis was originally called Caisson Disease of Bone. The term derives its name from a structure used in the building of bridge foundations. A caisson is a large cast-iron cylinder sealed at the top end with a series of interlocking compartments. When sunk on end to a river bed and filled with compressed air, the caisson provided an environment in which work could be done under dry conditions. The middle third of the cylinder was filled with masonry leaving only a narrow shaft through which a worker could descend from the air locks above to the working area below. The weight of the caisson caused it to sink as the ground beneath it was excavated. When bedrock was reached, the caisson could be filled with masonry. It then served as a platform for bridge construction.
The French engineer, Triger, first proposed the idea of using a caisson-like device in bridge construction in 1839. He was a pioneer of the use of compressed air in tunnel work, having successfully used the technology to keep water out of a coal mine shaft running beneath the Loire River. The first caisson was used in the construction of a bridge in 1851 by Hughes, an English engineer (Bert 1880).

**Recognition of decompression sickness.** The use of compressed air in industry actually originated with the invention of the diving bell in the 1500’s by Sturmius (Bert 1880). The first symptoms of decompression sickness were described in 1820 by Hamel and in 1826 by Colladen (Amako et al. 1974). Symptoms included musculoskeletal pains, neuralgias, respiratory distress, and sudden death. In 1845, Pol and Watelle recognized that symptoms occurred exclusively during decompression and published the first scientific paper on the subject (Bell et al. 1942).

Many theories were proposed as to the cause of the symptoms. The first theory suggested that the cooling of the air during decompression caused exhaustion and cold in the caisson worker. This, in turn, produced the decompression symptoms. A second theory suggested that increased air pressure caused the peripheral vessels to collapse, thereby forcing blood into the viscera and causing mechanical congestion. Boucquoy proposed in 1861 that one result of the release of this mechanical congestion was the liberation of gas contained in the viscera (Bell et al. 1942). However, it was Bert who more correctly proposed in 1871 that the cause of the decompression symptoms was the release of nitrogen bubbles from the tissues into the blood (Kahlstrom et al. 1939).
Recognition of dysbaric osteonecrosis. Dysbaric osteonecrosis was not described until 1911 by Bornstein and Plate (Bornstein and Plate 1911) and independently by Bassoe (Bassoe 1911). Bassoe reported eleven caisson workers with chronic joint pain and stiffness. In 1913, Bassoe described seven additional cases. In five of these cases, he found x-ray findings that were consistent with arthritis deformans. He characterized the observations as "late manifestations of decompression disease" (Bassoe 1913).

The study of dysbaric osteonecrosis in caisson and tunnel workers progressed quickly from the time of its recognition. The disease was described in divers by Grutzmacher in 1941 (Amako et al. 1974). By then, the practical use of the technology of diving had progressed from the first industrial diving bell in 1678, through the diving suit developed in the 1800's (Bell et al. 1942), to more contemporary diving equipment.

Epidemiology

The first systematic survey of compressed air workers was published in 1942 by Bell, Edson, and Hornick (Bell et al. 1942). Prior to that time, bone changes in compressed air workers were believed to be a rare occurrence found only in workers with significant symptomatology. The trio completed a radiological study of 32 asymptomatic compressed air workers, finding that 24 of them had lesions characteristic for osteonecrosis -- a prevalence of 75%.

Since that time, many surveys have been conducted to try to accurately assess the prevalence of dysbaric osteonecrosis in the compressed air and diving populations. Perhaps the most significant of these surveys was initiated by the Medical Research Council Decompression Sickness Panel, University of Newcastle upon Tyne,
England. In 1962, the group investigated workers involved in the construction of tunnels under the River Clyde. During the course of the study, the Research Council set up a registry for all compressed air workers and commercial divers known as the Decompression Sickness Central Registry (DSCR). The registry has functioned since 1970, collecting and cataloging radiological exams, medical histories, and occupational data.

**Prevalence of dysbaric osteonecrosis.** The most recent results from the Decompression Sickness Central Registry as well as multiple other surveys are presented in Tables 1 and 2.

<table>
<thead>
<tr>
<th>Author/Survey</th>
<th>Year</th>
<th>Number of Divers Examined</th>
<th>Number of Divers with Lesions</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zachariades et al. -- Greek, commercial</td>
<td>1990</td>
<td>38</td>
<td>30</td>
<td>78.9</td>
</tr>
<tr>
<td>Xue -- China, all types</td>
<td>1988</td>
<td>2101</td>
<td>249</td>
<td>11.9</td>
</tr>
<tr>
<td>Oiwa et al. -- Japan, shellfish</td>
<td>1987</td>
<td>37</td>
<td>20</td>
<td>54.1</td>
</tr>
<tr>
<td>Evans et al. (Decompression Sickness Central Registry) -- British, commercial</td>
<td>1981</td>
<td>4980</td>
<td>310</td>
<td>6.2</td>
</tr>
<tr>
<td>Hunter et al. -- US Navy</td>
<td>1978</td>
<td>934</td>
<td>16</td>
<td>1.7</td>
</tr>
<tr>
<td>Oiwa and Itoh -- Japan, diving school/commercial</td>
<td>1978</td>
<td>95</td>
<td>13</td>
<td>13.7</td>
</tr>
<tr>
<td>Wade et al. -- Hawaii, fish*</td>
<td>1978</td>
<td>20</td>
<td>13</td>
<td>65.0</td>
</tr>
<tr>
<td>Fagan and Beckman -- Gulf Coast, commercial</td>
<td>1976</td>
<td>30</td>
<td>8</td>
<td>26.7</td>
</tr>
<tr>
<td>Amako et al. -- Japan, shellfish</td>
<td>1974</td>
<td>450</td>
<td>268</td>
<td>59.6</td>
</tr>
<tr>
<td>Elliott -- British Royal Navy</td>
<td>1974</td>
<td>383</td>
<td>31</td>
<td>8.1</td>
</tr>
<tr>
<td>Kawashima -- Japan, divers treated for DCS**</td>
<td>1972</td>
<td>450</td>
<td>268</td>
<td>59.6</td>
</tr>
<tr>
<td>Graczyk -- Poland, commercial***</td>
<td>1970</td>
<td>67</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>Asahi -- Japan, fish</td>
<td>1968</td>
<td>79</td>
<td>15</td>
<td>19.0</td>
</tr>
<tr>
<td>Ohta et al. -- Japan**</td>
<td>1966</td>
<td>301</td>
<td>152</td>
<td>50.5</td>
</tr>
<tr>
<td>Nagai -- Japan**</td>
<td>1965</td>
<td>60</td>
<td>46</td>
<td>76.7</td>
</tr>
<tr>
<td>Kiryakov -- Bulgaria, commercial***</td>
<td>1964</td>
<td>-</td>
<td>-</td>
<td>65.0</td>
</tr>
<tr>
<td>Alnor -- Germany, commercial</td>
<td>1963</td>
<td>131</td>
<td>72</td>
<td>55.0</td>
</tr>
<tr>
<td>Kinoshita -- Japan, shellfish**</td>
<td>1961</td>
<td>15</td>
<td>9</td>
<td>60.0</td>
</tr>
<tr>
<td>Kimura -- Japan**</td>
<td>1959</td>
<td>21</td>
<td>8</td>
<td>38.1</td>
</tr>
<tr>
<td>Sjorahál**</td>
<td>1953</td>
<td>13</td>
<td>3</td>
<td>23.1</td>
</tr>
<tr>
<td>Herget**</td>
<td>1948</td>
<td>90</td>
<td>29</td>
<td>32.2</td>
</tr>
</tbody>
</table>

* (McCallum and Harrison 1982)
** (Amako et al. 1974)
*** (Elliott 1971)
DCS = decompression sickness
Table 2. Prevalence of Bone Lesions in Previous Surveys of Compressed Air Workers

