

**An experimental and computational investigation of the
mechanics of oxidatively damaged cortical bone with
applications to aging and disease states**

by

Faezeh Iranmanesh

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Examining Committee Membership

The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

Supervisor

Thomas L. Willett, Ph.D.

Associate Professor, University of Waterloo

Co-supervisor

John Montesano, Ph.D.

Associate Professor, University of Waterloo

External Examiner

Dr. Brent Edwards, Ph.D.

Associate Professor, University of Calgary

Internal Examiner

Dr. Nima Maftoon, Ph.D.

Associate Professor, University of Waterloo

Internal Examiner

Dr. Reem Roufail, Ph.D.

Continuing Lecturer, University of
Waterloo

Internal-external Examiner

Dr. Clifford Butcher, Ph.D.

Associate Professor, University of Waterloo

Author's Declaration

This thesis consists of material all of which I authored or co-authored (see Statement of Contributions included in the thesis). This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The following co-authors have contributed to the current work:

Professor Thomas. L. Willett and Professor John Montesano supervised this Ph.D. thesis. They edited the thesis. Mr. Jindra Tupy and Mr. Daniel D. Dapaah helped with the development of in-vitro oxidation model of bone. The method for chemical assay analyses including carbonyl assay and hydroxyproline assay were developed by Mr. Jindra Tupy and Mr. Daniel D. Dapaah. The human study the results of which were used for showing the physiological relevance of the carbonyls measured in this study (section 4.1.2) were done by Mr. Daniel D. Dapaah. Also the HIT-DSC tests performed on human ribated and diseased group, the result of which were used for validation of linear systems theory model in section 4.2.2.1 and 4.2.2.2 were done by Mr. Daniel D. Dapaah. The balance of the research is my own work.

Abstract

This thesis investigates the impact of oxidative damage on cortical bone quality through a series of experimental and computational analyses. The main hypothesis of this study was that oxidative damage, the result of oxidative stress, which is a significant factor in aging and various inflammatory diseases, degrades bone quality by damaging collagen, the dominant component of the bone organic phase. To investigate this hypothesis, the bone was treated with hypochlorous acid solution to induce physiological levels of oxidative damage in bovine cortical bone specimens.

A variety of techniques were employed to assess the effects of oxidation, including multiple sets of mechanical tests, carbonyl assays to quantify collagen carbonylation (the stable biomarker of oxidative damage), and thermo-analytical techniques to assess collagen nativity and connectivity. Mechanical testing revealed a significant degradation in bone yield and post-yield mechanical properties such as yield strength and ultimate strength at different strain rates with prolonged oxidation duration suggesting potential implications for bone fragility under oxidative stress conditions. Further assays and analyses focused on bone collagen quality confirmed trends in collagen damage and oxidative damage markers.

These findings were then integrated into a constitutive model to predict cortical bone behavior under oxidatively damaged conditions. Experimental validation, using single element verification and data from three-point bending fracture toughness tests, validated the model's efficacy in predicting mechanical behavior under varying oxidative damage levels. Utilizing this model, the study also explored the interplay between specimen size and the size of the microdamage process zone, a toughening mechanism observed in bone, showcasing a size-dependent growth trend in the MDPZ area with increasing specimen size. Interestingly, the findings indicated a saturation point in MDPZ growth, underscoring the complexity of bone tissue behavior in response to size and oxidative damage variations. Overall, this thesis contributes to understanding how oxidative damage impacts bone quality and offers insights into potential implications for aging and inflammatory diseases.

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My longstanding aspiration has always been to pursue higher education at a prestigious global university and contribute to a distinguished research team, addressing real-world challenges. As I reflect on this remarkable journey, it is essential to express my gratitude to those who have been by my side.

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Dedication

To My Family Members:

Jaleh & Masoud

Hojjat & Hossein

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List of Symbols

S	total cross-sectional area of the undamaged specimen
$\tilde{S}$	total cross-sectional area of the damaged specimen
D	Scalar Damage variable
σ	Cauchy stress second-order tensor
σ_{eff}	Effective stress second-order tensor
$\mathbb{E}$	elastic stiffness fourth-order tensor
ϵ	total strain tensor (second order)
ϵ^p	plastic strain tensor (second order)
κ	accumulated plastic strain
$\mathbb{C}$	Fourth-order compliance tensor
$\mathbf{M}_i$	structural tensors (second order)
$\mathbf{M}$	Fabric tensor
$\mathbf{u}_i$	principal directions of transverse isotropy or orthotropy
μ_{ij}	shear Lamé coefficient
ν_{ij}	Poisson's ratio
E_i	Young's modulus along axis i
Y	Rate-independent yield function
$\bar{Y}$	Rate-dependent yield function
ξ_{ij}	stress interaction coefficients
Y_i^+	tensile strength along axis i
Y_i^-	compressive strength along axis i

γ_{ij}	Shear strength
y_r	The equivalent saturation stress
k_s	Material curvature parameters
η	Viscosity parameter
m	Polynomial flux parameter
k_p	Parameter of the damage evolution law
$u()$	unit step function
x_{in}	system input signal
x_{out}	system output signal
$J(t)$	system's impulse response
α	represents the amount of instantaneous isometric tension developed per unit of collagen denaturation.
β	measures the connectivity of the denatured collagen network

Chapter 1: Introduction

1.1 Background

As the world's population ages, bone health becomes increasingly important to overall well-being. Current statistics [1] indicate that individuals aged 65 and above worldwide are one in every eleven people, a number projected to escalate to one in six by 2050 [2]. Bone health undergoes deterioration with advancing age, and the rise in aging-related diseases poses a threat to bone health, making it susceptible to damage and fractures, the most significant outcome of poor "bone quality", a term defined below. Fractures not only result in substantial physical and emotional costs for individuals and their families but also impose a considerable financial burden on healthcare systems [2]. In Canada alone, the estimated acute care cost for all fracture types is approximately \$1.2 billion [3].

Despite significant advancements in bone health research over the past few decades in terms of prevention, assessment, diagnosis, and treatment, there is still a lack of detailed understanding of the factors that deteriorate bone quality and the cause of bone disease and fractures [2]. Bone is a natural composite material made up of an organic phase (32-44% bone volume), an inorganic or mineral phase (33-43% bone volume), and water (15-25% bone volume) [4]. Bone quality, often assessed by bone mineral density (BMD), extends beyond BMD alone to include aspects of bone composition, structure, and properties that play roles in bone fracture resistance [5]. While low BMD correlates with increased fracture risk in the general population, its predictive value for an individual patient is limited [6]–[8]. This limitation becomes more evident in conditions like diabetes or chronic kidney disease, where patients may not exhibit low BMD but still experience higher fracture rates [9]–[11]. This highlights flaws in relying solely on BMD, as it only measures the mineral aspect of bone and neglects other non-BMD indices crucial for understanding bone fragility and explaining fractures [12].

While the mineral phase of bone, reflected in BMD, is widely understood to be a major contributor to the yield strength and stiffness of bone material [13], the organic phase of the bone plays an important role in the post-yield mechanical properties of bone [14]–[16]. In a study by Burr [17], it was suggested that collagen degradation can reduce the energy required to cause bone fracture (fracture toughness) and thus increase fracture risk. According to Zioupos et al. [15], there is supportive evidence that highlights the importance of collagen in bone toughening mechanisms. These findings suggest that the fragility of bones in aging could potentially be linked to alterations in the integrity of the collagen network. Wang et al. [14] indicated that bone collagen denaturation (melting) significantly reduces bone toughness and strength while bone stiffness is independent of collagen denaturation. Nyman et al.

[18] investigated the post-yield energy dissipation of cortical bone and found that this measure correlated with porosity, non-enzymatic crosslinking (pentosidine), and collagen content. Therefore, collagen damage may have a profound effect on bone fragility.

Alterations in bone collagen may occur as a result of translational and post-translational processes. Aging and disease are among the most important processes that can affect bone collagen by impacting both its content and structure [19]. Collagen, including both its enzymatic and non-enzymatic cross-linking, undergoes various post-translational changes with aging and disease. As previously stated, aging and certain chronic conditions such as diabetes [20][21] and chronic kidney disease (CKD) [22][23] are linked to heightened fracture risks. This susceptibility, which does not consistently align with the measured bone mineral density (BMD) reflecting only mineral status, underscores the significance of investigating the changes in the organic phase, significantly collagen, as the underlying cause of increased fragility.

In healthy bone, enzymatic collagen crosslinks enhance bone strength by increasing the stiffness and strength of collagen matrix [24]. Conversely, non-enzymatic cross-linking, including advanced glycation end-products (AGE), is widely believed to contribute to increased bone brittleness [25]–[29] although the causal association between the accumulation of AGEs and increased fragility is still inconclusive. Nonetheless, the effect of non-enzymatic collagen alterations on bone mechanical properties has been observed to be a significant feature in aging and chronic diseases. Therefore, a connection is anticipated between the decline in bone mechanical properties in aging and disease states and the changes in the organic phase, particularly collagen.

As indicated by prior research [30][15][31], a primary factor contributing to the accumulation of unfavorable AGEs in bone collagen is oxidative reactions. These reactions, generally called glycoxidation, not only lead to the formation of AGE cross-links in collagen but also diminish the beneficial enzymatic crosslinks [32]. Additionally, oxidation has the potential to damage collagen through chain fragmentation [33]. Oxidative stress plays a substantial role in triggering a variety of chronic diseases and age-related disorders [10], [34]–[36]. Collagen is a susceptible target for reactive oxygen species (ROS), potentially undergoing degradation through the collaborative actions of ROS and proteinases in the initial phases of inflammation [37]. Oxidative stress can alter the structure and function of long-lived proteins, such as collagen in bone, likely serving as the underlying cause of many pathologies that manifest over time. In the aging process and certain chronic diseases like diabetes,

non-enzymatic collagen crosslinking induced by glycation and/or oxidation can influence bone strength [24] and contribute to bone fragility.

Oxidative stress is a phenomenon that occurs when there is an elevated level of reactive oxygen species (ROS), leading to a disruption in the balance between the production of ROS or oxidants and cells' capacity to create a robust antioxidant response [38]. ROS can perform various physiological functions, such as cell signaling, and are typically produced as by-products of oxygen metabolism. However, environmental stressors (such as UV radiation, ionizing radiation, pollutants, and heavy metals) and xenobiotics (including anticancer drugs) significantly amplify ROS production [39]. While the human body possesses antioxidant systems, if the ROS level surpasses a certain threshold (dependent on the body's capacity for their removal), oxidative stress (OS) ensues. OS has the potential to induce oxidative damage to various components in the body, leading to chronic inflammation [40] and compromises cellular health [41]. Oxidative stress-triggered inflammation is the root cause of numerous chronic diseases. [40]. ROS have been widely implicated in the aging process and associated complications [42]. Notably, as age advances, various chronic diseases may emerge due to oxidative stress and chronic inflammation [43].

Increasing evidence indicates that the development of oxidative stress has a notable impact on bone biology, particularly bone collagen. Despite the demonstrated damage caused by oxidative stress to bone collagens [44][45], surprisingly little research has been conducted on the effects of oxidative stress on the deterioration of bone quality.

This study aims to investigate the impact of oxidative damage on cortical bone mechanical performance. Specifically, it focuses on situations such as aging and bone diseases where oxidative stress plays a significant role. The study utilizes diverse techniques, including mechanical testing, collagen assessment tools, and computational modeling to understand how oxidative damage affects the organic phase of the bone and its implications for the degradation of bone mechanical properties. In doing so, it seeks to uncover the mechanistic causes of bone fragility, an area that has been relatively underexplored in the existing literature.

1.2 Thesis Organization

This thesis is structured in a manuscript-based synoptic format. The overview of this dissertation includes a literature review (Chapter 2), an outline of research objectives and scope of the work (Chapter 3), a synopsis of the methods and results (Chapter 4), in depth discussions, conclusions, a

summary of contributions, and recommendations for future work (Chapter 5). The remainder of the thesis comprises four appendices, each containing either published or submitted peer-reviewed papers that elaborate on the conducted work and present the results of this research.

Chapter 2: Literature review

2.1 Bone structure and composition

2.1.1 Bone Structure

Bone is a nanocomposite material with a complex hierarchical structure which gives the body its support and stability [46]. Nature has intricately designed its mechanical properties to fulfill this specific physiological role. In a healthy state, bone exhibits exceptional mechanical characteristics, being stiff, strong, tough, and lightweight. These remarkable properties stem from the intricate hierarchical structure of bone and the complex arrangement of its components [4]. The structural scales of bone can be identified at different levels: (1) the macroscale involving cancellous and cortical bone; (2) the meso-scale (ranging from 10 to 500 μm) encompassing Haversian systems, osteons, and single trabeculae; (3) the microscale (1–10 μm) consisting of lamellae; (4) the sub-microscale (from a few hundred nanometers to 1 μm) comprising fibrillar collagen and embedded mineral; and (5) the nanoscale (below a few hundred nanometers) involving the molecular arrangement of constituent elements like mineral, collagen, and non-collagenous organic proteins [47] (Figure 1).

In cortical bone, the primary structural unit is the osteon or Haversian system, while trabecular bone consists of a porous network of trabeculae [48]. The basic microstructure units of cortical bone and trabecular bone are the single osteon or trabecula, respectively [49]. A typical osteon is a cylinder approximately 200 or 250 μm in diameter, with the wall composed of 20 to 30 concentric lamellae, each about 70 to 100 μm thick [48]. Moving to the sub-microscale, a single lamella (Figure 1) is comprised of mineralized collagen fibrils (MCFs) reinforced with hydroxyapatite (HA) crystals. Finally, at the nanoscale, the mineralized collagen fibril is a composite structure consisting of the organic matrix, inorganic phase, and water [4]. This hierarchically organized structure exhibits an irregular yet optimized arrangement and orientation of components, rendering bone material heterogeneous and anisotropic [50].

