Link Between Clinical Predictors of Heterotopic Ossification and Histological Analysis in Combat-Injured Service Members

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Background: Heterotopic ossification (HO) is a debilitating condition that occurs following traumatic injury and may restrict range of motion and delay rehabilitation. The timing and efficacy of surgical resection have varied widely, and there is a gap in knowledge between clinical predictors of HO recurrence and histological analysis.

Methods: Thirty-three service members seen at Walter Reed National Military Medical Center for symptomatic HO were enrolled in an institutional review board-approved study. Participants took oxytetracycline on four scheduled days prior to HO resection to determine the mineral apposition rate (bone growth rate).

Results: Detailed histological analyses included scanning electron microscopy with backscattered electron imaging and light microscopy. Data indicated that the mineral apposition rate of trauma-induced HO was approximately 1.7 μm/day at the time of operative intervention, which was 1.7 times higher than the rate in non-pathological human bone. The mineral apposition rate and postoperative alkaline phosphatase values were demonstrated to be positively and significantly related \( (p = 0.509, p = 0.026, n = 19) \). When the analysis was limited to patients with no more than a two-year period from injury to excision (thereby removing outliers who had a longer time period than their counterparts) and traumatic brain injury and nonsteroidal anti-inflammatory drugs (known correlates with HO development) were controlled for in the statistical analysis, the mineral apposition rate and recurrence severity were significantly related \( (p = -0.572, p = 0.041, n = 11) \).

Conclusions: Data demonstrated a link between benchtop research and bedside care, with the mineral apposition rate elevated in patients with HO and correlated with recurrence severity; however, a larger sample size and more clinical factors are needed to refine this model.

Level of Evidence: Prognostic Level II. See Instructions for Authors for a complete description of levels of evidence.

Heterotopic ossification (HO) is abnormal osseous tissue that occurs in muscles and periarticular regions following tissue injury and inflammation. Although ectopic bone may develop from rare genetic disorders, it is most frequently observed following orthopaedic trauma, burns, arthroplasty, spinal cord injury, and traumatic brain injury. HO has been reported in 20% to 30% of patients with spinal cord injury, in 10% to 20% of those with...
Fig. 1 Photographs of the amount of bone resected from the residual limb of a male service member with HO above the knee. This patient had sustained limb loss due to an injury from an IED blast when he was thirty-two years of age.

Fig. 2 Scanning electron microscopy with backscattered electron imaging showing newly formed bone (arrow) (Fig. 2-A); osteon formation (arrow), which is common with cortical bone (Fig. 2-B); and bone resorption (arrow) on a trabecular-type structure of bone (Fig. 2-C). The images suggest that bone fragments were displaced from the blast. Gray = bone, and black = pore space and soft tissue.
closed head injury, and following 16% to 53% of total hip arthroplasty procedures\textsuperscript{28,29}.

Most cases of HO in the general population appear minimal radiographically, are clinically asymptomatic, and do not necessitate operative intervention. However, for military service members injured by blasts in Afghanistan and Iraq, the prognosis has been quite different. These armaments generate extensive polytrauma, and hallmark injury profiles during overseas combat have included limb loss, traumatic brain and/or spinal cord injury, and HO\textsuperscript{1,30-32}. In fact, 1573 wounded service members sustained one or more major limb amputations in Operation Iraqi Freedom, Operation Enduring Freedom, and Operation New Dawn between 2000 and 2014\textsuperscript{33}; approximately 63% to 65% of these individuals developed posttraumatic HO\textsuperscript{2,6,10} and 20% to 40% required surgical excision\textsuperscript{6,34-36}. Symptomatic HO is problematic for service members since it delays rehabilitation regimens, causes pain, limits range of motion, and requires modifications of prosthetic limbs\textsuperscript{8,34}.

Wounded service members are a unique patient population given their relative youth, high fitness level prior to injury, and desire for aggressive rehabilitation in order to return to active duty or civilian recreational sports and activities\textsuperscript{37}. Therefore, when ectopic bone becomes evident (typically between one and twelve weeks after injury)\textsuperscript{38,39}, the patient may request that the mass be removed immediately. However, deciding when to excise HO requires careful consideration since delayed limb and patient immobilization may lead to muscle atrophy, disuse osteoporosis, cartilage erosion, and bone and fibrous ankylosis\textsuperscript{23,39}, whereas premature resection may lead to aggressive HO recurrence. While results of early resection have remained promising, premature surgical procedures often result in more florid ectopic bone regrowth.