<table>
<thead>
<tr>
<th>Author/Survey</th>
<th>Year</th>
<th>Number of Workers Examined</th>
<th>Number of Workers with Lesions</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xue -- China, caisson and tunnel</td>
<td>1988</td>
<td>159</td>
<td>10</td>
<td>6.3</td>
</tr>
<tr>
<td>Trowbridge (Decompression Sickness Central Registry) -- British, caisson and tunnel</td>
<td>1977</td>
<td>2200</td>
<td>383</td>
<td>17.4</td>
</tr>
<tr>
<td>Sealey -- Seattle, tunnel</td>
<td>1975</td>
<td>86</td>
<td>5</td>
<td>5.8</td>
</tr>
<tr>
<td>Kindwall -- Milwaukee, tunnel</td>
<td>1974</td>
<td>188</td>
<td>59</td>
<td>31.4</td>
</tr>
<tr>
<td>Rozahegyi et al.*</td>
<td>1963</td>
<td>66</td>
<td>15</td>
<td>22.7</td>
</tr>
<tr>
<td>Mungo et al*</td>
<td>1958</td>
<td>14</td>
<td>5</td>
<td>35.7</td>
</tr>
<tr>
<td>Cavigneaux et al.*</td>
<td>1949</td>
<td>125</td>
<td>47</td>
<td>37.6</td>
</tr>
<tr>
<td>Nicolas*</td>
<td>1949</td>
<td>20</td>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td>Bell et al -- New York</td>
<td>1942</td>
<td>32</td>
<td>24</td>
<td>75.0</td>
</tr>
</tbody>
</table>

*(Amako et al. 1974)*

The data varies widely. For example, the prevalence of bone lesions in divers ranges from 1.7% in US Navy divers (Hunter et al. 1978) to 78.9% in Greek commercial divers (Zachariades et al. 1990). The prevalence of bone lesions in compressed air workers is equally disparate, with a low of 5.0% as reported by Nicolas in 1949 (Amako et al. 1974) and a high of 75.0% as reported by Bell, Edson, and Hornick in 1942 (Bell et al. 1942).

There are several possible explanations for these observations. First, although the DSCR Radiological Panel suggested radiologic criteria for the diagnosis of dysbaric osteonecrosis in 1966, not all of the surveys have utilized those criteria (McCallum et al. 1966). Furthermore, a questionable lesion might be read as positive by one radiologist and negative by another. Not reflected in Tables 1 and 2 are those compressed air workers and divers with radiology findings suspicious for, but not diagnostic of, dysbaric osteonecrosis.

Second, studies that include divers involved in experimental dive protocols may not fairly represent the general compressed air and diving populations. Elliott
reported a higher than expected number of British Royal Navy divers with bone lesions. He suspected experimental dive protocols as the cause and suggested caution when drawing general conclusions (Elliott 1974).

Third, the regulation and monitoring of compression and decompression schedules have varied over time and among countries and organizations. If the incidence of dysbaric osteonecrosis is related to the adequacy of decompression, then one might expect the incidence of the disease to also vary. For example, the surveys of US Navy divers and British commercial divers reported the lowest incidence of bone lesions. Compared to other organizations, these two groups are highly regulated, follow set decompression protocols, and submit to regular disease surveillance exams.

It should also be noted that, while decompression tables are regularly scrutinized and modified to reduce the morbidity of compressed air and diving work, a decrease in morbidity over time is not guaranteed. This would suggest that other factors in addition to the adequacy of decompression are at work in determining the prevalence of the disease. These factors are discussed in the paragraphs which follow.

**Length of experience/number of exposures.** Data from the DSCR on compressed air workers suggests that the incidence of bone lesions is related to the length of compressed air experience or, more specifically, to the total number of hyperbaric exposures. The research panel found that among the Clyde tunnel workers, 8.6% of those with fewer than 300 hyperbaric exposures demonstrated bone lesions, whereas 30.4% of those with greater than 900 hyperbaric exposures had lesions (McCallum et al. 1966).
A 1981 report from the DSCR on the diving population published in 1981 found a similar connection between length of experience and occurrence of bone lesions. It estimated that the prevalence of bone lesions increases with the length of diving experience at a rate of approximately 0.5% per year (Evans et al. 1981).

While multiple exposures increase the risk of developing bone lesions, a single exposure can also cause the disease. In 1931, the submarine *Poseidon* sank in 120 feet of water following a collision. Five men escaped the boat, having waited for three hours while the boat filled with water. All five suffered from acute decompression sickness of varying degrees of severity. Three of the men were examined twelve years after the experience. Each demonstrated radiologic evidence of dysbaric osteonecrosis, although all denied any other compressed air exposures (James 1945).

**Age.** A factor closely related to length of experience is age. Early studies suggested that the prevalence of bone lesions increased with advancing age. However, the DSCR demonstrated that when age is analyzed independent of length of experience, there is no difference in the prevalence of bone lesions (Evans et al. 1981).

**Maximal pressure/depth.** Data from both the compressed air and diving populations has demonstrated that the incidence of bone lesions is related to the maximal pressure experienced or the maximal depth attained. The DSCR Research Panel reported that only 6% of its study population of compressed air workers who had experienced a maximal pressure of 18 pounds per square inch (psi) demonstrated bone lesions. In contrast, 29% of the workers who had experienced a maximal pressure of 34 psi had lesions (McCallum et al. 1966).
Similar data exists from the DSCR for the diving population. Bone lesions have not been observed in divers who have never been deeper than 30 meters. However, 15.8% of divers who have dived to greater than 200 meters have bone lesions (Evans et al. 1981).

**History of acute decompression sickness.** The occurrence of dysbaric osteonecrosis has been shown in several studies to be correlated with a history of acute decompression sickness, commonly known as the bends. Data from the Clyde tunnel survey suggested that those workers with a history of one or more attacks of the bends were more likely to have bone lesions than those with no history of the bends (McCallum et al. 1966).

Data from the diving population in the DSCR has provided similar results. While 10.7% of the divers with a history of acute decompression sickness had bone lesions, only 1.7% of the divers with no history of decompression sickness had lesions. The data also suggested that a history of multiple episodes of decompression sickness was worse than a single episode (Evans et al. 1981). These results are supported by several other surveys (Elliott 1971; Amako et al. 1974; Trowbridge 1977; Hunter et al. 1978).

While many studies support the correlation between dysbaric osteonecrosis and acute decompression sickness, it remains clear that bone lesions can appear without any history of the bends. Likewise, a history of acute decompression sickness does not predispose to the development of bone lesions. For this reason, many authors have suggested that acute decompression sickness and dysbaric osteonecrosis are not directly causally related, but rather are connected by a third, yet unknown factor.
Physical characteristics. The DSCR research panel examined the physical characteristics of its diving population. Those characteristics included body habitus, pulmonary function, and hemodynamic parameters. The divers with bone lesions were found to be heavier than those without lesions, as measured by weight and skinfold thickness. In addition, they were found to have a significantly higher mean packed cell volume, although the value was still within normal range (Evans et al. 1981). These observations are connected to several etiologic theories and will be discussed later.

Clinical Aspects

Symptomatology. Dysbaric osteonecrosis begins as a symptomless lesion of the head, neck, or shaft of the humerus, femur, or tibia. Lesions located away from a joint surface generally remain asymptomatic. Lesions in the head of the humerus and femur located directly beneath the articular surface are called juxta-articular lesions. They can account for as many as 71% of all lesions (Hills 1977), and can lead to significant morbidity as often as 40% of the time (Davidson 1989).

Juxta-articular lesions may collapse under the impact of normal weight bearing and physical activity. This leads to dysfunctional joint mobility and osteoarthritis. Symptoms include pain, stiffness, and limited range of motion. Such joint dysfunction represents a significant disability in young people who would otherwise expect to continue working for many years.

Clinical diagnosis. Diagnosis of asymptomatic lesions depends primarily on regular radiographic exams. The exams should include the proximal humerus and femur and the shafts of the femur and tibia. Exams should occur every one to
three years depending on the type and depth of compression work (Evans et al. 1981).

Radiographic diagnosis of bone lesions is based on the identification of altered bone density in the commonly affected areas. This change is usually an increase in density and results from the repair process activated in response to a necrotic focus. Radiological criteria for diagnosis and classification were formalized by Harrison in 1974, and are presented in Table 3.