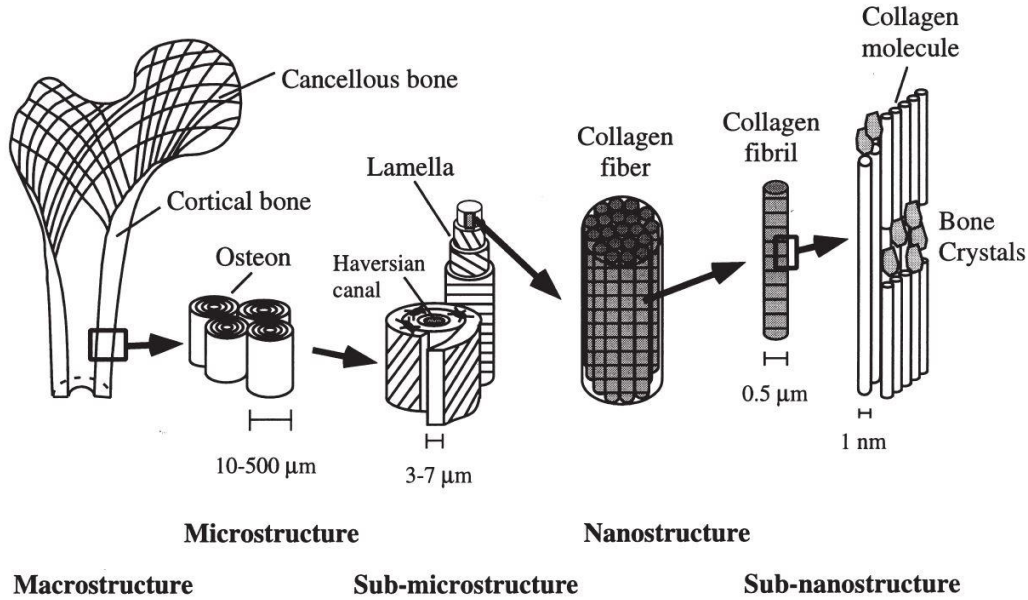


Figure 1. The hierarchical structure of bone [47]

2.1.2 Bone Composition

As stated earlier Bone is composed of inorganic phase, mineral phase and water [51][52]. The inorganic or mineral phase in bone consists of a poorly crystalline carbonated apatite, initially deposited as an amorphous calcium phosphate [53][54]. Minerals play a crucial role in dissipating fracture energy by inducing deformation in the organic matrix while maintaining the integrity of the composite structure [55][56].

The main element of the organic phase is collagen, predominantly collagen type I, which accounts for 90% of the total protein in bone [57], but other proteins, the so-called NCPs, accounted for ~5% of the total bone weight [58].

Collagen type I is a distinctive triple-helical molecule composed of two identical amino-acid chains (α_1 chain) and one distinct chain (α_2 chain) [58]. Each chain features a repetitive amino acid sequence predominantly consisting of proline and hydroxyproline amino acids, along with a consistent pattern of glycine residues (Figure 2). This sequential arrangement allows the intertwining of three polypeptide chains to form a triple-helix (Figure 2). Tropocollagen, the individual collagen molecules, has a length of approximately 300 nm and a diameter of 1.25 nm [59]. As illustrated in Figure 3, adjacent tropocollagens within a collagen fibril are organized in a staggered pattern [60].

Collagen fibrils are created by the assembly of collagen molecules through the formation of crosslinks. In mineralized tissues like bone, crosslinking stands out as a critical structural component in type I collagen, playing a pivotal role in establishing various mechanical properties, including tensile strength, fracture strength, and viscoelasticity [61]–[63]. Two types of crosslinks exist: enzymatic and non-enzymatic cross-links.

Enzymatic crosslinks, positioned at the ends of collagen molecules [53], establish connections with the adjacent molecules, providing the fibril with specific structural stability. Consequently, an increase in enzymatic crosslinking contributes to enhanced strength, stiffness and toughness in the bone. These crosslinks undergo a developmental process, starting as immature (referred to as divalent crosslinks) during early stages. Over time, a gradual enzymatic maturation process transforms them into fully mature, trivalent cross-links. The two most prevalent mature or trivalent cross-links are pyridinoline and deoxypyridinoline [64].

In contrast to the orderly formation of enzymatic crosslinks, non-enzymatic crosslinks result spontaneously from glycation and/or oxidative processes. Non-enzymatic glycation leads to the creation of advanced glycation end-products (AGEs). Pentosidine and vesperlysine are among the most common AGEs cross-links in bone. Elevated levels of non-enzymatic crosslinks is closely associated with a significant decline in the mechanical properties of bone, diminishing its strength and toughness [30]. Furthermore, they contribute to the stiffening of the tissue by constraining the deformation (plasticity) of collagen fibrils, albeit at the cost of increased brittleness [65][66]. Glycation marks the initial phase of an intricate sequence of reactions involving sugars and proteins. This process leads to the formation of advanced glycation end products (AGEs), which consist of adducts and cross-links that amass on proteins in tissues as we grow older and in certain health conditions like diabetes[67]. Over time, AGEs progressively accumulate in durable extracellular proteins like collagen. The accelerated formation of AGEs in tissue proteins due to factors such as high blood sugar levels, oxidative stress, or inflammation is associated with the development of aging, diabetes, and other chronic illnesses [67]. The accumulation of AGEs, including cross-links and adducts, in the collagenous tissues of individuals with diabetes and other bone-affecting chronic diseases, is a potential contributor to the observed skeletal fragility in this condition. Thus, in contrast to enzymatic crosslinks, AGEs accumulate in bone as a natural aspect of the aging process [30][68][69] [70].

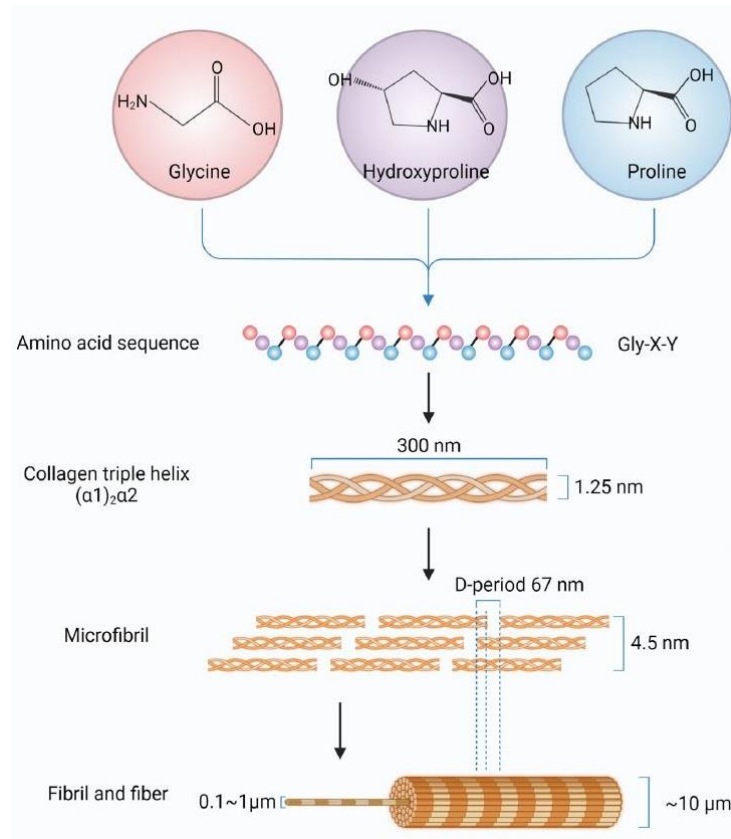


Figure 2. Hierarchical structure of type I collagen fiber [71]

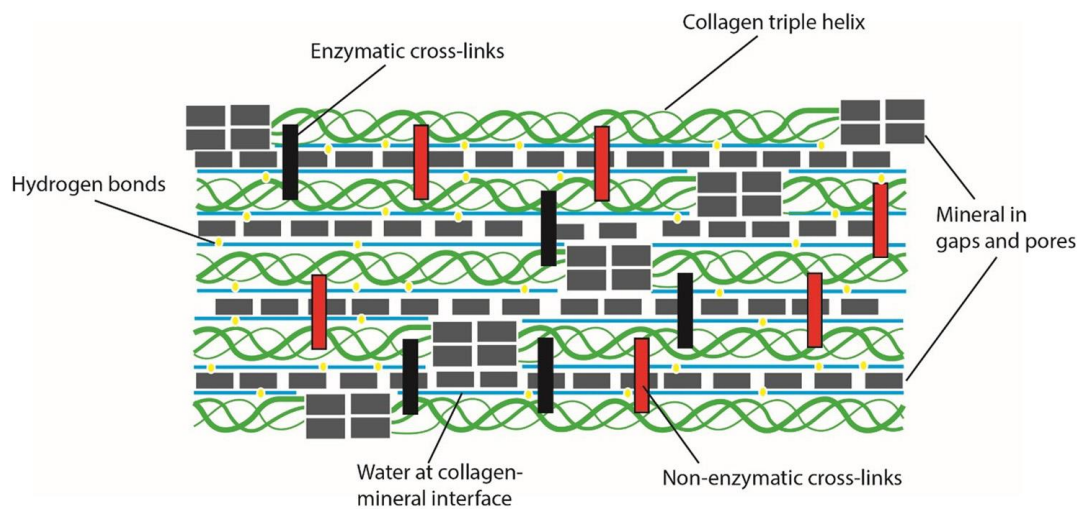


Figure 3. Hierarchical structure of mineralized collagen fibril [53]

Some studies explored the composition of bone by selectively removing either the organic or mineral phase. Their investigation revealed that bone samples maintain their structural integrity as independent structures in both scenarios. However, mechanical tests conducted on bone samples in these conditions demonstrated that the mechanical characteristics of demineralized and deproteinized bone are notably inferior to the original bone [72]–[74]. These outcomes underscore the idea that continuous phases in bone result from both collagen and minerals, suggesting that bone functions as an interpenetrating composite material with enhanced properties arising from the interplay between its two phases: proteins and minerals [4].

2.1.3 Bovine bone composition and structure

The hierarchical structure and composition of bovine cortical bone align closely with the information provided in sections 2.1.1 and 2.1.2, which remains relevant to cortical bone generally [54]. Nonetheless, distinctions exist between human and bovine cortical bone concerning the relative percentages of each phase, density, and cortical thickness [75]. Aerssens et al.'s research [76] indicates a slight 3% increase in ash content, reflecting mineral levels in cow bone compared to human bone. Conversely, collagen content shows an inverse correlation with mineralization, differing by less than 2%, while both bone types share similar amounts of extractable proteins. Notably, the tibial shaft of a cow is notably sturdier than that of a human, as outlined in the study [77] (refer to Figure 4).

In terms of microstructural organization, human bone is classified as Osteonal bone, which consists of osteons. Osteonal bone is highly dynamic and continuously remodels, enabling the repair of microdamage and regulation of calcium homeostasis [54]. In contrast, bovine bone is primarily categorized as plexiform bone, characterized by layers of lamellar bone arranged in a brick-like pattern with large, interconnected vascular channels. This type of bone tissue grows more rapidly and is less organized than Osteonal bone. Additionally, plexiform bone is less dynamic and undergoes minimal remodeling compared to Osteonal bone [78].

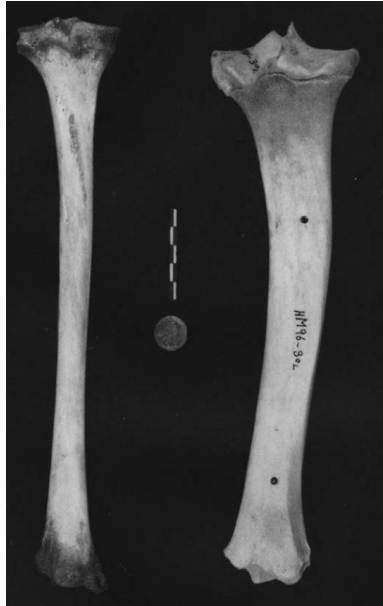


Figure 4. Comparison of the posterior view of a human tibia (left) and a bovine tibia (right) [77].

2.2 The Contribution of Mineral and Organic Phases to Bone Mechanical Behavior

It is widely known that the mineral content provides strength and stiffness to the healthy bones [13]. Minerals play a significant role in the dissipation of fracture energy by driving the deformation of the organic matrix while preserving the integrity of the composite structure [55][56]; Buehler [79] employed molecular dynamics simulations to observe that the minerals present in the gap region of bone's nanostructure result in localized plastic deformation near microcracks. This phenomenon contributes to energy dissipation and helps maintain the integrity of the nanostructure. The mineral composition in bones contributes to resisting mechanical forces. Studies have demonstrated that a reduction in bone mineral content is a significant factor leading to an elevated risk of osteoporotic fractures as individuals age [80].