Early attempts to correlate HO recurrence with clinical predictors have focused primarily on the timing of excision surgery and the severity of the neurological insult; however, these research studies were unsuccessful\textsuperscript{2,40}. Genêt et al. recommended
that “surgical excision of HO should be carried out as soon as it becomes troublesome, comorbid factors are under control and the HO is sufficiently constituted for excision.” While this recommendation is logical, it does not identify an objective measurement tool with which to define maturity and does not address the absence of histological markers for optimizing resection schedules. In an effort to bridge the gap between clinical and histological knowledge, our team planned a prospective research study of combat-injured service members who required removal of ectopic bone. The goals of this study were to use advanced imaging tools to (1) provide direct quantitative evidence of ectopic bone growth via the mineral apposition rate, percent osteoblasts, percent osteoclasts, and percent resting bone; (2) further understand HO architecture through scanning electron microscopy; and (3) assess relationships between ectopic bone severity/recurrence and demographic information and previously attributed predictors (neurological injury, use of anti-inflammatory drugs, etc.) in order to optimize surgical planning. Quantifying the metabolic rate of HO may validate conventionally used measures to determine ectopic bone development (preoperative alkaline phosphatase levels, nuclear scintigraphic activity [i.e., bone scanning], and radiographic evidence of HO maturity). It was hypothesized that an HO mineral apposition rate that is greater than traditional human bone remodeling ($1 \text{ mm/day}$) would be a predictor of HO recurrence.

Materials and Methods

Service members with symptomatic combat-injury-related HO treated between June 2012 and March 2015 at Walter Reed National Military Medical Center were included in this institutional review board-approved study. Participants were excluded if they were less than eighteen years of age, were allergic to tetracycline, were currently using tetracycline, or had used tetracycline within three months before enrollment. Orthopaedic surgeons referred patients for recruitment into the study after counseling them and confirming that surgical resection was necessary and planned. All participants signed an informed-consent document and received the standard of care, with the exception of being asked to take oxytetracycline (250 mg three times a day) on four separate dates prior to their scheduled surgery to determine their mineral apposition rate (i.e., bone growth rate). The dosing schedule varied slightly.
according to the participant’s clinical schedule, but typically consisted of a two-day dosing period, a minimum three-day hiatus, a two-day dosing period, a two-day washout period, and then surgical excision. The timing of surgical intervention was based on the clinical standard for assessing HO maturation (measurement of alkaline phosphatase levels, zonation and stability on serial orthogonal radiographs, and consideration of traumatic brain injury) and was at the surgeon’s discretion.

Clinical Evaluation
Demographic information and injury data were captured for each participant from electronic medical record systems. Specific recorded information included sex, age, date of injury, height and weight prior to injury, time from injury to HO excision, injury mechanism that caused the limb loss, serum alkaline phosphatase levels before and after the HO excision, history of nonsteroidal anti-inflammatory drug (NSAID) use, traumatic brain injury (coded as 0 [none], 1 [mild], 2 [moderate], or 3 [severe]), and HO anatomical location. (It should be noted that the postoperative serum alkaline phosphatase level was not collected and documented for all service members. We followed up with the health-care providers many times, but this information was not always loaded in the system and/or requested by the physician. Thus, that analysis was conducted using a smaller sample size.) Each patient’s radiographic data were blinded and were reviewed by an attending orthopaedic surgeon to assess HO severity prior to resection and recurrence three to six months postoperatively. Ectopic bone severity and recurrence were assessed on anteroposterior and lateral radiographs, with the amount of ectopic bone within the residual limb classified as 0% (none), <25% (mild), 25% to 50% (moderate), or >50% (severe).6