Table 3. Radiological Criteria for the Diagnosis and Classification of the Lesions of Dysbaric Osteonecrosis

<table>
<thead>
<tr>
<th>Juxta-articular lesions:</th>
<th>Head, neck, and shaft lesions:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 Dense areas with intact articular cortex</td>
<td>B1 Dense areas</td>
</tr>
<tr>
<td>A2 Spherical segmental opacities</td>
<td>B2 Irregular calcified areas</td>
</tr>
<tr>
<td>A3 Linear opacities</td>
<td>B3 Translucent areas and cysts</td>
</tr>
<tr>
<td>A4 Structural failures</td>
<td>B4 Cortical thickening</td>
</tr>
<tr>
<td>Translucent subcortical bands</td>
<td></td>
</tr>
<tr>
<td>Collapse of articular cortex</td>
<td></td>
</tr>
<tr>
<td>Sequestration of cortex</td>
<td></td>
</tr>
<tr>
<td>A5 Secondary degenerative osteoarthritis</td>
<td></td>
</tr>
</tbody>
</table>

(Harrison 1974)

The disadvantage of radiography is that bone lesions may not be diagnosed soon enough to prevent disabling complications. The earliest that radiography can detect bone abnormalities is four months following hyperbaric exposure (Davidson 1976). In addition, radiography may underestimate the size of a given lesion or fail to detect a lesion at all (Gregg 1976).

Alternative diagnostic modalities have been investigated. Radioisotope bone scans using $^{99m}$Technetium-labeled phosphorus compounds can detect a lesion within several weeks of hyperbaric exposure. However, the technique has a low specificity, with only 40% of identified lesions progressing to a positive radiographic
diagnosis. CT and MRI scans have also been investigated but, like radioisotope bone scans, these modalities have low specificity and are prohibitively expensive for disease surveillance (Davidson 1989).

**Pathologic diagnosis.** The first histologic descriptions of the lesions of dysbaric osteonecrosis were published by Kahlstrom et al. in 1939. The descriptions were of symptomatic juxta-articular lesions and coincident asymptomatic shaft lesions. Areas of necrotic bone including cystic areas filled with cell debris were noted. Separating these necrotic areas from the normal bone were fibrous tissues and sclerotic bone (Kahlstrom et al. 1939).

Subsequent studies of both symptomatic and asymptomatic lesions have demonstrated a similar pattern -- dead bone and marrow separated from living bone by a border of collagen and sclerotic bone. Dead bone is identified by the absence of osteocytes in the bone lacunae. Non-cellular bone structures usually remain intact. Dead marrow is identified by the lack of normal marrow architecture, the absence of nuclear staining, or the complete lack of cellularity.

Surrounding the areas of dead bone is a reactive area in which the living bone attempts repair. Some degree of resorption can occur. However, in the majority of cases, the repair process consists primarily of appositional deposition of new bone on the surface of the dead bone. This results in the encapsulation of necrotic areas with sclerotic bone.

**Treatment options.** Treatment is not required for asymptomatic lesions located away from a joint surface. Procedures designed to repair and revascularize juxta-articular lesions have not yielded significant success. When such a lesion
progresses to joint collapse and osteoarthritis, prosthetic joint replacement is the primary treatment. Unfortunately, the population this disease affects is composed mostly of young laborers, in whom joint prostheses have limited life span.

The best management of compressed air workers and divers begins with early detection of the potentially troublesome juxta-articular lesions. Those individuals in whom the lesions develop should limit their hyperbaric exposures to levels at which the risk of bone necrosis is minimal. Alternatively, such individuals should be counseled to seek other lines of work. This advice is particularly important because dysbaric osteonecrosis has been demonstrated to progress at all levels, including articular collapse and osteoarthritis, even in the absence of further hyperbaric exposure (Van Blarcom et al. 1990).

**Etiology**

**Physiology of diving.** At sea level the pressure exerted on the body by the surrounding air is equal to one atmosphere or 760 mm Hg. According to Dalton's Law, the pressure exerted by a mixture of gases such as air is equal to the sum of the pressures that each gas would exert individually, or to the sum of the partial pressures.

The partial pressure of a gas is equal to the total pressure times the fraction of the total that the gas represents. For example, air contains approximately 21% oxygen and 78% nitrogen. The partial pressure of oxygen is 0.21 X 760 mm Hg, or 160 mm Hg. The partial pressure of nitrogen is 0.78 X 760 MM Hg, or 593 mm Hg.

Gas dissolves in a liquid according to Henry's Law. The law states that the amount of a gas dissolved in a liquid is determined by the solubility of the gas in that liquid.
(the solubility coefficient) and by the partial pressure of the gas. At an equilibrium state, the liquid is considered to be fully saturated with the gas.

Gases also dissolve in the tissues of the body according to Henry's Law. Atmospheric gases enter the body through the lungs and are distributed throughout the tissues via the circulatory system. Diffusion gradients between the blood and the tissues allow movement of gas across barriers such as the endothelium and cell membranes. Different tissues have different solubility coefficients. For example, fatty tissues are capable of holding in solution five times as much nitrogen and four times as much oxygen as are nonfatty tissues.

In compressed air work, the air in closed compartments such as caissons or tunnels is made to be some pressure greater than one atmosphere. This increase in air pressure increases the partial pressures of the various gases. This in turn increases the volume of gas which can be dissolved in the blood and tissues.

In diving, water exerts a force on a diver which is related to the height of the column of water above him. The force, by its mechanical effects on the thorax, prevents the diver from breathing air at normal atmospheric pressures. The diver must therefore breathe compressed air to oppose the force of water. As in compressed air work, the end result is an increased volume of gas which is dissolved in the blood and tissues.

During compression and decompression, the equilibrium is disturbed and a new level of saturation must be reached. The rate of this change is dependent on the adequacy of the circulatory supply to the tissue and the ability of the tissue to absorb the gas. During decompression, a state of supersaturation is created. If
decompression is too quick, the gases do not have time to escape through the circulatory system to the lungs. The gases can coalesce into bubbles. It is these bubbles, especially those made of nitrogen or other inert gases, which have been shown to cause the symptoms of acute decompression sickness.

The tissues most at risk for developing damaging bubbles are those with a relatively poor blood supply and those with a high fat or lipid content. Tissues at greatest risk include subcutaneous tissues, the spinal cord, and the bone and bone marrow. Within the skeletal system, the long bones are thought to be at particularly high risk because they contain a high percentage of yellow or fatty marrow and have a relatively sluggish blood supply.

**Embolic gas bubbles.** Bornstein and Plate were not only among the first to describe dysbaric osteonecrosis, but they were also the first to propose an etiology for the disease. They suggested that gas bubbles could occlude one or more of a bone's nutrient arteries, producing ischemia and infarction of the tissue (McCallum et al. 1982). This theory was echoed by Christ in 1934 and Kahlstrom et al. in 1939 (Kahlstrom et al. 1939). It remains an attractive explanation for bone lesions because it would give dysbaric osteonecrosis and acute decompression sickness a common etiology -- embolic gas bubbles.

The formation of gas bubbles in decompression has been well documented. Using doppler ultrasound, Evans et al. monitored the appearance during decompression of gas bubbles. They detected circulating bubbles even in the absence of any symptoms of decompression sickness. These so called "silent bubbles" could potentially do damage to bones without giving any warning signs (Evans et al. 1972).
However, even Bornstein and Plate were not totally convinced by the embolic gas bubble explanation. They doubted that the obstruction of a single or even multiple arteries could explain the amount of necrosis or the symmetry of the lesions they had observed. Kahlstrom et al. suggested that with such extensive bone necrosis, damage to other tissues such as the brain, spleen, or kidney should also have been observed (McCallum et al. 1982). Furthermore, gas bubbles documented to be in the venous circulation would have to bypass the lungs to reach the nutrient arteries of the long bones.

**Extrinsic vessel obstruction by gas bubbles.** Kahlstrom et al. offered an alternative explanation. They suggested that nutrient vessels could be compressed by gas bubble formation within the trabecular spaces of the bone itself (Kahlstrom et al. 1939). The theory proposes that a gas bubble forming within the rigid confines of bone trabeculae could displace non-rigid medullary structures. This could result in a compromise of blood flow in or out of the bone and ultimately in an ischemic injury.

Gas bubbles have been documented within the medullary cavity and bone marrow sinusoids. Kawashima et al. reported the cases of four divers who died of acute decompression sickness and went to autopsy. The examination revealed sinusoids dilated by gas bubbles, platelets, and fat. The most extensive area of involvement was the subchondral layer of the femoral head, the same region in which juxta-articular lesions develop (Kawashima et al. 1977).