In contrary to the well-studied impact of the mineral phase on bone mechanical properties, the effects of the organic phase, including collagen and non-collagenous protein, are less understood. Nevertheless, there is a mounting body of evidence suggesting a crucial role for collagen in the mechanics of bone material. A noteworthy effort to comprehend the role of collagen independently of mineral was undertaken in a study by Wang *et al.* [14], where the direct influence of collagen on the mechanical behavior of bone was explored through the heating and denaturation of bone collagen. The

study's results indicate a significant decrease in bone toughness and overall strength with increasing thermal denaturation of collagen, while its elastic modulus is almost constant regardless of collagen denaturation. Similar investigations into the role of collagen in bone mechanical properties have been carried out by Zioupos et al. [15] and Willett et al. [16]. According to the findings in [16], the integrity of the bone collagen network emerges as a substantial contributor to the fracture toughness of cortical bone. Zioupos' research also lends support to the hypothesis regarding collagen's involvement in the toughening mechanism of bone, suggesting a potential correlation between age-related bone fragility and changes in bone collagen structure [15]. The proportion of denatured collagen relative to total collagen content was shown to be substantially correlated with both the tissue's fracture toughness and failure energy in a baboon-model investigations [81][82]. This implies that collagen serves as a key inhibitor of cracks in bone by stabilizing them and preventing unstable growth [16]. Additionally, collagen gives bone "ductility," or the ability to withstand strain beyond the yield point [83], [84].

Bone mechanical properties are influenced not only by collagen content but also by the amount of both enzymatic and non-enzymatic crosslinking. In general, enzymatic crosslinking is known to be a natural process for healthy collagen and has a beneficial effect on its mechanical properties, whereas non-enzymatic cross-linking results in a brittle collagen network that deteriorates bone mechanical properties when its accumulation exceeds normal repair [25]. Interfibrillar (pyrroles) cross-linking is also suggested to play a role in cortical bone bending and tensile strength [85][86]. On the other hand, non-enzymatic cross-linking including AGEs diminish the ductility of collagen fibers by impeding their deformation resulting in a decrease in bone strength [15] and toughness [30][16] due to tissue embrittlement [53]. Another well-established effect of AGE build-up is the age-related decline of post-yield energy absorption ability of bone [87]–[89] making the bone more fracture-prone by inhibiting stress relaxation and facilitating crack formation [90]. Investigating the functional role of AGEs in pathophysiology poses challenges due to the diverse pathways and numerous intermediates involved in AGE formation [91][92]. Over time, various AGEs in the form of cross-links or adducts have been identified [93]. Notably, AGE cross-links have garnered attention in bone research for their impact on making collagen fibrils more brittle through collagen over-stabilization, consequently compromising the bone's natural toughness[94][95]. Recent studies suggest that adducts, from measurable AGEs, are significantly more abundant than cross-links by one or two orders of magnitude [32].

In addition to enzymatic and non-enzymatic changes in collagen, non-collagenous proteins (e.g., osteopontin, osteocalcin) make up 10% of the bone's organic matrix, may also affect bone mechanical

properties by contributing to the stabilization of micro-damage. When force is applied, these components stretch, dissipate energy and prevent harmful crack formation and propagation by breaking sacrificial bonds between neighboring collagen fibrils [96].

While the majority of existing clinical tools evaluate bone material through the identification of decreased bone mineral density, significant alterations in the matrix, especially within the organic phase of bone, contribute to a decline in bone quality. [97].

2.3 Age and disease-related alterations in the mechanical properties of cortical bone

Age-related degradation in the mechanical properties of cortical bone have been shown in a number of studies [30], [98]–[102]. Typically, these changes are linked to specific milestones or decades throughout the lifespan. Based on literature, human bone reaches maturity by the age of thirty to thirty-five years, after which it begins to deteriorate [88], [99], [101], [103], [104]. After 35 years, the elastic modulus declines by 2.3% per decade [99], and starting in the third decade, the flexural strength of cortical bone declines by around 3.7% [99]. Ultimate tensile strain drops by around 10% per decade [100] and fracture toughness declines by about 4% [105] per decade. Beginning in the third decade, the bone strength declines by around 2-5% every decade [106]. The nonlinear energy associated with the onset of fracture or J -integral decreases by 3%, and the work to fracture declines by around 8.7% per decade [99].

Age-dependent alterations in the mechanical properties of cortical bone are attributed to a number of factors, including increased porosity [101], accumulation of microdamage [107], hypermineralization [87], elevated levels of advanced glycation end products (AGEs) [30], and reduced amounts of non-collagenous proteins [108].

Several health conditions, can also impact the mechanical properties of bones, consequently elevating the risk of fractures for patients [29], [109]–[111]. Based on clinical observations, it is widely acknowledged that diseases impacting the material properties of bone tissue via altering its mineral

phase—such as osteomalacia¹ or osteopetrosis²—escalate the risk of fractures. However, it is also evident that diseases that primarily affect the collagen phase, without any underlying changes to tissue mineralization, can similarly heighten the risk of fractures. For instance, in a mouse model of osteogenesis imperfecta (OI), collagen defects resulted in a 60% reduction [112][113] in post-yield deformation of bone, subsequently diminishing the fracture toughness [114].

In type 2 diabetes (T2D), patients may exhibit normal or even higher clinical bone mineral density (BMD), while susceptibility to fractures among T2D patients is higher than in the general population [29]. This suggests that, instead of affecting bone mineral density, diabetes is conjectured to diminish bone quality, reflecting the aspects that contribute to the fracture resistance of bones, apart from bone mineral density [29]. The factors behind the heightened vulnerability to bone fragility in type 2 diabetes (T2D) are complex and not fully understood. However, several studies have underscored the importance of advanced glycation end products (AGEs) and oxidative stress as key contributors to skeletal fragility in T2D, both of which occurs in the collagen phase [9]–[11], [24], [115].

Clinical evidence also suggests that fracture risk is elevated in patients with chronic kidney disease (CKD) [110], [116], [117]. In a rat model of CKD, the mechanical properties of bone at all tissue levels were deteriorated, however, as with diabetes, there are no noticeable differences in mineral content between normal and CKD-affected bone [118]. Research on animal models of progressive CKD [22][23] indicates that although various factors contribute to changes in bone mechanical properties in CKD, modifications in collagen cross-linking are notable in late-stage CKD. These modifications encompass both reduced levels of enzymatic cross-links and the accumulation of non-enzymatic advanced glycation end-product crosslinks [22][23].

In addition to OI, T2D and CKD, degraded and/or denatured collagen is prevalent in various other conditions such as bone tumors [119], , and even osteoporosis [120] leading to the degradation of bone mechanical properties in these pathologies.

Age- and disease-related alterations in bone are significantly influenced by changes in the amount and structure of collagen [19]. With aging and disease, collagen, including both its enzymatic and non-

¹ Osteomalacia, is a metabolic bone disorder characterized by the inadequate mineralization of bone tissue .

² Osteoporosis is a bone disease that develops when bone mineral density and bone mass decreases.

enzymatic cross-linking, experiences a variety of post-translational changes. It has been observed that the density of collagen crosslinks varies with age and certain diseases (such as diabetes), which has an impact on the tissue's mechanical properties [121]. In healthy bone, enzymatic collagen crosslinks enhance bone strength by increasing the stiffness of collagen fibrils. However, with aging, and some chronic diseases, non-enzymatic collagen crosslinking caused by glycation and/or oxidation affect bone strength [24]. Studies on genetically muted collagen demonstrated the important role of collagen (the main part of the organic phase of the bone) in bone's ability to sustain post-yield deformation and absorb energy before fracture [112]. Also, some studies have shown that age-related reduction in the fracture toughness of bone correlates significantly with changes in the bone collagen network integrity and connectivity and supports the idea that collagen has a toughening role in bone mechanics [30][15].

One important phenomenon which is common in collagen-affecting disorders and aging process, requiring further investigations, is oxidative stress. As previously discussed, numerous alterations in the mechanical properties of cortical bone associated with aging and disease are linked to increased levels of AGEs. Accumulate of certain AGEs in bone collagen with aging and disease, suggests a potential connection between AGE accumulation and the aging/disease processes. However, as indicated by prior research [30][15], the high levels of AGE crosslinks and adducts in bones affected by age and certain diseases (e.g., osteoporosis, diabetes, and renal osteodystrophy) likely signify chronic, elevated oxidative stress rather than being the direct cause of compromised bone quality and fracture resistance [34]–[36], [44], [122], [123].

Oxidative stress plays a role in various age-related conditions and chronic diseases [10], [34]–[36], serving as a probable contributor to complications in longer-lived proteins, such as collagen in bone, and can consequently alter their structure and function. Despite oxidative stress and the resulting damage being demonstrated to influence bone cell function and harm different collagens [45], there has been limited research on the subsequent degradation of bone mechanical properties.

2.4 Definition of oxidative stress

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) in the body and the biological system's capacity to remove these species through an effective antioxidant response (redox). The body's antioxidant system is made up of components including superoxide dismutase (SOD), glutathione peroxidase, reduced glutathione (GSH), and vitamins E and C [36]. Radical and non-radical oxygen species produced by the partial reduction of oxygen are known

as reactive oxygen species (ROS) and include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\bullet$) [38]. Free radicals typically exhibit instability and high reactivity due to the tendency of unpaired electrons to form pairs with other electrons [124]. While ROS are typically generated as by-products of oxygen metabolism and serve various physiological roles, such as cell signaling, they can have detrimental effects on tissues when their concentration increases beyond a threshold [125]. Environmental stressors like UV rays, ionizing radiation, pollutants, and heavy metals, along with xenobiotics such as antiproliferative drugs, significantly elevate ROS production. This heightened production disrupts the balance, resulting in oxidative stress and consequent damage to cells and tissues [39].

An overabundance of free radicals generated during conditions of oxidative stress results in damage to proteins, lipids, and nucleic acids, posing a serious threat to cell health and contributing to the development of diseases [41]. The involvement of reactive oxygen species (ROS) in aging and related complications is widely acknowledged [42]. Oxidative stress is widely recognized for its involvement in the pathogenesis that commonly arises with aging or lifestyle-related diseases, including cancers, neurodegenerative disorders, arthritis, atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, malignancies [124].

There is growing evidence that reactive oxygen species have a specific impact on bone biology as well which will be investigated in further details in the next section.

2.4.1 Effects of oxidative stress on bone biology

Osteoporosis (OP) and Type 2 diabetes (T2D) are chronic diseases of bone metabolism and glucose respectively both of which negatively affect bone and increase fracture risk [126][127].

The chronic bone disease known as osteoporosis, which is defined by the loss of bone mass, degeneration of bone tissue, and an increased risk of fracture, is brought on by an imbalance in the activities of osteoblasts, cells that are responsible for bone formation, and osteoclasts, cells in charge of bone resorption [128]. Some investigators have suggested that osteoporosis is associated with biochemical markers of oxidative stress. Oxidative stress has an important role to play in increased osteoclastic (bone resorption) and decreased osteoblastic (bone formation) activities leading to a reduction on skeletal mass [129].

Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are the primary forms of reactive oxygen species (ROS) associated with osteoclastogenesis³[130]. The superoxide anion has been shown to accelerate bone resorption, as reported by Garrett et al. [131], who were among the first to link ROS and osteoclast activity. ROS are concentrated at the interface between bone and osteoclasts, leading to the production of superoxide within the osteoclast [132]. Additionally, some other studies have revealed that hydrogen peroxide is the primary ROS responsible for promoting the formation and activity of osteoclasts [133]. While early studies suggested a direct role for ROS in bone resorption, later research has proposed that ROS may indirectly promote osteoclast formation and activity through activation of signaling pathways [134]. Furthermore, ROS not only enhance osteoclast activity, but also inhibit the cell differentiation of osteoblasts⁴ [135].

In the context of T2D, individuals with this condition are more prone to fractures compared to those without diabetes. Interestingly, their bone mineral density (BMD) values usually fall within the normal or even higher range. However, the primary reasons behind the fragility of their bones are often linked to the accumulation of advanced glycation end products (AGEs) and oxidative stress [24]. These conditions, either directly or indirectly (by fostering microvascular complications, for example [24]), detrimentally impact the structural ductility of bone and have adverse effects on bone turnover. Consequently, this results in compromised bone quality rather than a reduction in BMD [24]. AGEs, elaborated upon extensively, represent a type of non-enzymatic cross-link that can develop because of various processes such as a high-fat diet, oxidative stress, and prolonged periods of hyperglycemia [136][137]. Both in-vivo and in-vitro experimental investigations (utilizing diverse mouse models of diabetes and human studies) affirm that AGEs accumulate within the bone. This accumulation results in modifications to bone turnover, the composition of the bone matrix, and, ultimately, compromises bone strength [138][33]. However, some critics argue that the observed association between AGEs accumulation and decreased bone strength is simply correlative and not necessarily causative. [33]. Therefore, in order to definitively determine the effect of AGEs on the bone fragility of T2D and non-T2D people, further prospective investigations in sizable T2D samples are necessary [24].

³ The process by which osteoclasts, cells responsible for bone resorption, are formed and matured within the body.

⁴ A type of bone cell responsible for the formation of new bone tissue during bone growth and repair.

Oxidative stress emerges as another significant contributor to bone fragility in individuals with type 2 diabetes (T2D). While it does promote the formation of advanced glycation end products (AGEs), oxidative stress can arise in T2D through several different pathways. These include the polyol pathway, activation of membrane NADPH-oxidase through protein kinase C, and dysfunction in the mitochondrial electron transport chain[139]–[142]. Actually, the oxidative mechanisms that lead to the generation of free radicals and other ROS are worsened by hyperglycemia/hyperinsulinemia and poor fatty acid metabolism in diabetes [141][142]. This is especially common in the mitochondria of many cell systems, and it may cause a variety of systemic problems, such as brittle bones and an increased fracture risk necessitating further investigation [143].