Histological Evaluation
Following surgical intervention, HO samples were deidentified and were processed for postoperative analysis. All specimens were photographed, radiographed, fixed in formalin, dehydrated in ascending grades of ethanol, and embedded in polymethylmethacrylate according to standard laboratory procedures (Fig. 1)43,44. Two-millimeter slices were sectioned, and HO sections were ground, polished, and sputter-coated with carbon to increase conductivity for analysis. Three HO specimens from each patient were analyzed with scanning electron microscopy with backscattered electron imaging at 10 to 2000x magnifications (Figs. 2 through 6). Following scanning electron microscopy, the HO cross sections were ground to a thickness of approximately 75 μm, polished, and prepared for analysis of the mineral apposition rate. To calculate the bone growth rate, three bone sections that had double labeling were randomly selected, and sixty-three total data points were measured at an average of 200x magnification using a mercury lamp microscope. The width of the newly mineralized bone layer was calculated in micrometers per day (μm/day), by measuring the distance between the midline of two parallel fluorescent labels with...
the equation: mineral apposition rate (μm/day) = Σₙₑ(ε/π)(4/n)t, where Σₙₑ = sum of all of the measurements between double labels, ε = the micrometer calibration factor (mm), π/4 = obliquity correction factor, n = total number of measurements, and t = time interval (days). The numbers of double and single labels were also counted to assess the metabolic activity of the resected HO bone (Fig. 7).

Each bone slide was stained using Sanderson bone stain, and thirty images were analyzed with light microscopy to calculate the percentages of osteoblasts, osteoclasts, and resting bone by determining the proportion of quiescent and metabolically active bone (Fig. 8).

Results

Demographic Information

Forty-six service members were initially enrolled in this research study and met the inclusion criteria. However, thirteen subjects were excluded because they did not adhere to the tetracycline dosing schedule, which would have prevented assessment of the mineral apposition rate, or because an infection or comorbid injury required immediate surgical intervention. Thus, thirty-three service members who developed symptomatic HO following combat-related trauma were included in the study. Thirty-two (97%) of the subjects were male, and the average height and weight (and standard deviation) prior to injury were 69 ± 3 in (175 ± 7.6 cm) and 186 ± 23 lb (84 ± 10.4 kg), respectively.

The subjects were a mean of 27 ± 6 years of age at the time of injury, and the ectopic bone was resected at a mean of 13 ± 9 months after the traumatic insult. Of the thirty-three injuries, twenty-five (76%) were caused by an improvised explosive device (IED); five (15%) were caused by a gunshot, suicide bomber attack, or training injury; two (6%) were sustained in a motor vehicle accident; and one (3%) was caused by a rocket propelled grenade. Twenty patients (61%) had transfemoral amputation; six (18%), transtibial amputation; four (12%), upper-extremity amputation; and three (9%), hip disarticulation. Twenty-two (67%) of the subjects had a traumatic brain injury, which was mild in twenty, moderate in one, and severe in one.

The alkaline phosphatase level averaged 111 ± 44 IU/L preoperatively (recorded one to three months prior to the HO resection) and 85 ± 26 IU/L postoperatively (measured three to six months after the HO resection). As scored on the preoperative radiographs, fifteen (45%) of the HO cases were mild, ten (30%) were moderate, and eight (24%) were severe. Radiographic review more than three months after the HO resection showed no signs of recurrence in twenty-five subjects (76%), a minimal and not clinically relevant amount of ectopic bone in four (12%), and a mild amount that required further observation in four (12%).
Histological Findings

Histological data indicated that the mineral apposition rates in the HO specimens averaged 1.7 ± 0.5 μm/day (range, 1.1 to 3.7 μm/day), which was approximately 1.7 times higher than previously reported rates (1.0/μm/day) in non-pathological human bone at the time of surgical intervention41,42 (Fig. 9).

Scanning electron microscopy images showed HO in varying stages of remodeling with average percentages of osteoblasts, osteoclasts, and resting bone of 28.1% ± 14.9%, 8.3% ± 5.8%, and 63.6% ± 19.8%, respectively.

Backscattered electron imaging demonstrated that HO was a composite structure of cortical bone (Fig. 2-B) and cancellous bone (Fig. 6-B), with bone chips and newly formed woven bone (Fig. 5-C). A wide range of mineralization was noted within the HO structure, with low areas suggesting recent appositional bone formation. Eroded resorption fronts along the periphery of bone fragments complemented the ongoing remodeling (Fig. 3-A). Osteon-type structures along with woven bone formation demonstrated the complexity of remodeling and bone formation that had occurred.

Bivariate Pearson correlation coefficients (p) indicated a significant association between the mineral apposition rate and the HO anatomical location (p = 0.353, p = 0.047, n = 32), with the highest rates observed in upper extremities (2.6 ± 1.1 μm/day; range, 1.5 to 3.7 μm/day), followed by the pelvis/hip (1.8 ± 0.3 μm/day; range, 1.6 to 2.1 μm/day), femur (1.6 ± 0.2 μm/day; range, 1.1 to 2.0 μm/day), and tibia (1.4 ± 0.3 μm/day; range, 1.1 to 2.0 μm/day).