The principal argument against this theory is one of time. An ischemic state must persist for 12 to 48 hours for death of the bone to occur (Solomon 1985). This would require an extravascular bubble to remain intact for the same duration. A
conservative decompression profile is designed to allow enough time for dissolved gases to escape the body without coalescing into bubbles and causing symptoms. It is unlikely that any decompression profile could sustain an intramedullary bubble for the required amount of time without producing symptoms of acute decompression sickness.

*Gas bubble-induced bone microfracture.* An alternative consequence of intramedullary bubble formation has been proposed by Fraser et al. The theory suggests that gas bubbles formed in the confined spaces of bone trabeculae could exert enough pressure on the surrounding bone to cause it to fracture. If the fractures were extensive, they could disrupt the general blood supply as well as nutrient transport to osteocytes within the bone, leading to bone death.

This mechanism of injury has been observed in monkeys suffering from inner-ear decompression sickness. Histologic examination of the temporal bone encasing the semicircular canals revealed evidence of extensive microfracture and the disruption of blood flow. Long-term pathologic follow-up demonstrated necrotic bone surrounded by a sclerotic margin indicative of an incomplete repair process. The findings were similar to those seen in dysbaric osteonecrosis (Fraser et al. 1984).

Evidence for this mechanism of bone injury has not been documented in the classic locations for dysbaric osteonecrotic lesions. It remains unclear whether or not a sufficient number of microfractures could be induced to cause the extent of bone involvement which has been observed.
Embolic lipid particles. Following the line of reasoning that bone necrosis is a result of ischemic injury caused by vascular obstruction, researchers have suggested that other embolic particles might have an important role. Fat emboli have been seen as the most likely culprits, since fat emboli are known to be produced during decompression. In addition, experiments have demonstrated that lipid particles injected into arteries supplying the long bones can result in necrosis of the tissue (Jones et al. 1974).

While the existence of fat emboli has been clear, the origin of these emboli has been elusive. Femoral bone marrow disruption with release of lipid particles was originally thought to be the source. Two additional theories have since been postulated. The first theory suggests that expanding gas bubbles in the liver might induce the release of lipid droplets into the circulation (Hills 1977). The second theory suggests that a gas bubble might serve as a nidus for the aggregation of the normally circulating lipids, resulting in a particle of sufficient size to obstruct the microcirculation of bone (Clark et al. 1969).

Epidemiological and experimental data also support the idea that lipids play an important role in the pathogenesis of dysbaric osteonecrosis. As has been previously mentioned, compressed air workers and divers with bone lesions tend to have greater than average weight and skin-fold thickness. In an experimental correlate, Chryssanthou observed that obese mice subjected to a hyperbaric environment had a higher incidence of bone lesions than did their thin siblings (Chryssanthou 1976).

Arguments against the theory of fat embolization begin with the fact that bone necrosis has not been shown to occur as a direct result of decompression-induced
fat emboli. Contrary to what might be predicted, ischemic damage secondary to fat emboli does not occur with the same frequency in other organs as it does in bone. Also, as in the case of gas bubble embolization, fat particles must reach the arterial circulation to have any damaging effects.

**Thromboemboli.** The observation of changes in platelets following hyperbaric exposure leads to the suggestion that platelets might be involved in the pathogenesis of dysbaric osteonecrosis. Martin et al. were the first to document these platelet changes in men. They observed a fall in platelet number which reached its nadir three days after the hyperbaric exposure. They hypothesized that the missing platelets had aggregated in the microvasculature (Martin et al. 1972).

Giry et al. further explored this phenomenon by using radioisotopes to label platelets in the bone marrow and spleen. They observed increased platelet consumption as well as increased platelet production immediately following hyperbaric exposure. At one day after exposure, platelet consumption had further increased while platelet production had returned to normal levels. The researchers were unable to determine the method of platelet consumption, although microvasculature aggregation and splenic resorption were the prime suspects (Giry et al. 1977).

Other researchers have focused on the interaction between platelets and gas bubbles. Philip et al. have described a mechanism whereby platelets and other blood components might become stuck to the surface of a gas bubble. They suggest that when plasma proteins, particularly fibrinogen, interact with a gas-blood interface, they are altered such that they have increased reactivity. The proteins become a sticky coating for the gas bubble that attracts platelets and
other blood components. The end result is a particle large enough to be trapped in the microvasculature that will remain even after the gas has dissipated (Philip et al. 1972).

While gas bubbles have been observed in association with platelet clumping in acute decompression sickness, they have not been directly associated with the bone lesions of dysbaric osteonecrosis. Attempts to prevent the occurrence of bone lesions using anticoagulants and platelet function inhibitors has been largely unsuccessful (Chryssanthou 1978). As is the case with other embolic mechanisms, the theory of bubble induced thromboemboli fails to explain the occurrence of symmetric lesions in bone and the absence of infarction in other organs.

**Increased blood viscosity.** The altered plasma proteins induced by gas bubbles exhibit a related consequence -- an overall change in blood flow through the microvasculature. Several researchers have suggested that platelet, erythrocyte, and lipid aggregates increase the viscosity of the blood, thereby reducing flow (Lee et al. 1971; Guest et al. 1974). The reduction in blood flow in the vascularly challenged regions of the long bones could result in a significant hypoxic insult.

Researchers have also noted an increase in the hematocrit upon decompression. This is thought to be a hemoconcentration effect induced by fluid shifts from the vasculature to the interstitial spaces (Wells et al. 1971; Guest et al. 1974). The exact cause of the fluid shifts is unknown, although an osmotic effect produced by gas supersaturation of the tissues has been proposed (Hills 1977). Regardless, hemoconcentration further increases the viscosity of the blood.
It remains unclear whether the decrease in blood flow and tissue perfusion caused by increased blood viscosity has a significant effect on bone. As was noted previously, this change in hemodynamics would have to persist for 12 to 48 hours for irreversible damage to occur.

**Vasoactive effects.** A change in hemodynamics unrelated to blood viscosity has also been observed. Bond et al. measured intra-arterial pressures in the femurs of dogs. Following an injection of a gas bubble, they documented an initial constriction followed by a prolonged dilatation. They hypothesized that a gas bubble generated during decompression could have a similar effect. The vascular reactivity occurred in the small, noninnervated arteries, and it caused a measurable change in blood flow. The group suggested that this, in combination with microemboli, might significantly obstruct small nutrient arteries (Bond et al. 1965).

The direct cause of the vasoconstriction is unknown. Bond et al. suggested that the circulating gas bubbles might induce vasoconstriction through direct injury of the endothelium. In contrast, Chryssanthou proposed that the gas bubbles, in interacting with blood components, might cause the release or activation of vasoactive substances (Chryssanthou 1978).

**Vessel intimal damage.** Stegall et al. have documented damage to the endothelial lining from gas bubble trauma. An acute response to such an injury would include thrombus formation along the exposed surface (Stegall et al. 1978). A more chronic response to repeated injury might be the proliferation of the intimal cells ultimately resulting in stenosis of the vessel. A narrowed vessel
lumen would accentuate the ischemic effects of any of the mechanisms previously discussed (Chryssanthou 1978).

**Oxygen toxicity.** All of the previously discussed etiologic theories have focused on the decompression phase of hyperbaric exposure. However, increased partial pressure of oxygen in the tissues would have its maximal effect during the compressed phase of hyperbaric exposure. Several researchers have speculated that oxygen might have a direct toxic effect on bone and marrow cells, eventually resulting in bone death.

Oxygen has its toxic effects through the generation of oxygen free radicals -- hydrogen peroxide, superoxide, and hydroxyl radical. Cells have a detoxification mechanism in the form of superoxide dismutases, catalases, and peroxidases. The increased tissue saturation by oxygen in the compressed state might, in the short term, overwhelm the ability of the cells to counteract oxygen's toxic effects. Oxygen radicals would be free to inflict damage on cell and organelle membranes and components of the intracellular and extracellular matrix.

Several studies have examined oxygen radical effects on marrow fat cells. Walder et al. have demonstrated both in vitro and in vivo that fat cell swelling occurs in a hyperoxygenated environment. They postulate that the radicals damage the sodium pump, the cell is unable to maintain normal intracellular ionic concentrations, and the osmotic movement of water causes the cell to swell (Pooley et al. 1981; Walder et al. 1987). They further suggest that this fat cell swelling might through external compression of blood vessels, impede blood flow into the bone.
Others have speculated that oxygen radicals might in a similar fashion damage osteocytes or other bone cells, potentially resulting in their death (Jones 1987). No evidence for this mechanism of bone injury exists at this time.