As previously mentioned, patients with chronic kidney disease (CKD) have a greater frequency and risk of fractures compared to healthy individuals. Particularly, dialysis patients are around four times more likely to have a hip fracture than those of the same sex and age in the rest of the population [144][145]. Healthcare professionals and researchers concur that fracture risk factors in CKD are intricate due to the numerous abnormalities present in CKD patients, which may heighten fracture risk [146]. However, akin to diabetes, the predictive value of clinical BMD in an individual CKD patient is notably limited, underscoring the importance of considering the decline in bone quality as a potential contributor to the higher fracture risk in CKD patients [147]. Bone fragility in CKD is attributed to a number of uremia-related conditions, such as severe secondary hyperparathyroidism, skeletal resistance to parathyroid hormone, and problems with bone mineralization [146]. Uremia exacerbates the loss of bone volume, disrupts microarchitecture, and intensifies the deterioration of material properties in bone, often involving abnormal bone cells or an excess of oxidative stress [146]. It is well recognized that uremic conditions produce an environment with excessive oxidative stress leading to a disruption in osteoblastic/osteoclastic activities [148].

As stated earlier in section 1.1, collagen, which is the primary structural protein found in bone tissue and has a long lifespan, can be targeted by reactive oxygen species (ROS) and broken down by the combined actions of ROS and proteinases during the early stages of inflammation [37]. Various types of ROS can impact collagen. For example, the superoxide anion can cause fragmentation within the helical segment of the collagen molecule. Additionally, in environments with high levels of oxygen, hydroxyl radicals can break down collagen into smaller peptides, with a specific focus on proline or 4-hydroxyproline residues [37]. Furthermore, collagen experiences major structural alterations when it comes into contact with hypochlorous acid (HClO), a potent oxidizing agent generated at inflammatory

sites by neutrophils, monocytes, and osteoclasts [37]. Research in [37] reveals a discernible drop in the amount of α -1 and α -2 chains in collagen that has been exposed to hypochlorous acid. Additionally, it appears to promote the creation of new cross-links, albeit it is yet unclear what exactly these cross-links include [37]. Consequently, collagen may be impacted by reactive oxygen species in a number of ways, including fragmentation or cross-linking. These distinct outcomes may indicate different underlying pathological conditions, with collagen fragmentation being associated with inflammatory degradation and collagen cross-linking potentially related to the aging process and its associated complications [37].

Despite extensive research on oxidative stress and its effects on various tissues, little is known about how oxidative stress specifically affects bone collagen and consequently bone quality. As observed, the available literature primarily focuses on the effects of oxidative stress in relation to bone cell function, such as osteoblast and osteoclast activity, but the direct impact on the structural integrity of the bone matrix remains poorly understood. This lack of knowledge represents a significant gap in understanding how oxidative mechanisms affect bone quality, particularly in relation to its organic phase, predominantly collagen.

2.4.2 Biomarkers of oxidative stress

Biomarkers of oxidative stress can be grouped into molecules that undergo modifications due to interactions with reactive oxygen species (ROS) in the microenvironment, and components of the antioxidant system that adjust in response to increased redox stress [149]. Examples of these susceptible molecules, affected by excessive ROS in vivo, include lipid, protein, and DNA molecules [149]. As previously outlined, oxidative stress is considered a prominent contributor to aging and age-related diseases. Therefore, biomarkers of oxidative stress serve as crucial instruments for evaluating the state and advancement of diseases [150].

Under normal or oxidative stress conditions, proteins (such as collagen in bone) represent a broad target for reactive oxygen species (ROS), scavenging most (50%–70%) of the produced ROS [151], due to their rapid reaction rates with ROS and their abundance in cells, extracellular tissues, and body fluids. When amino acid residues react with ROS, oxidative modification to proteins happen, leading to the generation of protein oxidation products [150][152]. More reactive species cause damage across various sites in both side-chains and backbones of proteins, whereas less reactive species exhibit greater selectivity towards targeted residues [153]. The array of reaction sites results in a diverse spectrum of

posttranslational protein modifications, consequently altering composition, folding, net charge, and the hydrophobicity/hydrophilicity of proteins [154].

Protein carbonylation, the creation of protein nitrotyrosin, s-nitrosylation, glycooxidation, s-glutathionylation, oxidation of sulfur-containing residues, and the hydroxylation of aromatic and aliphatic groups are the primary changes that happen to proteins as a result of oxidation [155][153]. Furthermore, oxidation can lead to the scission of polypeptide chains and the creation of protein-based crosslinks. These modifications may be permanent or reversible [152], [153], [155]. While permanent alterations may be a factor in pathological conditions and numerous diseases, reversible modifications are mostly important in physiological processes and comprise signaling systems, or "redox signaling" [156]. Permanent changes of proteins include the formation of carbonyls and protein nitrotyrosine, breakage of the histidine and tryptophan rings, and hydrolysis of the peptide bonds in the presence of proline [150].

Chemically stable protein oxidation products that develop in significant quantities, and correlate with disease severity show promise as potential biomarkers of oxidative damage [156]. Protein carbonylation is an irreversible protein modification that occurs through three mechanisms, including direct oxidation of protein-bound amino acids, oxidative scission of the protein backbone, which primarily affects glutamyl side chains, and incorporation of carbonyls from glycooxidation or lipoxidation [157]. This alteration can also occur on different amino acid residues, such as proline, lysine, histidine, threonine, and arginine [155], [158]–[161]. Carbonylated proteins are commonly used as biomarkers for detecting oxidative protein degradation caused by aging and disease. Notably, carbonylated proteins have significant benefits over other indicators of protein oxidation due to their early production and stability [162][163]. Additionally, this change is simpler to detect since it impacts several amino acids [164]–[166], exhibiting a substantially higher magnitude than modifications that are limited to single amino acid residues [159][160].

The measurement of protein carbonylation is commonly done through the spectrophotometric method [167][168], utilizing the chromogenic reaction between carbonyl groups and 2,4 dinitrophenylhydrazine (DNPH). However, a debate has arisen regarding the sensitivity and reproducibility of the DNPH-based assay for carbonyl groups. Some scholars [169][170] have challenged the validity of the DNPH-based spectrophotometric assay due to the lack of observable age-related changes in protein carbonyl levels. Subsequently, methods involving Western blots have been

devised to detect specific proteins displaying elevated carbonylation. These techniques involve derivatizing carbonyl groups with either DNPH [171]–[175] or dioxigenin-hydrazide [176], and then using antibodies against DNPH or digoxigenin-hydrazide to identify the derivatives. Another approach involves derivatizing carbonyl groups with avidin-biotin staining [177] or biotin-hydrazide, with the identified derivatized protein detected using FITC-labeled avidin [178].

More recently, fluorescence-based assays, initially suggested by [179] and [180], have gained traction for directly quantifying protein carbonylation by derivatizing carbonyl groups with fluorescein-5-thiosemicarbazide (FTC). This method has the benefit of removing the requirement for antibodies or other conjugation-based techniques.

While protein carbonyl detection methods have been extensively used to assess protein carbonylation in various materials like cells, tissues, or bodily fluids [178], [181]–[183], to our knowledge, protein carbonylation has never been quantified in bone collagen, particularly using fluorescence-based assays. Furthermore, the utilization of this biomarker as a biomarker of inflammatory disease development represents a novel approach not documented in prior literature.

In order to obtain a comprehensive evaluation of collagen molecules and determine whether they had undergone any alterations due to oxidative damage, thermo-analytical techniques, including differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) testing, are required to evaluate the effect of oxidative damage on bone collagen quality in terms of collagen network integrity and connectivity. These techniques will be reviewed in the next section.

2.5 Review of thermo-analytical techniques for evaluating collagen quality

Differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) testing are two thermo-analytical techniques usually employed to evaluate quality of collagen in bone tissue in terms of collagen network stability, nativity, and connectivity.

DSC is a powerful method for the measuring the temperature and heat flow associated with phase transition of materials. In DSC, a sealed sample and an empty reference sample are subjected to a consistent heating rate (in a constant heating rate test). The procedure records differences in heat quantities or heat flow, charted over time or temperature (Figure 5-A) [184]. When the sample undergoes melting or denaturation, which occurs when testing collagen or other proteins, the sealed pan absorbs more heat compared to the reference pan. Consequently, an endothermic peak emerges in

the temperature-dependent heat flow graph (Figure 5-B), offering insight into the sample's thermal characteristics.

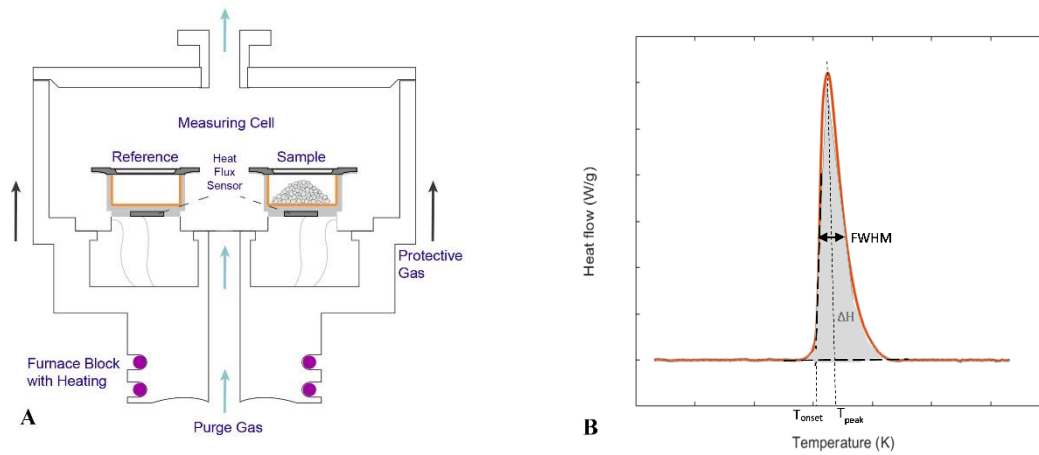


Figure 5. A) Schematic of DSC apparatus. B) a typical flow vs. temperature curve obtained by DSC test [185]

Typically, scientists employ DSC to obtain significant information into the thermal denaturation (melting) process, especially concerning large molecules such as collagen, when subjected to heat. The denaturation endotherm of melted collagen typically yields the following information: the onset of denaturation temperature in the DSC endotherm (T_{onset}), the temperature at maximum recorded heat flow (T_{peak}), the enthalpy of denaturation (ΔH), and the full width at half maximum (FWHM) of the denaturation endotherm.

The Hydrothermal Isometric Tension (HIT) testing method, pioneered by Allain, Lelous, and their collaborators [186][187], offers another approach to assess the quality of collagenous tissues across various types, including skin [186], tendon [188], heart valve [189], pericardium, and bone collagen [16]. One intriguing aspect of collagen exploited in HIT testing is its response to temperature changes, wherein it contracts as temperature gradually rises. This phenomenon occurs at a specific temperature known as the denaturation temperature (T_d), marking a structural transition from collagen triple helices to random amorphous coils [15]. Consequently, the collagen sample shrinks, however due to its constant length constraint it generates isometric force instead (refer to Figure 6). This denaturation temperature serves as an indicator of the collagen's thermal stability [190][191]. In addition to thermal stability, HIT testing offers insights into the collagen network's integrity, including intermolecular network connectivity, and crosslinking [192][16]. The maximum rate of isometric tension generation

[16] (Max. Slope) and the half-life of load decay under isothermal conditions [193] (typically at 90°C) are two reported measures for collagen network's integrity and connectivity.

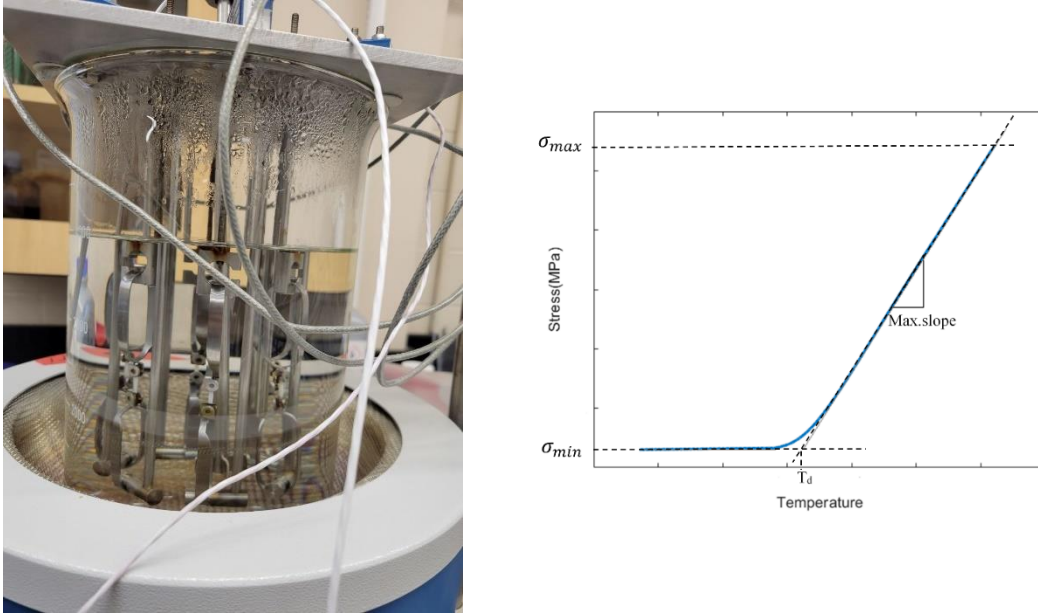


Figure 6. A) Schematic of a Hydrothermal Isometric Tension (HIT) testing apparatus; B) Typical curve obtained by HIT testing measuring the specimen's thermal stability (T_d) and network connectivity (Max. Slope).