A direct relationship between histological markers and clinical predictors was established, with a significant association between the mineral apposition rate and postoperative alkaline phosphatase levels (p = 0.509, p = 0.026, n = 19) and between the preoperative alkaline phosphatase levels and the percentage of osteoblasts (p = 0.522, p = 0.004, n = 29). The number of double labels counted during the analysis of the mineral apposition rate was significantly related to the preoperative alkaline phosphatase levels (p = 0.430, p = 0.032, n = 25). Furthermore, the mineral apposition rate and the time from injury to excision were significantly and directly related (p = 0.399, p = 0.024, n = 32).

No significant relationship was found between the mineral apposition rate and severity of HO recurrence (p = −0.285, p = 0.120, n = 31). However, when the analysis was limited to patients with no more than a two-year period from injury to HO excision (thereby removing outliers who had a substantially longer time period than their counterparts) and traumatic brain injury and NSAID use (known correlates with HO development) were controlled for in the statistical analysis using an analysis of covariance, the mineral apposition rate and recurrence severity were found to be significantly related (p = −0.572, p = 0.041, n = 11).

Linear regression analysis indicated a significant relationship between mineral apposition rate and several predictors. Only an average 2.4% error between the predicted and actual mineral apposition rates was found when the following equation was used: mineral apposition rate = 2.362 + 0.007 × alkaline

Fig. 7
The mineral apposition rate analysis for the patient whose micrographs are shown in Figure 2 demonstrated that the HO bone was remodeling at a rate of 1.8 ± 0.6 μm/day. The arrow points to a double label used to calculate the mineral apposition rate.

Fig. 8
Histological analysis used to calculate the percentage of osteoblasts, osteoclasts, and resting bone. A sample was prepared with Sanderson rapid bone stain and then was captured (Fig. 8-A); pink = bone, blue = tissue/cells, and white = pore space. The image was next traced using a software program to determine active and quiescent areas of remodeling (Fig. 8-B). It was then color-coded for easy recognition (Fig. 8-C).
phosphatase level (IU/L) prior to the surgery to remove the HO – 0.008 × weight of the patient (lb) prior to injury + 0.177 × traumatic brain injury classification (0 to 3 according to the scoring system noted above) (Fig. 10).

Discussion

While the peer-reviewed literature is replete with suggestions that HO is more metabolically active than non-pathological bone\textsuperscript{28,45-47}, we are not aware of any previous study

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Mineral apposition rates (MARs) in \(\mu\text{m}/\text{day}\) calculated in this study. Note that all values exceeded the value of \(1.0 \ \mu\text{m}/\text{day}\) for non-pathological bone reported in the peer-reviewed literature\textsuperscript{42}.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig10.png}
\caption{Comparison between the actual mineral apposition rates (MARs) that were calculated and the rates that were predicted using the linear regression model developed in this study. There was an average error of 2.4\%.}
\end{figure}
directly quantifying this activity. The mineral apposition rate has been used to evaluate the growth rate of human and animal bone but has not been utilized to examine ectopic bone growth. The current study suggests that ectopic bone growth is approximately 1.7 μm/day in traumatically injured patients, a rate 1.7 times higher than the rate in non-pathological human bone. However, it is important to note that previous analysis of the mineral apposition rate in humans emphasized growth at the bone-implant interface in patients with a cementless total joint replacement; therefore, age-matched control data were not available for direct comparison with our results. Additional data analysis is necessary to confirm these results in a more controlled manner before recommending alterations to current HO surgical procedures.

Detailed backscattered electron imaging in our study showed HO as a cortical and cancellous hybrid with varying degrees of vascularity and mineralization. This finding may have an impact on clinical practice since one of the main ways of deciding when to remove ectopic bone is to wait for the formation of a well-defined neocortex, which suggests HO maturity. Our study indicates that in some cases this may lead to a protracted time period prior to excision—i.e., if only trabecular bone forms.

The most important findings from this study include the observation that there may be a direct link between clinical predictors of HO and postoperative histological analysis. When data were analyzed on an aggregate level, the mineral apposition rate was found to be directly correlated with postoperative alkaline phosphatase levels. This association is physiologically sound because osteoblasts actively deposit unmineralized bone (as indicated by the elevated mineral apposition rates) and these cells also release alkaline phosphatase, which assists in the calcification process. This tightly coupled bone remodeling process clearly occurs in HO as well as non-pathological bone, as confirmed through single and double fluorochrome labeling.