**Collagen damage.** An alternative effect of oxygen radical generation in the hyperbaric environment is damage to collagen, a major component of bone matrix. Oxygen radicals are known to alter and degrade collagen in a number of tissues (Bulkley 1983).

Richmond et al. studied collagen metabolism in bone following hyperbaric exposure. They observed changes in the number and type of crosslinks in collagen which was accompanied by increased derangement of the collagen fibers (Richmond et al. 1977).

In similar experiments, Brickley-Parsons et al. noted changes in the genetic type of collagen that was being synthesized. They found a higher proportion of a repair type collagen in their dived subjects. They proposed that the bone's response to injury caused by hyperbaric exposure was to synthesize the repair collagen. However, this collagen was not resorbed as occurs in healed fracture sites, but rather it contributed to the development of an osteonecrotic lesion (Brickley-Parsons et al. 1980).

The disordered collagen's exact contribution to bone necrosis is unknown. Chryssanthou has suggested that the altered collagen has a decreased structural integrity, making it susceptible to fracture (Chryssanthou 1978). A possible explanation for decreased structural integrity is that the altered collagen
improperly complexes with the other matrix components resulting in abnormal matrix mineralization (Hills 1977).

The primary argument against oxygen toxicity as the cause of dysbaric osteonecrosis is the observation that subjects treated with hyperbaric oxygen therapy do not demonstrate the characteristic bone lesions (Hills 1977).

**Carbon dioxide toxicity.** Hills has speculated that elevated levels of carbon dioxide in bone during hyperbaric exposure might have a damaging effect. He suggests that increased carbon dioxide in the extracellular fluids of bone might stimulate bone demineralization or resorption. He cites as circumstantial evidence the observation that the incidence of bone lesions among the tunnel projects varies widely, and that the amount of carbon dioxide released from the earth at a particular site might be the cause (Hills 1977).

**Osmosis.** Hills has proposed a theory which involves the osmotic effects of dissolved gases. He has observed in vitro that water can be moved in and out of bone tissue using the transient gas concentration gradients that occur during pressure changes (Hills 1974). He speculates that similar events could occur within bone during the compression and decompression phases of hyperbaric exposure.

Hills further suggests that these fluid shifts would leave dissolved minerals in a supersaturated state. These minerals might "seed spontaneously," resulting in a breakdown in bone matrix mineralization. If these "seeds" were to grow in compromising spaces, bone cells or blood vessels might be damaged (Hills 1977).
Bone Structure and Physiology

A brief review of bone structure and physiology is presented as background for the histomorphometry methods used in this research as well as for the discussion that follows.

**Bone function.** The skeletal system has three primary functions. First, it serves as the structural framework onto which muscles attach, allowing for a diverse range of movements. Second, it serves a protective role for internal organs as well as serving as the location for hematopoietic tissue and fatty marrow. Third, it serves a metabolic function as the primary body reserve of calcium and phosphate.

**Macroscopic structure.** The skeletal system is composed of flat bones and long bones, which differ primarily in their embryologic origin and development. Long bones can be divided into three anatomic portions. The epiphyses lie at each end of the long bone and are usually covered by a layer of cartilage which forms the articular surface. The diaphysis is the midshaft portion. The metaphyses connect the epiphyses to the diaphysis. The growth plate lies between the epiphysis and the metaphysis.

The external surface of bone is covered by a layer of osteogenic cells called the periosteum. Beneath the periosteum lies the cortex, a layer of compact bone of variable thickness. Lining the internal surfaces of bone is the endosteum. In the region of the diaphysis, the internal cavity of the bone is filled with marrow. In the regions of the epiphysis and metaphysis, the internal cavity is filled with trabecular or spongy bone and marrow. The cortex serves a primarily structural function whereas the trabeculae serve a primarily metabolic function.
**Microscopic structure.** Bone is composed of cellular and non-cellular elements. The non-cellular elements include collagen and non-collagenous proteins, hydroxyapatite, and a ground substance of carbohydrate protein complexes. Most are produced by osteoblasts, although some are plasma proteins which are absorbed into newly forming bone. When complexed together, these elements form the calcified bone matrix.

The cellular elements of bone include osteocytes, osteoblasts, and osteoclasts. Osteocytes are found in lacunae within the calcified bone. They are derived from osteoblasts that have become trapped in the calcifying matrix. They are metabolically active, maintaining contact with neighboring cells through long cell processes.

Osteoblasts are the bone forming cells. They are derived from bone marrow stromal stem cells. When activated, they differentiate into a cuboidal cell with a round nucleus, basophilic cytoplasm, and a prominent Golgi complex. They are found in clusters where they synthesize the non-cellular bone components. Once secreted, the components become osteoid tissue. When they are finished secreting, osteoblasts become either osteocytes, trapped in the calcifying osteoid, or lining cells.

Osteoclasts are bone resorbing cells. They are derived from hemopoietic cells of the mononuclear-phagocytic line. When activated, osteoclasts become giant multinucleated cells, with a prominent Golgi complex, abundant secretory vesicles, and a ruffled border along the apical surface. They form a resorbing compartment along the bone surface into which they secrete protons and various
enzymes. As the bone is externally digested and subsequently phagocytized, a divot forms in the bone surface called a Howship's lacunae.

Osteoclasts acidify the extracellular resorbing compartment by the active transport of hydrogen ions across the apical membrane. Protons are generated by cytosolic carbonic anhydrase from H$_2$O and CO$_2$. Alternatively, protons are provided by sodium-hydrogen exchangers in the basolateral membrane. The acidity serves two functions. First, it dissolves hydroxyapatite crystals within the matrix. Second, it activates lysosomal enzymes and collagenase which degrade and digest matrix components.

**Bone physiology.** Bone is formed and resorbed in several distinct processes. Bone growth occurs as a part of the normal maturation of the skeletal system and is driven by growth hormones. In long bones, interstitial growth and resorption occurs at the growth plate, resulting in elongation of the bone. Appositional growth occurs along the periosteal surface. When matched by resorption along the endosteal surface, a larger diameter diaphysis is the result.

Bone modeling is a process of resorption and formation involving the periosteal and endosteal surfaces of cortical bone. It occurs as a normal part of bone growth. Bone modeling also occurs as a specific response to local stressors. The stressors can be mechanical or metabolic in nature and stimulate either bone resorption or bone formation. Bone modeling alters the shape or mass of the bone to better accommodate the local influences (Schultheis 1991).

Bone remodeling is a process involving cortical and trabecular bone in which bone resorption and formation occur as a part of normal bone turnover and renewal.
While it can be influenced by local stressors, remodeling is an ongoing process. If the amount of resorption equals the amount of formation, the process is said to be balanced. In the normal adult, formation does not exactly match resorption, resulting in a slight loss of bone mass with each cycle of bone turnover.

Bone remodeling is performed by groups of osteoclasts and osteoblasts which work as a unit. The cells are "coupled" such that bone formation occurs only at a site of previous resorption. The sequence of events in the remodeling process are as follows: activation of a resting unit, differentiation of osteoclasts, a resorption phase, a reversal phase, a formation phase, and lastly, a return to the resting phase (Baron 1993).

Bone remodeling is regulated by a variety of systemic and local factors. Systemic factors include parathyroid hormone, calcitonin, vitamin D, insulin, growth hormone, glucocorticoids, sex steroids, and thyroid hormones. Local factors include growth factors, prostaglandins, and cytokines. Activation of bone remodeling requires both the influence of systemic factors as well as the local production of regulatory factors by osteoblasts, osteocytes, and stromal cells (Canalis 1993). It is this intercellular dependence that couples resorption to formation in bone remodeling.
II. STATEMENT OF PURPOSE

Clinical and experimental evidence suggest that not every hyperbaric exposure will result in permanent bone damage and that partial repair of bone lesions can occur. Radiologic diagnosis of dysbaric osteonecrosis depends on the identification of areas of altered bone density -- alterations caused by increased bone cell activity. Despite these facts, prior research on the earliest bone changes in dysbaric osteonecrosis has focused on bone cell death rather than bone cell activity. These studies have relied on production of ischemic insults sufficient to result in histologic evidence of osteocyte death within six hours (Rösingh et al. 1969).