One of the key challenges associated with using the HIT test to evaluate collagenous tissue quality is the absence of a mathematical model capable of interpreting HIT test results across the temperature range from ambient to 90°C. Although Le Lous et al. [186] offered insights by investigating the relationship between paired HIT and DSC test outcomes, a definitive model remains elusive. Their study revealed a significant correlation between the kinetics of tension variation, reflected in the first part of the HIT curve, and the kinetics of collagen denaturation observed in DSC tests. This correlation arises from the underlying principles of both HIT and DSC tests. In HIT tests, the generation of isometric tension depends on the total number of denatured collagen molecules, whereas in DSC tests, the rate of heat flow into the sample corresponds to the rate of collagen denaturation [186]. While their analysis involved integrating the DSC endotherm, resulting in a curve that closely matched the HIT curve up to a specific point (typically until denaturation completion around 75°C), it failed to predict the trajectory of the HIT curve beyond this point.

During the latter phase of an HIT test, as the temperature surpasses T_d (corresponding to the last part of the HIT test), the denatured collagen exhibits rubber elastic properties, behaving in accordance with

the theory of rubber elasticity. This phenomenon contributes to the additional isometric force observed in the latter portion of the HIT curve, as demonstrated by Flory et al.[194]. During this phase, the stresses generated are directly proportional to the absolute temperature.

Beyond the methods outlined for evaluating collagen, another useful tool for understanding the mechanisms underlying bone fragility and the contribution of the organic phase is computational and constitutive modeling. Therefore, it is imperative to conduct a thorough review of the constitutive behavior of bone material.

2.6 Cortical bone's constitutive behavior

Bone is a natural composite material with a hierarchical structure as fully reviewed in section 2.1.3. Owing to its multiscale structure, cortical bone exhibits anisotropic mechanical properties with respect to its main directions: longitudinal (which runs parallel to a long bone's main axis) and transverse (which runs perpendicular to it). The anisotropic mechanical properties of bone have been extensively explored through various experiments conducted at both macro-scale [195][196][197] and micro-scale [198], utilizing a range of mechanical and non-mechanical techniques [199][200]. The longitudinal elastic modulus of the human long bone is approximately 17.4 GPa (20.4 GPa for bovine bone), and the transverse elastic modulus of the same bone is about 9.6 GPa (11.7 GPa for bovine bone) [106].

In addition to the anisotropy of the bone mechanical properties, cortical bone exhibits strength asymmetry, resulting in variations in its yield and ultimate properties between tensile and compressive loading modes [195], although the elastic modulus only slightly changes among tension and compression [195] (refer to Figure 7). According to [63] and [201], it has been noted that the compressive ultimate strength of bovine cortical bone in the transverse direction is approximately three times higher than that in tension. The significantly elevated levels of ultimate stress and strain under compression suggest the activation of distinct deformation mechanisms under varying loading scenarios. One of the reasons for this significant difference is attributed to the intricate nature of failure in compression (compared to tension) in which a specimen can still carry load even after experiencing considerable deformation. However, in tension, failure occurs immediately upon reaching the material's strength [202]. According to [203], in compression shear mechanisms come into play. In addition, research conducted in [63] explored damage mechanism involved in compression and proposed a potential interaction involving slippage between collagen and the mineral phase, resulting in reversible deformation. In [201], damage mechanisms in both tension and compression were studied and observed

a diffuse damage pattern was observed in tension, whereas compression showed a less severe crosshatch type of damage, enabling greater energy dissipation.

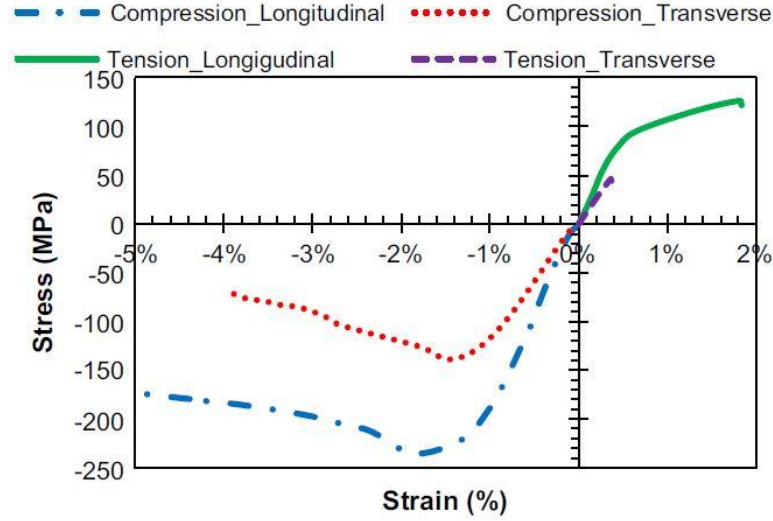


Figure 7. A typical tensile and compressive stress-strain curves for cortical bone in longitudinal and transverse directions [197].

To assess and characterize the macroscopic constitutive behavior of cortical bone, the best approach is to analyze a typical uniaxial quasi-static cyclic stress-strain curve [204]. In Figure 8 a tensile load-unload test result conducted in longitudinal direction of cortical bone at a low strain rate is depicted.

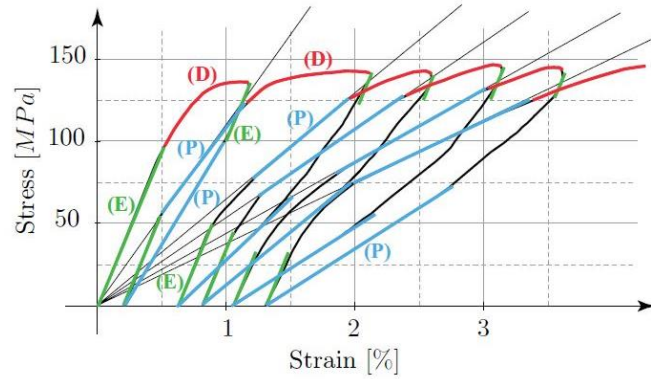


Figure 8. Quasi-static cyclic tensile stress-strain curves for cortical bone: (E) Pure elastic regime (D) damage mode (P) plastic mode [204]

Based on Figure 8, the material behavior of cortical bone can be categorized into three deformation regimes. The material deforms in a linear elastic manner during the first regime (at the start of each new cycle, denoted by the letter E). In the second regime marked by D, corresponding to the damage mode, the stress-strain response becomes nonlinear as voids and microcracks form, leading to a loss of material continuity and degradation in stiffness and other mechanical properties, as observed in the reloading curves. The third regime, identified as the plastic mode (abbreviated as P), involves permanent strains without further extension of damage [205]. When bone is loaded beyond the yield point, it experiences two mechanisms of energy dissipation: plasticity and damage. As mentioned, plasticity results in permanent deformation upon unloading, while damage is linked to a decrease in stiffness as a result of the emergence of microcracks [206][207].

Additionally, the load-unload curve reveals clear evidence of a viscous effect in the behavior of bone material, although not explicitly shown here [54]. The capacity of the bone to dissipate energy within the elastic range of deformation, the dependency of stiffness, yield point, and strength on the strain rate, and the occurrence of stress relaxation and creep behavior in both compact and trabecular bones are all attributed to the viscous component of the mechanical behavior of the bone [204].

Elasticity, plasticity, damage, viscosity, and creep are characteristics of the constitutive mechanical behaviors of bone tissue. They are consistently present but vary in prominence based on factors such as hydration level, molecular composition, loading rate, and microstructure of the specific bone sample. For instance, bone tissue lacking physiological water content tends to be more brittle and less viscous compared to hydrated bone [208].

The damaging behavior observed in cortical bone manifests through the creation of microcracks (leading to reduced stiffness), energy dissipation, permanent strains, and eventually fracture [209][210]. Bone damage may be induced by the shear failure of the organic matrix, such as the sliding of the microfibrils or collagen molecules on one another [211][212]. When subjected to cyclic loading, bones may endure fatigue damage and failure, characterized by microcrack formation, debonding, void expansion, and collagen fiber rupture [211][212].

Consequently, even though damage accumulation has not been frequently included in constitutive models of bone, it is a well-established feature of the nonlinear behavior of bone material. The most obvious explanation is that the constitutive models are not applicable under most situations. For example, under physiological loading conditions, creep damage or low-cycle fatigue damage—that is,

damage from high strains—is improbable. High-cycle fatigue damage, or damage that builds up at low strain amplitudes, is likely to happen frequently but is typically repaired by natural bone replacement. Nonetheless, there are a few good reasons to consider building computational models for the damaging behavior of the bone, as elaborated in the subsequent paragraphs [54].

One reason for this is because damaging processes are mostly responsible for the nonlinear behavior of the bone. It can be argued that critical rates of damage accumulation, rather than yield or failure stress, provide a more realistic illustration of the maximum allowed working stresses for bone [210][213]. As a result, models that predict damage accumulation may be able to offer more accurate estimations of the probability of failure for a given load.

The second justification is based on the reality that fatigue failure will occur in real life. Consequently, the capability to forecast fatigue failure becomes highly desired for practical applications. For the class of clinical problems that are commonly called fragility fracture, both the problems of creep damage and fatigue damage are important [214]. The capacity to anticipate the impact of damage on functional load-bearing activities and to foresee the effects of alterations in bone structure and organization on damage accumulation holds importance for both clinical medicine and biomedical science.

Another justification for the advantage of these models lies in their capacity to incorporate damage into frameworks elucidating the interplay between bone biochemistry and mechanical conditions. In normal bone biology, the degree to which damage accumulation plays a role is still controversial. Nonetheless, it is apparent that under certain circumstances, damage does impact bone biology [54]. Conversely, in specific scenarios like aging and bone-related diseases, where the underlying mechanisms of bone fragility have received limited investigation, these models could enhance our comprehension of the relationship between bone biochemistry and mechanical conditions. Furthermore, the accurate modeling of the damage accumulation process is imperative for developing damage-repair models that assess the role of damage in relation to other factors and for creating practical tools to predict damage and fractures on an individualized basis for patients [48].

The upcoming section provides an overview of constitutive models devised to examine damage accumulation in cortical bone. Specifically, the focus is on models categorized as continuum damage mechanics (CDM) models. Therefore, it is essential to commence with an examination of continuum damage mechanics theory.

2.6.1 Overview of the continuum damage mechanics theory

Continuum damage mechanics is a mechanical theory that uses a continuum mechanics perspective to examine the processes of damage and failure in materials [215]. Kachanov [216] was the first to establish CDM by introducing a scalar damage variable to address the creep failure of metal alloys. But Kachanov's initial CDM model lacked a precise physical definition for damage. Therefore, Rabotnov [217][218] expanded upon this theory to imbue the damage variable with physical significance. Rabotnov redefined the scalar damage variable, considering it as a measure of the reduction in load-carrying effective area resulting from microcrack development. In this context, the damage variable, denoted as D , can be readily defined in terms of the total cross-sectional area of undamaged (S) and damaged ($\tilde{S}$) materials.

$$D = \frac{S - \tilde{S}}{S} \quad (1)$$

Where $D = 0$ represents no damage to the specimen, whereas $D = 1$ denotes fractured material.

Rabotnov also proposed a hypothesis regarding the influence of damage on creep rate, $\dot{\epsilon}$, using material constants B and n [217][218].

$$\sigma = (1 - D) \left(\frac{\dot{\epsilon}}{B} \right)^{1/n} \quad (2)$$

Quantifying the material's damage is the primary challenge in CDM [219]. Some researchers have defined the damage variable as the ratio of damaged area to total cross-section [220][221], while others have utilized the concept of effective stress to introduce the damage variable [222][223]. Lemaitre suggested an alternative approach based on the reduction of elastic stiffness [224], proposing an elastic constitutive law for damaged material. On the basis of the strain equivalency hypothesis, he put forth the following elastic constitutive law for a damaged material: "*The deformation behavior of the damaged material is represented by the constitutive laws of the undamaged material with the true stress replaced by the effective stress.*" [225]. Based on Lemaitre's approach, the effective stress is defined as:

$$\sigma_{\text{eff}} = E_0 \epsilon, \quad (3)$$

Or, equivalently,

$$\sigma = E\varepsilon, \quad (4)$$

Where E_0 and $E = (1 - D) E_0$ represent the material's Young's modulus in its original undamaged and its damaged condition following loading, respectively. Consequently, the damage variable in Lemaitre's theory is reformulated as the reduction of Young's modulus in the isotropic case.

$$D = \frac{E_0 - E}{E_0} \quad (5)$$

Subsequently, Lemaitre's theory has been further explored by other researchers [226][227]. The damage variable can be defined either as a scalar variable for an isotropic material or a tensor for an anisotropic material [228][229]. In its simplest form, i.e., under ideally isotropic conditions, the damage variable is a scalar [225], [230]. Later, Lemaitre et al. [231] expanded the initial isotropic model to include partial closure of microcracks under compressive pressures and the anisotropy of damage into consideration. The damage variable in the presence of anisotropy is a 2nd order tensor, the evolution of which is linked to the principal directions of the plastic strain rate.

In the most general anisotropic scenario, the damage variable emerges as a 4th order non-symmetric tensor due to the notion of strain equivalency, according to Chaboche's phenomenological theory for creep-damage, which is based on rigorous thermodynamic foundations [232][233]. The anisotropic damage variable in the theory developed by Murakami and Ohno [234] is represented by a symmetric tensor of rank two. By assuming the presence of a hypothetical, mechanically identical, undamaged configuration, the effective stress idea is extended to three dimensions in this case, leading to the definition of the damage variable. Murakami further extended this concept to describe the general anisotropic state of internal damage in solids, particularly in elastic-brittle materials [235].

The composite nature of bone suggests that anisotropic models will be necessary to describe bone damage [236]. However, the concepts of the isotropic damage model are still fairly useful in many scenarios that correspond to the standard procedure of mechanical testing along the principal material axes. Scalar damage variables are preferred because they are straightforward and limit practical problems to proportional tests that examine a single direction in the strain space. In these situations, even if the damage is directional, the scalar damage variable is sufficient to explain the observed behavior [54].