In our study, ectopic bone manifested most rapidly in the upper extremity, followed by the pelvic region, above the knee, and finally below the knee, in wounded soldiers following limb loss. As we previously noted in detail, the use of IEDs and battlefield tourniquets may in part explain the increased amount of ectopic bone noted in wounded soldiers during the recent conflicts. A blast injury drastically changes the microenvironment in the residual limb (pH, oxygenation, perfusion, etc.) and may trigger a cascade of chemotactic agents and up-regulation of vascular endothelial growth factor, transforming growth factor-beta, fibroblast growth factor, and glucose transporters. However, previous reports have indicated that increased HO volume most notably occurs in areas with increased muscle mass (and higher resident mesenchymal progenitor cell counts), whereas the present study suggests that HO progression may be linked with the anatomical location, affecting the upper extremity more rapidly than the lower limb.

The mineral apposition rate was determined to be positively and significantly correlated with the time from injury to excision. This indicates that, as the time period lengthened, the bone growth rate (mineral apposition rate) increased as well. This relationship seems counterintuitive since one would expect that a more protracted period would eventually lead to quiescent or so-called mature bone formation. This phenomenon is likely attributed to (1) patients with extensive polytrauma/comorbid injuries requiring a longer period before the HO could be excised (this included a unique subset of self-selected patients) and (2) the fact that some of these individuals had concurrent fractures or neurological insult, which may have confounded the analysis of the alkaline phosphatase levels. To test this principle, our team restricted the time from the injury to the excision to only one year (to limit the analysis to the patients with the most acute injuries); when we did so, the relationship between mineral apposition rate and surgical timing was nearly significant and inverse ($p = -0.426, p = 0.061, n = 20$). The latter association is more logical and reaffirms the need for further analysis with an increased sample size and more medical information from each patient.

When the mineral apposition rate and HO severity were analyzed on an aggregate level, no statistical relationship was found. However, when the analysis was limited to only patients with at least a two-year period between the injury and the HO excision and the data were controlled for known HO correlates (traumatic brain injury and NSAID usage), the relationship was significant but inverse. This indicates that, as the mineral apposition rate increased, the likelihood of severe HO decreased. As noted above, there are probably exogenous factors in this model that necessitate further investigation since one would expect fewer instances of recurrence with less metabolically active bone.

The mineral apposition rate equation developed in this study (mineral apposition rate = $2.362 + 0.007 \times$ alkaline phosphatase level at the time of surgery – $0.008 \times$ weight of the patient prior to injury + $0.177 \times$ traumatic brain injury classification) provides additional insight to help surgeons plan their surgical procedures. However, one must understand that, while this is a tool for decision-making, ultimately the optimal time to resect symptomatic HO must still be a clinical decision. Although the alkaline phosphatase level was shown to correlate with HO in the present study, ectopic bone growth has been previously associated with sex, genetic factors, bioelectric signals, infection, zone of injury, and age. It is important to recognize that a series of factors probably contributes to HO development and there is not one single catalyst. Furthermore, while this investigation was performed to provide additional understanding of HO, it represents only a small subset of the service members injured in Afghanistan and Iraq and does not include civilians who sustained traumatic injuries and subsequent ectopic bone growth. More clinical investigation and a carefully designed, physiologic, and translatable large animal model may be required to accurately reflect combat situations (including tourniquet and wound vacuum usage/duration, positive infection signals, controlled blasts, etc.) to further understand the etiology and pathophysiology of HO.

In conclusion, HO remains a frequent and troublesome clinical complication following both military and civilian trauma. While some patients do not develop HO following traumatic injury or present asymptotically, others may experience florid symptomatic growth within a residual limb or periarticular space. Therefore, developing a link between clinical predictors and histological findings has tremendous value and may help surgeons improve their planning of HO excision and refine patient
counseling regarding recurrence risk. Data from this study highlight some direct relationships between benchtop and bedside; however, additional factors must be further investigated to directly correlate mineral apposition rate with the development and recurrence of HO. This study demonstrated a positive and significant relationship between the mineral apposition rate and postoperative alkaline phosphatase values in soldiers who developed HO following traumatic injury.

References