This investigator's hypothesis is that the bone changes seen in dysbaric osteonecrosis begin not as a response to ischemic or necrotic bone tissue, but rather as a response to the hyperbaric exposure itself. Bone changes in response to an unusual environmental condition are not exclusive to hyperbarics. The field of aerospace medicine has uncovered two such conditions -- hypobaric exposure and space flight.

In moving from sea level to high altitude, a pilot experiences changes in air pressure similar to those experienced by a diver upon decompression. Hypobaric exposure can result in a modified form of acute decompression sickness. Of interest to the field of dysbaric osteonecrosis is the observation that the incidence of bone pathology in altitude decompression disease is close to zero (Behnke 1971). The reason why bone damage occurs in hyperbaric but not hypobaric exposure is unknown. Researchers have speculated, however, that the physiologic differences
between hyperbaric and hypobaric exposure are the keys to determining the pathogenesis of dysbaric osteonecrosis.

Space flight offers an entirely different although better understood situation. During space flight, astronauts are subjected to a weightless environment. Without the effects of gravity, the mechanical forces on bones are greatly reduced. This reduction results in the stimulation of bone turnover. However, for a number of reasons, bone resorption is favored over bone formation. The result is a net loss of bone mass. Similar processes are thought to be involved in the bone loss observed in prolonged bedrest and osteoporosis (Schultheis 1991; Vico and Alexandre 1992).

This experiment was undertaken to answer the question of whether or not, in those hyperbaric exposures that seem to have no significant effect on bone, changes in bone cell activity are actually occurring. To that end, this study was designed as follows:

1. To subject experimental animals to a hyperbaric environment that would be unlikely to result in bone necrosis after a single exposure but that would predictably lead to classic dysbaric osteonecrotic lesions after multiple exposures.

2. To measure bone cell activity as it responds to the single hyperbaric exposure.
III. METHODS

Subjects
Thirty male Sprague-Dawley rats (average weight 220 g) were used. They were housed no more than three animals per cage and fed a normal rodent diet with tap water ad libitum. The animals were obtained one week prior to the start of the experiments and kept in a common room in the Yale University BCMM Animal Care Facility. Upon return to the facility following the dive, the animals were placed in an isolation room.

The experimental dives were conducted at the Naval Submarine Medical Research Laboratory, Groton, CT. The animals were transported from New Haven to Groton on the morning of the dives by private vehicle and were returned promptly following the conclusion of the dives. During transport and the experimental dives, five animals were housed in each cage.

Fluorescent Labels
The animals were divided into three groups of ten. Groups A and B were designated as experimental groups and Group C as the control group. Each animal was given two 0.25 cc intraperitoneal injections of calcein (Merck: 25 mg calcein/mL of a 2% NaHCO₃ solution in 0.15M saline) followed by one 0.25 cc intraperitoneal injection of alizarin red complexone (Sigma Chemical Co.: 25 mg alizarin red/mL of a 2% NaHCO₃ solution in distilled water).

The schedule of fluorescent label injections is summarized in Table 4. The first calcein dose (1C) was given on Day 1, the second calcein dose (2C) was given on
Day 5, and the alizarin red dose (R) was given 36 hours following the second calcein dose on Day 6.

Table 4. Experimental Protocol Summary

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<tr>
<th>Day #</th>
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**Experimental Dives**

The hyperbaric chamber at the Groton Naval Submarine Medical Research Laboratory was manufactured by the Bethlehem Corporation (model 18365HP, series 76.1011). The chamber has an internal volume of 3.8 square feet which accommodates two standard-size rat cages (Figures 1 and 2). Three separate dives were conducted, one for each experimental group.

Groups A and B were subjected to identical dive profiles. Air was the only gas mixture used in the dives. The profile consisted of a compression phase to 120 ft (~5 atmospheres) at a rate of 1 ft/sec, a twelve hour stay at 120 ft, and a decompression phase to 0 ft at a rate of 1 ft/sec. Group C was placed in the chamber for a twelve hour dive, but no compression was done. A dive time of twelve hours was chosen because it is the maximum shift length that would be expected of a compressed air worker or professional diver.
Figure 1. Hyperbaric chamber at the Naval Submarine Medical Research Laboratory, Groton, CT.

Figure 2. Interior of hyperbaric chamber with two animal cages.
Groups A and C completed their dives on Day 6, twenty-four hours after the second calcein injection. They received the alizarin red injection immediately following the dive. Group B completed the dive on Day 6 as well, but thirty-six hours after the second calcein injection. They received the alizarin red injection immediately prior to the dive. The chronological relationship between injections and dive times is shown in Table 4.

Air quality in the chamber was maintained by the following three mechanisms:

1. The chamber was continuously purged via a slow continuous air leak from the exhaust valve, while maintaining a constant chamber pressure.
2. Every fifteen minutes, a vigorous purge was effected by fully opening both inlet and outlet valves for one minute.
3. A soda lime product called Sodasorb (Dewey and Almy Chemical Division) was placed in the chamber to absorb carbon dioxide.

Air quality within the chamber was monitored using a portable oxygen analyzer (Teledyne) which measured the percent oxygen in air released from the exhaust valve. Readings were taken every fifteen minutes, and oxygen content was maintained above 20% for the duration of the dives. Equipment for the measurement of carbon dioxide was not available.

Temperature was also continuously monitored during the dives. Because the increased density of air in a dive chamber conducts heat away from an animal more efficiently than under normobaric conditions (Webb 1970), the temperature was maintained at 85° F ± 4°. The rats were monitored visually during the dives through two ports located in the top of the chamber. They were provided adequate food and water during the twelve-hour period.
Following decompression, the animals were observed for signs of decompression sickness. In rats, those signs include hyperventilation, puritis, ataxia, stiffness, paralysis, or movements indicating joint discomfort.

**Specimen Collection and Processing**

Groups A and C were sacrificed twelve hours following the completion of the dive, using ether anesthesia and spinal cord transection. Group B was sacrificed immediately following the completion of the dive, by Ketamine anesthesia and decapitation. The alternative method of sacrifice for Group B was used because of restrictions at the dive site regarding volatile gases. Both femurs were harvested from each animal for further processing.

The femurs were fixed in 40% alcohol, progressively dried in increasing concentrations of alcohol, and embedded in methyl methacrylate. The bones were not decalcified. Longitudinal sections 4 μm thick were cut through the center of each femur and stained with toluidine blue. Additional 10 μm sections were cut and kept unstained.