2.6.2 Overview of the constitutive models of bone

Given the intricate nature and dynamic behavior of bone tissue, constitutive material models for bone can be categorized based on several factors: (a) Length scale, which refers to the size of the representative volume element (RVE) and includes considerations of structures such as cortical bone, trabecular bone, single trabeculae, and whether the material is homogeneous or heterogeneous. (b) Material symmetry, which encompasses properties like isotropy or orthotropy. (c) Loading capabilities, such as the range of strain amplitudes (small or large), the nature of loading (monotonic or cyclic), and the strain rates (low or high). (d) Constitutive response, which includes elastic, plastic, viscous, damage, fracture, and densification behaviors [208]. Typically, these models are integrated into finite element tools to facilitate the study of intricate geometries, material distributions, and loading scenarios.

Usually, the constitutive models have a specific predictive range. Formulating constitutive models that capture a broad spectrum of material behavior for predictive purposes presents mathematical challenges and is very demanding to validate experimentally. Consequently, constitutive models are developed to suit specific applications, describing reality within an accepted level of accuracy for those particular contexts. In the following, previously developed constitutive models for bone are reviewed with a focus on the models most relevant to this thesis.

Fondrk et al. [237][238] introduced the initial damaging viscoelastic model for bone material under tensile loading. While this model effectively predicts bone tensile behavior until strain reversal, neither damaging elastic nor viscoelastic models can adequately predict behavior under strain reversal regimes, failing to account for residual strain or stress accumulation [31]. To tackle this issue, Fondrk proposed a model [213][238] incorporating rate-dependent plastic behavior. Given the evident rate effects on cortical bone, particularly nonlinear creep behavior, viscoelastic models seem more suitable [54]. Fondrk's model stands as one of the most successful attempts in elucidating the mechanical behavior of cortical bone tissue under cyclic overloading conditions [238]. The model is a 1-D damage model based on two internal state variables. However, this model solely addresses bone axial tensile behavior, lacking distinct compressive behavior consideration. Additionally, interpreting and experimentally calibrating internal state variables poses challenges. Moreover, there's a dearth of a 3D generalization of the model, essential for finite element analysis [204].

Krajcinovic et al. [239] introduced a noteworthy endeavor to model bone tissue. Their fundamental microstructural model quantifies damage by assessing the ratio of pulled-out osteons to the total osteon count. This model offers valuable insights into microdamage mechanisms [204].

Among the more recent studies, the model developed by Zysset and Wolfram [240] captures the rate-independent inelastic material behavior of the cortical bone through microcrack opening and closing. While the results aligned effectively with experimental data, the model is constrained to one dimension and does not capture the viscous behavior of the bone material. Reisinger et al. [241] introduced another recently developed model for bone tissue, a new 1D model developed to capture both plastic and viscous behaviors. This model was validated using single bone trabeculae, with parameter fitting conducted based on cyclic tensile load experiments. However, the damaging effect has been neglected in this work which is a major drawback.

Ng et al. [242] also proposed a brittle damaged plasticity model to assess the damaging mechanism of cortical bone under compressive loading. Bone material has been considered a quasi-brittle material and the viscous effect has been neglected in this model. In another study, Lei et al [243] proposed a two-term constitutive model for capturing the strain rate effect and viscoelastic properties of cortical bone material under high strain rate. In this model there is no explicit inclusion of either plasticity or damage behavior.

Zysset [244] and Zysset and Curnier [245] proposed the most comprehensive damage model to date in their application to bone. While initially employed for trabecular bone, it possesses sufficient generality to extend to cortical bone. This three-dimensional model operates without assuming small strains, making it an entirely general model, and has been integrated into a Finite Element Analysis (FEA) algorithm for assessing damage accumulation around total hip arthroplasty components within femurs [246]. It stands as one of the most realistic depictions of inelastic strain accumulation under complex loading, capable of predicting experimental loss of compliance and residual strains.

Garcia et al. [205] expanded upon the early model of Zysset and Curnier to encompass both cortical and trabecular bone, incorporating architectural variables for differentiation. This model relies on a 3D elastic-plastic constitutive law to predict bone's macroscopic mechanical behavior under load-unload test though it lacks consideration for bone's viscous behavior, necessitating further refinement.

Schwiedrzik and Zysset [247] extended the model of Garcia et al. to accommodate strain-rate dependency in post-yield behavior. They proposed an anisotropic elastic-viscoplastic damage

constitutive model for bone, implementing Perzyna's formulation [248] for viscoplasticity and introducing a scalar function of accumulated plastic strain to model damage. A polynomial flow rule was also introduced to capture material behavior post-viscoplastic yielding.

Notably, the model by Schwiedrzik and Zysset comprehensively captures bone's evolution from elastic to viscoplastic to fracture modes, applicable across various length scales. It accounts for bone porosity, mineral and organic phase cohesion, as well as viscoplastic and damage dissipation mechanisms. As the present study builds upon this model, it is pivotal to briefly review the Schwiedrzik et al. model as it serves as the foundation for the current research, with the constitutive model in the present study representing an extension of the aforementioned model.

A common challenge in constitutive modeling is to define the onset of nonlinearity or the choice of failure criterion. Therefore, before getting to the Schwiedrzik et al. model, a review of the most common yield criteria applicable to bone is necessary.

2.6.2.1 Review of yield criteria in application to bone

Numerous studies, including FE analyses, have been implemented in recent decades to predict bone failure. Von Mises [249]–[252], although designed for isotropic materials in non-porous media, was one of the basic failure criteria used in most of these works. However, this criterion is not accurate for bone, because it fails to consider anisotropy, and it does not account for the varying strength in uniaxial tension and compression.

Empirical findings underscore the high anisotropy of bone and its varying strengths in compression and tension. Consequently, any chosen bone failure criterion should accommodate these variations. Attempts to address these concerns have introduced alternative failure criteria aimed at capturing bone's distinct behaviors in compression and tension, including modified versions of von Mises [253], Hoffman [250][253], modified Hoffman [253], maximum stress [253], maximum strain [253], Mohr-Coulomb [253], modified Mohr-Coulomb [253], Drucker-Prager [254][63]. However, these have fallen short of fully resolving the issue.

Only a handful of criteria have taken bone anisotropy into account, such as Hill's criterion [255], the Tsai-Wu criterion, and subsequent extensions thereof. For instance, Cowin [256][257] proposed a strength criterion for porous materials, an extension of the Tsai-Wu criterion [249] incorporating the

fabric tensor concept. Similarly, Hashin [258] proposed a failure criterion model as an extension of existing anisotropic failure criteria.

An investigation of the yield behavior of cortical bone under combined loading circumstances combining torsion, compression, and tension was conducted by Cezayirlioglu and colleagues [259]. They looked at the Tsai-Wu criterion [260], Hill's criterion [255], Ragahav's criterion [261], and the traditional Von Mises criterion, which are the four failure criteria that have been used for composite materials. Of these, the Tsai-Wu criterion aligned best with test results, although Hill's criterion provided reasonable precision in defining individual compression-torsion or tension-torsion regimes [259].

However, criticism has been levied against the applicability of Hill and Tsai-Wu criteria to cortical bone. In Hill criterion, acquiring all necessary parameters necessitates pure shear tests in the principal material configuration, in addition to direct compression/tension tests which is not easy to conduct on bone tissue. Apart from these experimental limitations on parameter determination, the Hill criteria assumes the compression and tension strengths to be identical, an assumption that is not relevant to bone tissue. Despite these limitations, Hill and Tsai-Wu criteria remain the most prevalent failure criteria for cortical bone, with their parameters measured and reported in various studies [259], [262], [263].

In the constitutive model presented by Schwiedrzik et al. [247], an eccentric elliptical isotropic yield surface was defined to simplify material identification, akin to the Tsai-Wu criterion.

2.6.2.2 Review on the anisotropic elastic-viscoplastic damage model of Schwiedrzik and Zysset

Schwiedrzik and Zysset [247] devised a model wherein bone's dissipative mechanism is described as viscoplastic deformation coupled to damage. Illustrated in Figure 9, the rheological model comprises a damageable elastic spring in series with a viscoplastic component, featuring a plastic pad and a dashpot in parallel.

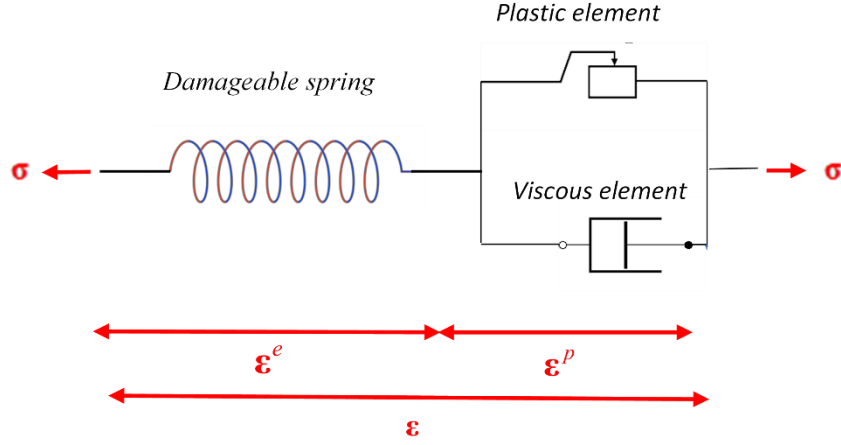


Figure 9. Rheological model of elasto-plastic solid damage proposed by Schwiedrzik and Zysset [247]

In the purely elastic range, the model's behavior remains independent of the strain rate. However, within the viscoplastic range, plastic strains amass in a viscous manner according to a Perzyna-type formulation [248]. This model relies on two internal state variables: $\boldsymbol{\varepsilon}^p$, a second-order tensor encompassing the inelastic strains, and κ , representing the accumulated plastic strain. The assumption is made that damage accumulation is coupled with plasticity through a damage function $D(\kappa)$ that diminishes all components of the elasticity tensor. This assumption is reasonable for bone since inelastic deformation in bone typically coincides with damage initiation, suggesting simultaneous onset.

The constitutive equation is presented as follows [247]:

$$\boldsymbol{\sigma} = (1 - D(\kappa))\mathbb{E} : (\boldsymbol{\varepsilon} - \boldsymbol{\varepsilon}^p) \quad (6)$$

Here, $\boldsymbol{\sigma}$ represents the stress tensor, $\mathbb{E}$ denotes the elastic stiffness tensor, $\boldsymbol{\varepsilon}$ signifies the total strain tensor, $\boldsymbol{\varepsilon}^p$ stands for the plastic strain tensor, and D represents the damage variable defined by [244][264]:

$$D(\kappa) = 1 - e^{-k_p \kappa} \quad (7)$$

$$0 \leq D(\kappa) \leq 1$$

Where k_p is a constant and κ represents the accumulated plastic strain, enabling the measurement of the plastic deformation path as given by [247]:

Assuming the cortical bone is a transversely isotropic material, the compliance tensor $\mathbb{C} = \mathbb{E}^{-1}$ is defined as follows:

$$\mathbb{C} = \begin{pmatrix} \frac{1}{E_1} & \frac{-\nu_1}{E_1} & \frac{-\nu_{31}}{E_3} & 0 & 0 & 0 \\ \frac{-\nu_1}{E_1} & \frac{1}{E_1} & \frac{-\nu_{31}}{E_3} & 0 & 0 & 0 \\ \frac{-\nu_{31}}{E_3} & \frac{-\nu_{31}}{E_3} & \frac{1}{E_3} & 0 & 0 & 0 \\ 0 & 0 & 0 & \frac{1}{2\mu_{31}} & 0 & 0 \\ 0 & 0 & 0 & 0 & \frac{1}{2\mu_{31}} & 0 \\ 0 & 0 & 0 & 0 & 0 & \frac{1+\nu_1}{E_1} \end{pmatrix}$$

The elastic properties of the transversely isotropic material of cortical bone are described by the five elastic constants E_1 , E_3 , ν_1 , ν_{31} , and μ_{31} , where indices 1 and 3 represent the transverse and longitudinal directions, respectively.

An anisotropic quadric yield criteria defined in 3D stress space is the rate-independent yield function [265].

$$Y(\boldsymbol{\sigma}, \kappa) = \sqrt{\boldsymbol{\sigma} : \mathbb{A} : \boldsymbol{\sigma}} + \mathbf{A} : \boldsymbol{\sigma} - r(\kappa) \quad (8)$$

The hardening function, $r(\kappa)$, in this formulation is an isotropic hardening function that is assumed to be a clear function of the total plastic strain:

$$r(\kappa) = 1 + (1 - \gamma_r)g(\kappa) \quad (9)$$

$$g(\kappa) = 1 - e^{-k_s \kappa}$$

The yield to ultimate properties ratio, denoted by γ_r , and the curvature of the exponential hardening function, represented by k_s , are the material constants in this formulation.

Additionally, $\mathbf{A}$ is a second-order tensor and $\mathbb{A}$ is a fourth-order tensor in eq. (8), which define the yield criterion's shape, orientation, and origin [247][265].

(10)

$$\begin{aligned}
\mathbb{A} = & \sum_{i=1}^3 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \mathbf{M}_i \otimes \mathbf{M}_i - \sum_{i,j=1; i \neq j; j \neq 3}^3 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j \\
& - \sum_{i=1; j=3}^2 \frac{1}{4} \left(\frac{1}{Y_j^+} + \frac{1}{Y_j^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j + \sum_{i,j=1; i \neq j}^3 \frac{1}{2T_{ij}^2} \mathbf{M}_i \otimes \mathbf{M}_j \\
\mathbf{A} = & \sum_{i=1}^3 \frac{1}{2} \left(\frac{1}{Y_i^+} - \frac{1}{Y_i^-} \right) \mathbf{M}_i
\end{aligned}$$

In eq. (10), the uniaxial tensile and compressive yield stresses in the primary material directions are represented by Y_1^+, Y_2^+, Y_3^+ and Y_1^-, Y_2^-, Y_3^- respectively, and shear yield stresses are denoted by T_{12}, T_{23} and T_{13} . Additionally, the coefficients ξ_{12}, ξ_{23} and ξ_{13} denote interaction coefficients [265]. Again, based on the assumption of transversely isotropy for cortical bone, the material properties in the two transverse directions are assumed to be equivalent ($\begin{bmatrix} \cdot \\ \cdot \end{bmatrix}_1 = \begin{bmatrix} \cdot \\ \cdot \end{bmatrix}_2$). The second order tensor, $\mathbf{M}_i$, in eq (10), which helps in defining the anisotropy of the material (in this case transversely isotropy), is defined using $\mathbf{u}_i$, the unit vectors of the principal material coordinate system of cortical bone.

$$\mathbf{M}_i = \mathbf{u}_i \otimes \mathbf{u}_i \quad (11)$$

During viscoplastic flow, Perzyna viscoplastic materials' stress state may be outside of the rate-independent yield surface. In the inelastic regime ($\dot{Y} \geq 0$), the rate-dependent yield surface may be defined by stating a new condition confining the viscoplastic flow. This condition is an extension of the rate-independent yield condition ($\dot{Y} = 0$).

$$\bar{Y} = Y + \frac{m}{2} - \left(\frac{m^2}{4} + \frac{\eta}{\Delta t} \frac{\Delta \kappa}{h} \right)^{\frac{1}{2}} = 0 \quad (12)$$

In eq. (12), η and m are two parameters that determine viscoplastic flow. η represents viscosity, while m represents the degree of non-linearity in viscoplastic strain evolution. In addition, h denotes the norm of the normal of the yield surface. As expected, when η approaches zero, the rate-dependent yield surface, turns into the rate-independent yield surface.

2.6.3 Constitutive modelling of materials under extreme conditions

Since the constitutive modeling of diseased bone under oxidative damage, a biochemical phenomenon, has never been done before, a literature review was conducted to discover the relevant literature. This review focused on constitutive models of materials under severe environmental conditions such as moisture, radiation, and temperature. Irradiation is specifically relevant in this case because it causes severe oxidative damage to materials.

Mohammadi et al. [266] devised a micromechanical model to describe how temperature and UV radiation affect the constitutive behavior of cross-linked polymeric systems like elastomers. The goal of this model was to predict the impacts of photo-oxidative aging on the mechanical response of cross-linked systems as well as their inelastic responses such as permanent set and Mullins effect. The suggested model is a generalized micromechanical model for both thermo-oxidative and photo-oxidative aging. As a result, the model's parameters are divided into two sets. One set is simply affected by temperature, whereas the other is affected by both temperature and UV light intensity.

Wang et al. [267] suggested a multiscale finite element model to predict radiation's influence on the compressive stress response of silicon rubber foams, using a constitutive model for the rubber material before and after gamma radiation.

Jia et al. [268] modified the Hyperfoam constitutive model [269]–[271] to characterize the mechanical behavior of silicon rubber foam under gamma-irradiation. This paper introduces the effect of gamma-irradiation on the mechanical properties of silicon rubber foams (which change the microstructure by crosslinking and scission reactions of a polymer chain) indirectly by establishing relationships between model parameters and irradiation dose.

A constitutive crystal plasticity model is introduced and formulated in [272] to describe the inelastic deformation of irradiated bcc ferritic/martensitic steels. This study presents an approach utilizing mechanism-based internal state variables (ISVs) coupled with continuum crystal plasticity to simulate inelastic deformation in bcc materials affected by radiation damage at ambient and intermediate homologous temperatures during quasi-static and creep loading conditions.

Shakiba et al. [273][274] suggested a constitutive model based on CDM theory to simulate moisture's influence on the reaction of moisture-sensitive materials like asphalt and concrete. The model was then coupled to the mechanical constitutive relationships in order to describe the moisture-mechanical

response of asphalt concrete. The authors proposed two internal state variables: ϕ (mechanical damage density) and ω (moisture damage), which describe the material's deterioration due to moisture.

Murakami and Mizuno [275] expanded upon prior research [276] by integrating irradiation and material damage effects into their constitutive equation for irradiation-influenced thermal creep. Neutron irradiation's impact on thermal creep is characterized by two factors: neutron flux, represented by ϕ , and neutron fluence, denoted by $\Phi = \int \phi dt$. To accommodate irradiation effects on thermal creep and creep damage, the coefficients within the constitutive and evolution equations under unirradiated conditions are substituted with material functions corresponding to ϕ and Φ .

Chapter 3: Research Objectives and Scope

3.1 Research Objectives

The primary goal for bone quality researchers is to unveil the underlying physiological and pathological factors that lead to bone fragility and fractures. By delving into these factors, they seek essential knowledge to identify determinants of poor bone quality to mitigate the risk of fractures. This study endeavors to explore the hypothesis that oxidative damage to bone collagen, prevalent in aging and diseases, causes degradation of bone collagen quality, thereby deteriorating bone quality and presumably increasing bone fracture risk.

To advance toward the research goal, and based on the observed gaps in the literature, the following three key objectives were set:

- **Objective 1:** Investigate the effects of physiological levels of oxidative damage generated in vitro on bone collagen and, consequently, cortical bone mechanical behaviour.
- **Objective 2:** Propose a mathematical model of HIT- DSC testing of bone collagen to aid in bone collagen quality assessment.
- **Objective 3:** Generate and validate a constitutive model for cortical bone that captures the effects of physiologically relevant levels of oxidative damage observed in aging and diseases.

3.2 Research Scope

To fulfill the objectives of this research project, the following tasks and sub-tasks were defined.

3.2.1 Task 1: In vitro study of the effects of physiological levels of oxidative damage on cortical bone quality

To experimentally explore the impact of physiologically relevant oxidative damage on bone quality in vitro, bovine cortical bone specimens were exposed to a new oxidative damage model using neutral hypochlorous acid as the source of oxidation. The oxidized bone specimens underwent thorough characterization through various experimental methods outlined in the following subtasks:

- **Sub-task 1-1: Mechanical Testing**

- 1) To test the hypothesis that the mechanical properties of bone degrade as a result of in vitro physiologically relevant oxidative damage multiple sets of mechanical testing were conducted.

These tests aimed to capture the effect of oxidation on various bone constitutive behavior, including yield and post-yield properties, as well as its viscosity and damage.

➤ **Sub-task 1-2: Chemical assay analysis**

The second sub-task was carried out to verify oxidative damage of the collagen by measuring its carbonylation, the stable biomarker for detection of oxidative damage in proteins. In this part, two different chemical assays were conducted on all specimens that underwent mechanical testing.

➤ **Sub-task 1-3: Thermo-mechanical Analyses**

The final sub-task was conducted to investigate how oxidative damage degrades bone collagen by affecting the thermal stability and integrity of the collagen network. This investigation involved conducting DSC and HIT tests on specimens previously subjected to mechanical testing.

3.2.2 Task 2: Develop a model of HIT-DSC testing to aid the interpretation of HIT data during bone collagen quality assessment

As mentioned in the Task 1, thermo-mechanical analyses including DSC and HIT were among the techniques used in this study for evaluation of the bone collagen quality when exposed to oxidation. To establish a connection between DSC and HIT test results and improve the interpretation of the HIT data for assessing collagen quality using physics-based parameters, a linear systems model of HIT-DSC testing is proposed. This task incorporates two sub-tasks including:

➤ **Sub-task 2-1: Propose the linear systems theory model to bridge DSC and HIT**

In order to accomplish this task, a simple linear systems theory model of HIT testing was proposed to combine the denaturation kinetics from DSC measurements with a new model for isometric tension generation.

➤ **Sub-task 2-2: Verification of the model using two human bone collagen datasets**

In a later stage, to test the validity of the model, a more diverse dataset of HIT-DSC measurements was included. This dataset encompassed various types of human bone collagen samples, including those from healthy individuals, donors with different types of diseases, as well as ribose-treated specimens.

3.2.3 Task 3: Constitutive Modeling and Size Effect Analysis of Oxidatively Damaged Cortical Bone with Application to aging and disease states

A computational damage mechanics (CDM)-based constitutive model is proposed based on a previously developed model [247] to model the degradation of cortical bone mechanical properties and damage resistance under the effect of oxidative damage. In pursuit of this objective, the subsequent sub-tasks were outlined and executed:

➤ **Sub-task 3-1: Integration of Oxidative Stress Effects into the Constitutive Model**

Integrate the impact of oxidative damage into the constitutive model using the experimental findings from task 1 and the model created in task 2. Implement this model into a user-material subroutine (UMAT) for use with the commercial finite element solver Abaqus.

➤ **Sub-task 3-2: Experimental validation of the model**

In this step, the fully implemented model was validated against 3-point single edge notched bending (SENB) test results in two states: unoxidized control and gamma-irradiated (extreme oxidation).

➤ **Sub-task 3-3: Exploring the size effect on the microdamage process zone (MDPZ) using the established constitutive model**

In this step, the fully established model was used to explore the effects of specimen size variation and oxidative damage on the development of the MDPZ.

Chapter 4: Synopsys of the thesis

4.1 In vitro study of the effects of physiological levels of oxidative damage on cortical bone quality

This study section investigates how in vitro oxidative damage affects bone quality using a combination of techniques. It was hypothesized that oxidative damage, induced by neutral hypochlorous acid solution in vitro, would cause poor bone quality by damaging the bone collagen. To test this hypothesis, bovine cortical bone specimens were oxidized using neutral hypochlorous acid solution, a potent physiological oxidizing agent. The oxidized bone specimens characterized using the following methods: 1) Multiple sets of mechanical testing to measure changes in bone tissue behaviour as a result of oxidation. 2) Carbonyl assay to quantify bone collagen carbonylation, a key marker of oxidative damage. 3) Thermo-analytical techniques (DSC and HIT testing) to measure bone collagen integrity and connectivity. Figure 10 offers a graphical overview of this section. Also note that a more detailed presentation of the presented result in this section can be found in Appendix A.

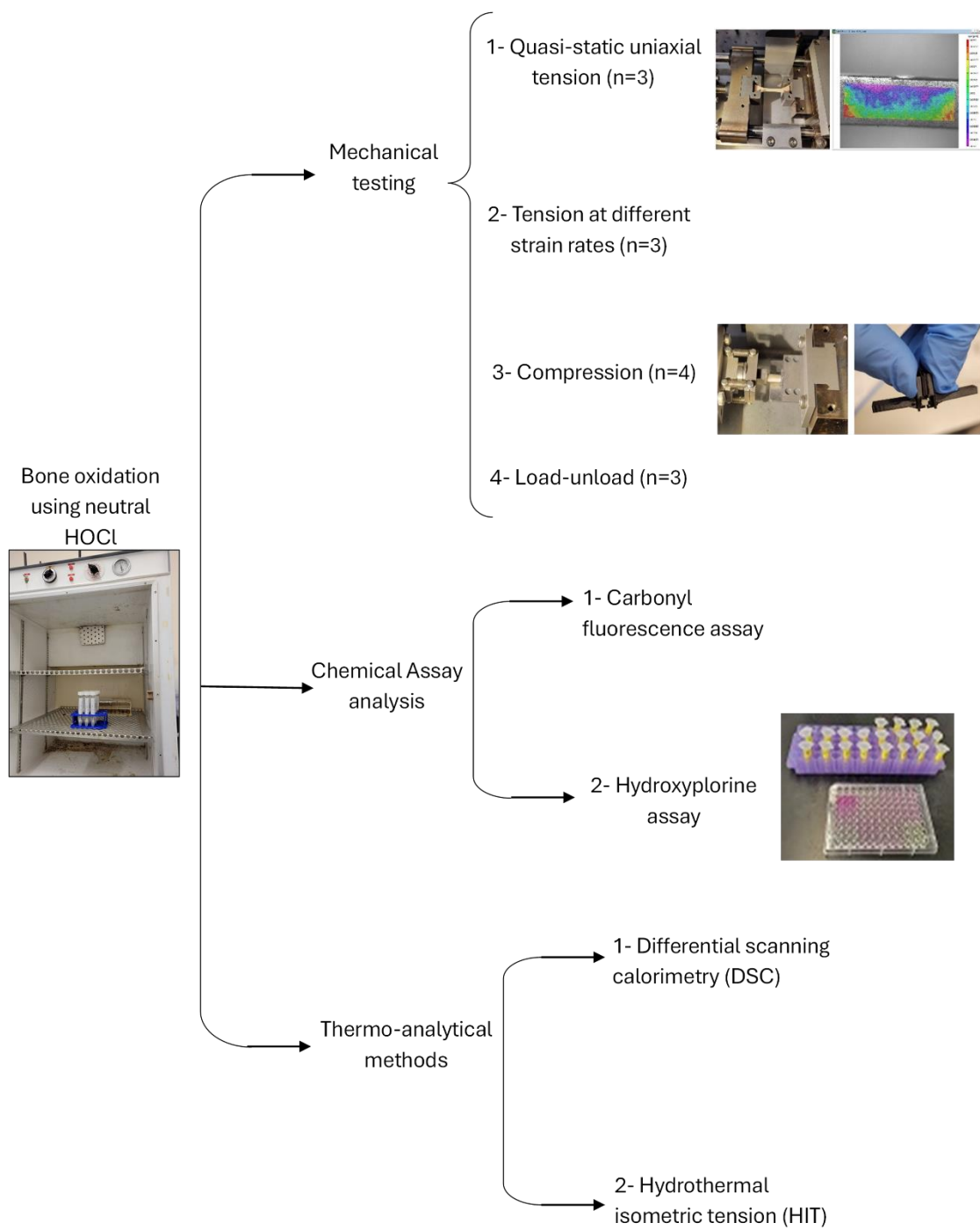


Figure 10. The graphical overview of the study depicting the different experimental methods used.