**Histomorphometry**

The histomorphometry parameters used in this study are summarized and defined in Table 5. The parameters were measured and calculated according to the methods described by Parfitt using a Nikon Labophot microscope and the OsteoMeasure Analysis System by OsteoMetrics (Parfitt 1988).
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<td>Bone volume/total volume</td>
</tr>
<tr>
<td>Osteoid Volume to Total Volume</td>
<td>OV/TV</td>
<td>(%)</td>
<td>Osteoid volume/total volume</td>
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<td>OV/BV</td>
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<td>Osteoblast Surface to Bone Surface</td>
<td>Ob.S/BS</td>
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</tr>
<tr>
<td>Osteoblast Surface to Osteoid Surface</td>
<td>Ob.S/OS</td>
<td>(%)</td>
<td>Osteoblast surface/osteoid surface</td>
</tr>
<tr>
<td>Osteoclast Surface to Bone Surface</td>
<td>Oc.S/BS</td>
<td>(%)</td>
<td>Osteoclast surface/bone surface</td>
</tr>
<tr>
<td>Mean Trabecular Thickness</td>
<td>Tb.Th</td>
<td>(µm)</td>
<td>Mean trabecular thickness</td>
</tr>
<tr>
<td>Mean Osteoid Thickness</td>
<td>O.Th</td>
<td>(µm)</td>
<td>Mean osteoid thickness</td>
</tr>
<tr>
<td>Osteoblast Volume Density</td>
<td>N.Ob/T.Ar</td>
<td>(/mm²)</td>
<td>Number of osteoblasts/total area</td>
</tr>
<tr>
<td>Osteoblast Bone Surface Density</td>
<td>N.Ob/B.Pm</td>
<td>(/mm)</td>
<td>Number of osteoblasts/bone parameter</td>
</tr>
<tr>
<td>Osteoblast Osteoid Surface Density</td>
<td>N.Ob/O.Pm</td>
<td>(/mm)</td>
<td>Number of osteoblasts/osteoid parameter</td>
</tr>
<tr>
<td>Osteoblast Index</td>
<td>N.Ob/Ob.Pm</td>
<td>(/mm)</td>
<td>Number of osteoblasts/osteoblast parameter</td>
</tr>
<tr>
<td>Osteoclast Volume Density</td>
<td>N.Oc/T.Ar</td>
<td>(/mm²)</td>
<td>Number of osteoclasts/total area</td>
</tr>
<tr>
<td>Osteoclast Bone Surface Density</td>
<td>N.Oc/B.Pm</td>
<td>(/mm)</td>
<td>Number of osteoclasts/bone parameter</td>
</tr>
<tr>
<td>Mineralizing Surface (pre-dive) to Bone Surface</td>
<td>MS.C/BS</td>
<td>(%)</td>
<td>Mineralizing surface (calcein only)/bone surface</td>
</tr>
<tr>
<td>Mineralizing Surface (peri-dive) to Bone Surface</td>
<td>MS.R/BS</td>
<td>(%)</td>
<td>Mineralizing surface (alizarin red only)/bone surface</td>
</tr>
<tr>
<td>Mineral Apposition Rate Pre-dive</td>
<td>MAR.1C-2C</td>
<td>(µm/d)</td>
<td>Mean interlabel thickness (first calcein to second calcein)/4 days</td>
</tr>
<tr>
<td>Mineral Apposition Rate Peri-dive</td>
<td>MAR.2C-R</td>
<td>(µm/d)</td>
<td>Mean interlabel thickness (second calcein to alizarin red)/1.5 days</td>
</tr>
</tbody>
</table>
Light histomorphometry was performed at a magnification of x250 on the undecalcified 4 μm sections stained with toluidine blue (Figure 3). Osteoclasts (Figure 4), osteoblasts, and osteoid tissue (Figures 5 and 6) were identified. All measurements were made in the trabecular bone of the distal femur epiphysis.

Fluorescent histomorphometry was performed at a magnification of x250 on the undecalcified and unstained 10 μm sections. The fluorescent labels were seen using UV light (Figure 7). Measurements were made in the trabecular bone of the distal femur epiphysis (Figure 8) and along the endosteal surface of the middle third of the femur diaphysis (Figure 9).

Results are expressed as the mean ± one standard deviation. Comparisons among the three experimental groups were made using the one-way analysis of variance (ANOVA) test. Differences were considered significant if p < 0.05 by the Scheffe F-test.

**Contributors**

The experimental work was designed and performed by the investigator, with the assistance of staff in the Orthopaedic Histology Lab, Yale University, and at the Naval Submarine Medical Research Laboratory, Groton. Specific activities performed by those staff members are as follows:

- Nancy Troiano, MS -- block sectioning, light histomorphometry
- Richard Darrell, BS -- fluorescent label preparation, specimen fixation and embedding, block sectioning
- Chief William Staples, USN -- dive chamber preparation.
Figure 3. Undecalcified 4 μm section of distal femur epiphysis stained with toluidine blue, demonstrating trabecular bone (TB) and bone marrow (M). Magnification = x125.

Figure 4. Osteoclasts (arrows) along trabecular surface. Magnification = x250.
Figure 5. Trabecular bone with osteoblasts (open arrows) and osteoid (outlined by small arrows). Magnification = x250.

Figure 6. Active osteoblasts (arrows) with granular cytoplasm on osteoid surface. Magnification = x500.
Figure 7. Undecalcified and unstained 10 µm section of distal femur epiphysis, demonstrating trabecular bone (TB), bone marrow (M), and fluorescent labeling. Magnification = x125.

Figure 8. Epiphyseal trabecular bone (TB) demonstrating triple-labeling with calcein (1C and 2C) and alizarin red (R). Magnification = x250.

Figure 9. Femur mid-diaphysis demonstrating cortical bone (CB), bone marrow (M), and endosteum with triple-labeling (1C, 2C, and R). Magnification = x250.
IV. RESULTS

Experimental Dives
The dive protocols were executed as planned. During the dives, the animals were observed in the normal activities of eating, hygiene, and rest. Following decompression, the animals and their cages were found to be completely soaked with water. It is suspected that small changes in pressure during the dive caused the water bottles to leak. The animals did not show any signs of acute decompression sickness immediately following the dive or during the subsequent twelve hours.

Animal Exclusion
Prior to sacrifice, each animal was weighed. It was noted that three of the animals in the control group (Group C) weighed significantly less than the remainder of the animals. Mean weight in grams of the three low-weight animals in Group C was 168 ± 11 while the mean weight of the remaining 27 animals was 268 ± 14. The three low-weight animals were housed in the same cage for the majority of the experiment. Although there was no evidence that they were sick, an illness is the most likely explanation for their weight loss. Therefore, these three animals were excluded from data analysis.

Light Histomorphometry
Group B was sacrificed immediately following the experimental dive. Any differences between Group B and Group C should reflect changes occurring in the bone during the twelve-hour dive. Group A was sacrificed twelve hours following the experimental dive. Any difference between Group A and Group C should reflect changes occurring in the bone in the immediate post-dive period. As noted
above, Group C experienced a simulated experimental dive and served as the control group.

The results of the measurements made on the distal femur epiphysis using light microscopy are summarized in Table 6. With the exception of the osteoclast parameters, the bone parameters were not significantly different among the three groups.

<table>
<thead>
<tr>
<th>Table 6. Light Histomorphometry</th>
<th>Group A (n = 10)</th>
<th>Group B (n = 10)</th>
<th>Group C (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Volume to Total Volume — BV/TV (%)</td>
<td>22.8 ± 3.1</td>
<td>24.0 ± 3.2</td>
<td>23.9 ± 5.6</td>
</tr>
<tr>
<td>Osteoid Volume to Total Volume — OV/TV (%)</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Osteoid Volume to Bone Volume — OV/BV (%)</td>
<td>2.7 ± 1.1</td>
<td>2.5 ± 0.8</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Bone Surface to Volume Index — BS/BV (mm²/mm³)</td>
<td>36.5 ± 3.8</td>
<td>34.0 ± 2.6</td>
<td>36.0 ± 6.3</td>
</tr>
<tr>
<td>Osteoid Surface to Bone Surface — OS/BS (%)</td>
<td>21.6 ± 7.7</td>
<td>20.2 ± 4.5</td>
<td>19.1 ± 3.9</td>
</tr>
<tr>
<td>Osteoblast Surface to Bone Surface — Ob S/BS (%)</td>
<td>6.6 ± 3.1</td>
<td>4.9 ± 2.2</td>
<td>5.1 ± 1.5</td>
</tr>
<tr>
<td>Osteoblast Surface to Osteoid Surface — Ob.S/OS (%)</td>
<td>29.7 ± 7.7</td>
<td>23.8 ± 6.4</td>
<td>26.8 ± 6.3</td>
</tr>
<tr>
<td>Osteoclast Surface to Bone Surface — Oc S/BS (%)</td>
<td>2.1 ± 1.0 *</td>
<td>2.4 ± 1.4 *</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Mean Trabecular Thickness — Tb.Th (µm)</td>
<td>55.3 ± 5.6</td>
<td>59.2 ± 4.8</td>
<td>57.1 ± 10.4</td>
</tr>
<tr>
<td>Mean Osteoid Thickness — O.Th (µm)</td>
<td>3.3 ± 0.5</td>
<td>3.4 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Osteoblast Volume Density — N.Ob/T.Ar (1/mm²)</td>
<td>44.6 ± 16.7</td>
<td>35.4 ± 16.4</td>
<td>36.4 ± 9.7</td>
</tr>
<tr>
<td>Osteoblast Bone Surface Density — N.Ob/B.Pm (1/mm)</td>
<td>6.6 ± 2.7</td>
<td>5.3 ± 2.5</td>
<td>5.3 ± 1.7</td>
</tr>
<tr>
<td>Osteoblast Osteoid Surface Density — N.Ob/O.Pm (1/mm)</td>
<td>30.2 ± 6.0</td>
<td>25.5 ± 8.0</td>
<td>28.1 ± 7.2</td>
</tr>
<tr>
<td>Osteoblast Index — N.Oh/Oh.Pm (1/mm³)</td>
<td>103.4 ± 10.4</td>
<td>107.1 ± 10.7</td>
<td>104.9 ± 8.1</td>
</tr>
<tr>
<td>Osteoclast Volume Density — N.Oc/T.Ar (1/mm²)</td>
<td>3.9 ± 2.1</td>
<td>4.5 ± 2.8 *</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>Osteoclast Bone Surface Density — N.Oc/B.Pm (1/mm)</td>
<td>0.6 ± 0.3</td>
<td>0.7 ± 0.3 *</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Significant differences as compared to control (Group C) by Scheffe F-test: p < 0.05

Osteoclast Surface to Bone Surface (Oc.S/BS) represents the percent of total bone surface covered by osteoclasts. Both Group A (2.1 ± 1.0) and Group B (2.4 ± 1.4) demonstrated a statistically significant increase in this value as compared to the control (0.8 ± 0.3). The small difference between Group A and Group B was not significant. These findings indicate an increase in osteoclast number which began during the dive and continued into the immediate post-dive period.
Osteoclast Volume Density (N.Oc/T.Ar) and Osteoclast Bone Surface Density (N.Oc/B.Pm) represent the number of osteoclasts per unit total area and bone surface respectively. Group B demonstrated a statistically significant increase in these values as compared to the control. Although Group A also demonstrated a marked increase in these parameters as compared to the control, the difference was not statistically significant.

**Fluorescent Histomorphometry**

The experiment was designed such that, for the mineralization parameters, each group would have an internal control in addition to the external control. The first two calcein injections were completed well before the experimental dives, and reflect pre-dive bone mineralization dynamics. The alizarin red injections were given either immediately before or immediately following the experimental dives, and reflect peri-dive bone mineralization dynamics.

The results of the measurements made on the distal femur epiphysis and on the endosteal surface of the middle third of the femur using fluorescent microscopy are summarized in Table 7.

<table>
<thead>
<tr>
<th>Table 7. Fluorescent Histomorphometry</th>
<th>Group A (n = 10)</th>
<th>Group B (n = 10)</th>
<th>Group C (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epiphyseal results:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralizing Surface (pre-dive) to Bone Surface -- MS.C/BS (%)</td>
<td>62.4 ± 7.0</td>
<td>63.6 ± 6.5</td>
<td>60.0 ± 9.7</td>
</tr>
<tr>
<td>Mineralizing Surface (peri-dive) to Bone Surface -- MS.R/BS (%)</td>
<td>13.5 ± 9.7</td>
<td>6.3 ± 3.9</td>
<td>6.5 ± 4.7</td>
</tr>
<tr>
<td>Mineral Apposition Rate Pre-dive -- Ep.MAR 1C-2C (μm/d)</td>
<td>2.3 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Mineral Apposition Rate Peri-dive -- Ep.MAR 2C-R (μm/d)</td>
<td>2.8 ± 0.4</td>
<td>2.6 ± 0.3</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td><strong>Endosteal results:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral Apposition Rate Pre-dive -- En.MAR 1C-2C (μm/d)</td>
<td>6.2 ± 1.4</td>
<td>7.2 ± 1.3</td>
<td>6.1 ± 2.0</td>
</tr>
<tr>
<td>Mineral Apposition Rate Peri-dive -- En.MAR 2C-R (μm/d)</td>
<td>7.3 ± 1.1</td>
<td>7.8 ± 1.0</td>
<td>6.4 ± 1.8</td>
</tr>
</tbody>
</table>
No statistically significant differences were noted among the three groups for any parameter. In addition, no statistically significant differences were noted between pre-dive and peri-dive values for the mineral apposition rate. Differences noted between pre-dive and peri-dive values for mineralizing surface to bone surface are a reflection of experimental design.
V. DISCUSSION

Summary of results. The effects of hyperbaric exposure on bone physiology observed in this experiment can be summarized as follows:

1. The absolute and relative numbers of osteoclasts increased as a result of the hyperbaric exposure, suggesting an alteration in bone resorption rates.
2. The increase in osteoclasts occurred during the compressed state and persisted for twelve hours following decompression.
3. The absolute and relative numbers of osteoblasts, the volume of osteoid, and the rate of mineralization showed no change as a result of the hyperbaric exposure, suggesting no alteration in bone formation rates over the time period observed.

The increase in the observed number of osteoclasts can be accounted for by two possible mechanisms. It could represent either an increased production of osteoclasts, in which case the extra osteoclasts would be new cells. Alternatively, it could represent a prolongation of the osteoclasts' active life span, in which case the extra osteoclasts would be old cells. There is no way to determine from this experiment which mechanism is active.

Activation of bone resorption. While the increase in osteoclast number suggests an alteration in bone resorption rates, other possibilities exist. For example, an alternative interpretation of the experimental observations might be that bone resorption is not changed. In this case, a decrease in osteoclast activity would have to accompany the increase in osteoclast number. Although this interpretation is indeed possible, it is unlikely. Therefore, for purposes of this discussion, it will be
assumed that the increased number of osteoclasts represents an activation of bone resorption. Several possible explanations for this activation can be proposed.

First, the bone may be responding in a normal manner to bone injury caused by hyperbaric exposure. This injury might take the form of microfractures or damaged and disordered collagen. Intra-trabecular gas bubbles released during decompression have been implicated in previous studies as a cause of microfractures. In addition, oxygen radicals generated in the compressed state are known to alter and degrade collagen. The activation of bone remodeling could be an attempt at repair.

Second, hyperbaric exposure could cause errors in the regulatory mechanisms of bone remodeling. Osteoclast regulation is dependent on intercellular communication within the bone microenvironment. It is possible that localized hypoxia could compromise the function of osteoblasts, osteocytes, and stromal cells, resulting in an alteration in their ability to communicate with osteoclasts. Cellular damage from oxygen radicals and osmotic fluid shifts could cause similar effects.

Third, osteoclast activity could be altered through direct effects on the osteoclast or its precursors. Osteoclast function is dependent on the generation of a high hydrogen ion concentration in the resorptive compartment. Arnett et al. demonstrated in vitro that an increase in the partial pressure of carbon dioxide results in an increase in the number and size of osteoclast resorptive sites (Arnett et al. 1994). In a hyperbaric environment, the partial pressures of all gases, including carbon dioxide, are increased.
Resolution of changes in bone cell activity. It is clear that the length of this experiment did not accommodate the full natural history of the observed changes. Further changes or a return to normal bone cell activity would be expected at some point beyond the twelve-hour post-dive mark. In the remodeling process, bone resorption occurs in conjunction with bone formation. Although evidence of bone formation was not observed in this experiment, the possibility that formation would occur was not ruled out.

Two scenarios can be postulated for the resolution of the process initiated by the hyperbaric exposure. In the first scenario, the increase in bone resorption would be followed by an increase in bone formation. The resultant increase in bone turnover rate, depending on how long it persisted, could result in a net bone loss and increased bone fragility.

In the second scenario, the increase in bone resorption would occur independent of an increase in bone formation. This would represent an uncoupling of resorption and formation. Unopposed resorption results in net bone loss and increased bone fragility.

In both scenarios, the end result is bone loss and fragility. Fortunately, these conditions have not been documented in the compressed air or diving populations. This suggests that a recovery or repair mechanism must exist to counter the physiologic effects on bone of hyperbaric exposure.

The possible existence of such a recovery mechanism naturally leads to the question of what might occur if the mechanism failed. The incidence of dysbaric osteonecrosis is positively correlated with the total number of hyperbaric
exposures. The cumulative effects of hyperbaria could actually reflect the overloading and failure of a normal recovery mechanism.

**Summary.** This study took a novel approach to the investigation of dysbaric osteonecrosis. The experiment demonstrated changes in bone cell activity in response to hyperbaric exposure. Several possible explanations for the changes were proposed. These explanations included the effects of hyperbaria on bone microstructure, on the regulation of bone remodeling, and on individual bone cells. The potential long term effects of the changes, although not observed, were also discussed.

This researcher suspects that the changes in bone cell activity documented in this experiment represent the initial steps in the bone damage that eventually results in dysbaric osteonecrosis. Further investigation may lead not only to a better understanding of the pathogenesis of dysbaric osteonecrosis but also to new approaches for disease surveillance and prevention.
VI. REFERENCES


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