4.1.1 Sub-task 1-1: Mechanical Testing

As depicted in Figure 10, this study included four different sets of mechanical tests. All mechanical experiments were conducted using a Psylotech μ TS mechanical testing frame (1.6 kN load cell capacity, Psylotech Inc., Evanston, IL, USA), installed on a vibration isolation table (Vision Isostation, Newport Corp., USA) (refer to Figure). High-resolution images of the specimens were captured with an Olympus microscope equipped with a 5-megapixel camera (Point Grey, 5 MP, 2/3" detector). These images were analyzed using VIC 2D digital image correlation (DIC) software. Specimens were prepared for DIC by applying speckle patterns with 4-5 μ m toner powder particles (Xerox Phaser 6000) to create speckle patterns for DIC strain measurement. The tests were all conducted under displacement control.

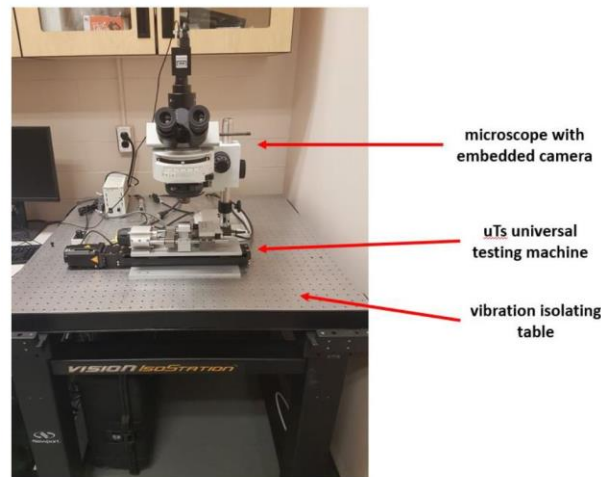


Figure 11. Psylotech μ TS testing system with vibration table and microscope [277]

4.1.1.1 Sample preparation and oxidation

Bovine steer (male bovine) tibiae aged 12–30 months were sourced from a local farm. Specimens were prepared by removing flesh and isolating the posterior diaphysis using a wood band saw. Using a vertical mini-CNC mill (model 5410, Sherline products, Vista, California, US) dog-bone shaped specimens were cut (with gauge dimensions of 12 mm $\times$ 2.4 mm $\times$ 3 mm) for tensile and load-unload tests, and small cuboids (7 mm $\times$ 2.4 mm $\times$ 3 mm) were prepared for compression tests from the posterior part of the diaphysis (see Figure 12). Since the direction of osteons are more consistent in the posterior part of the bone, cutting all specimens from this section reduces the variability among them.

All specimens are aligned with the bone's longitudinal axis. Surfaces were polished using a series of diamond discs and polycrystalline diamond suspension (45 μm , 15 μm , 9 μm , 0.5 μm) (MetaDi Supreme, Buehler, IL, USA) to remove imperfections, and specimens were stored in saline soaked gauze at -20°C.

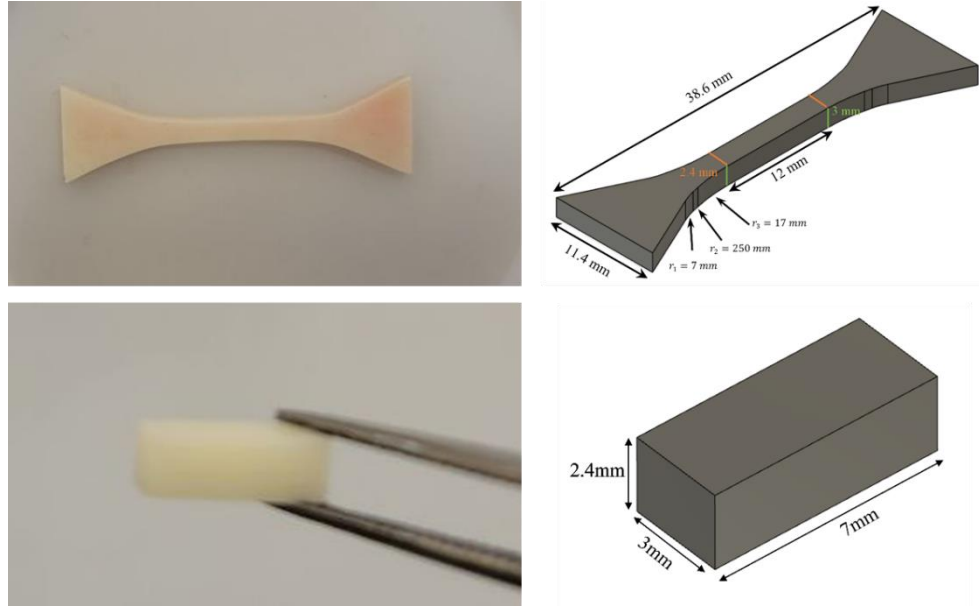


Figure 12. Geometry of the tensile and compression testing specimens.

To generate oxidative damage, specimens were exposed to neutral hypochlorous acid (HOCl) solution, prepared by mixing 5% Acetic acid (HOAc) and 5.5% Sodium Hypochlorite (NaClO), for 48 and 96 hours at 37°C. The control group was immersed in PBS under the same conditions. Samples were rinsed and submerged in PBS for 3-4 hours before mechanical testing.

4.1.1.2 Uniaxial tension test

The initial experiments involved quasi-static uniaxial tensile tests on nine control and oxidized bone specimens at three different time intervals (Control, 48 hr, 96 hr), performed at a minimal displacement rate of 13 $\mu\text{m/s}$ (average strain rate $\dot{\epsilon}_0 = 2 \times 10^{-4} \text{s}^{-1}$). These tests were designed to assess the impact of oxidation on tensile mechanical properties such as elastic modulus, yield strength, ultimate strength, and ultimate strain.

To evaluate how cortical bone responds to different levels of oxidation under varying stress rates, additional uniaxial tensile tests were conducted on 18 more specimens (n=3 per group) at two higher

strain rates (medium rate $\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$, high rate $\dot{\epsilon}_2 = 0.02 \text{ s}^{-1}$). These strain rates correspond to physiological levels that the body typically experiences during everyday movements. Given that the goal of this study was to investigate how oxidative stress affects the mechanical properties of bone, these rates were appropriate for the intended analysis. As a result, the study did not focus on the effects of higher strain rates, which are more relevant to impact loading events, such as those occurring during accidents.

Flat, dog-bone-shaped samples were gripped securely and tested at room temperature, with data for low and medium strain rates recorded at 10 Hz and high strain rate data at 40 Hz. Figure 13 shows the tensile test setup, illustrating the grips and specimens, and a typical strain field analyzed via digital image correlation (DIC).

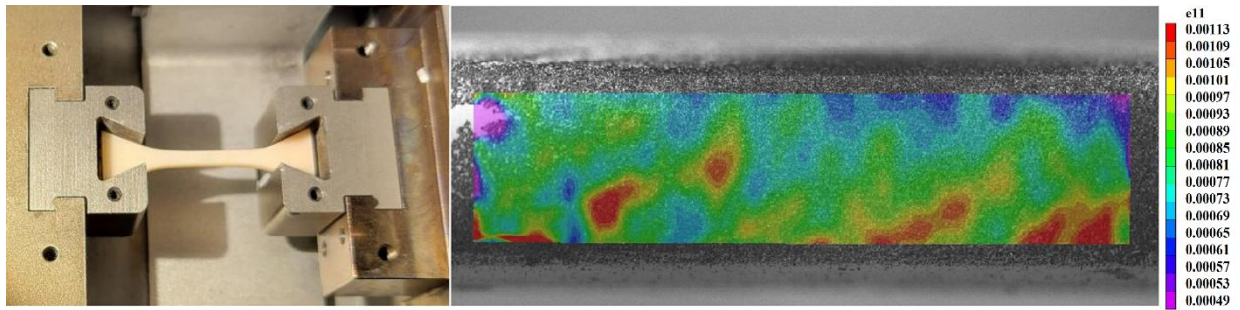


Figure 13. Tensile testing setup (left) and bone specimen surface with the DIC strain field in the gauge section (right).

The results of the tensile tests were analyzed in two ways. First, stress-strain curves for different oxidation exposures at each strain rate were compared and statistically analyzed using one-way ANOVA followed by Tukey's post hoc test with 0.05 level of significance (See Figure 14 and Table 1). Notably, the yield strength in Table 1 has been estimated using the conventional 0.2% strain offset criterion [204]. In addition to the one-way ANOVA test, the repeated measures one-way ANOVA has also been applied to the experimental results to investigate the statistically significant difference between the results (Table 1). Since the specimens used in each oxidation group came from different tibias (to account for tibia-to-tibia variation), a repeated measures one-way ANOVA was also conducted to assess significant differences.

In Figure 13, while the strain field is predominantly uniform (as indicated by the mostly green color, with small red and purple patches), the minor inhomogeneities observed could be attributed to several factors. Given that the material under study is biological tissue, it likely exhibits intrinsic

inhomogeneities such as microstructural variations, which can create localized stress concentrations and lead to non-uniform strain fields. Additionally, DIC depends on tracking the speckle pattern applied to the specimen's surface. If the speckle pattern is not fully uniform (as seen in some areas, such as those with purple regions), the DIC algorithm may not accurately capture the strain field, resulting in apparent inhomogeneities.

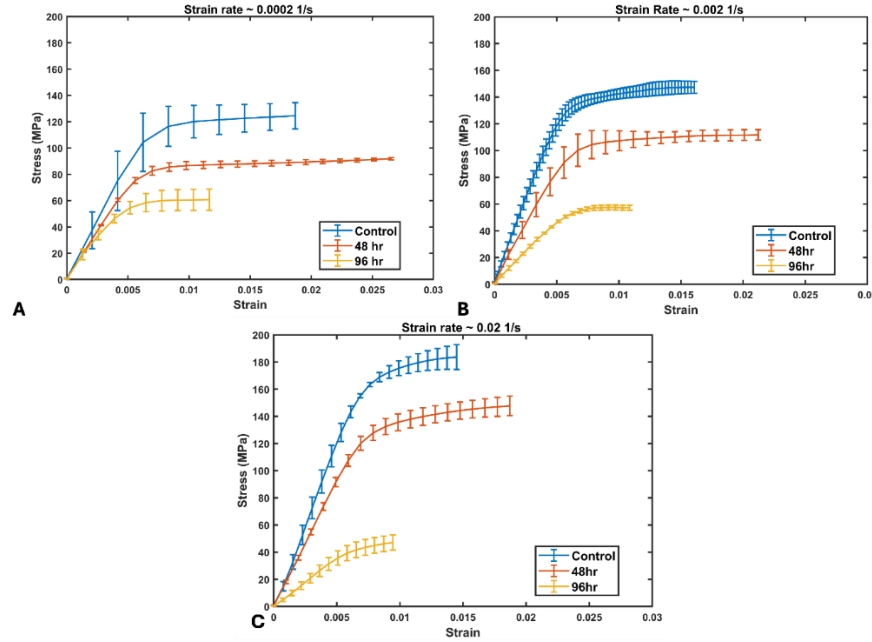


Figure 14. Stress-strain curves for the A) low strain rate, B) medium strain rate C) and high strain rate tensile test specimens at different incubation time points.

Table 1. Tensile mechanical properties measured at each strain rate for the control and oxidised groups

Strain rate		Modulus (GPa)	Yield Strength (MPa)	Ultimate Strength (MPa)	Ultimate strain ()
$\dot{\epsilon}_0 = 0.0002 \text{ s}^{-1}$	Control	18.0 (± 3.9)	119 (± 12)	125 (± 10)	0.0204 (± 0.0003)
	48 hr	14.5 (± 2.1)	85 (± 15)	92 (± 16)	0.0263 (± 0.0023)
	96 hr	13.0 (± 1.4)	59.4 (± 8.5)	60.5 (± 8.1)	0.0132 (± 0.0023)
	p-values (One way ANOVA)	0.368	0.021	0.015	0.004
	p-values (RM One way ANOVA)	0.538	0.045	0.044	0.016
$\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$	Control	23.4 (± 1.4)	128 (± 13)	150 (± 14)	0.016 (± 0.002)
	48 hr	17.0 (± 2.7)	106.1 (± 9.3)	111.7 (± 3.6)	0.021 (± 0.002)
	96 hr	9.39 (± 0.27)	56.9 (± 2.1)	57.2 (± 1.8)	0.011 (± 0.004)

	p-values (One way ANOVA)	p < 0.001	p < 0.001	p < 0.001	p = 0.273
	p-values (RM One way ANOVA)	0.003	0.001	< 0.001	0.17
$\dot{\epsilon}_2 = 0.02 \text{ s}^{-1}$	Control	24.6 (± 1.2)	169.1 (± 3.2)	186 (± 11)	0.015 (± 0.003)
	48 hr	15.8 (± 3.0)	118 (± 22)	139 (± 29)	0.019 (± 0.001)
	96 hr	7.2 (± 1.1)	45.1 (± 5.3)	49.9 (± 5.2)	0.010 (± 0.003)
	p-values (One way ANOVA)	p < 0.001	p < 0.001	p = 0.011	p = 0.059
	p-values (RM One way ANOVA)	0.005	0.002	0.005	0.069

Second, stress-strain curves for each strain rate at different incubation times were examined. Figure 15 (A-C) illustrates stress-strain curves for control, 48 hr, and 96 hr specimens at various strain rates.

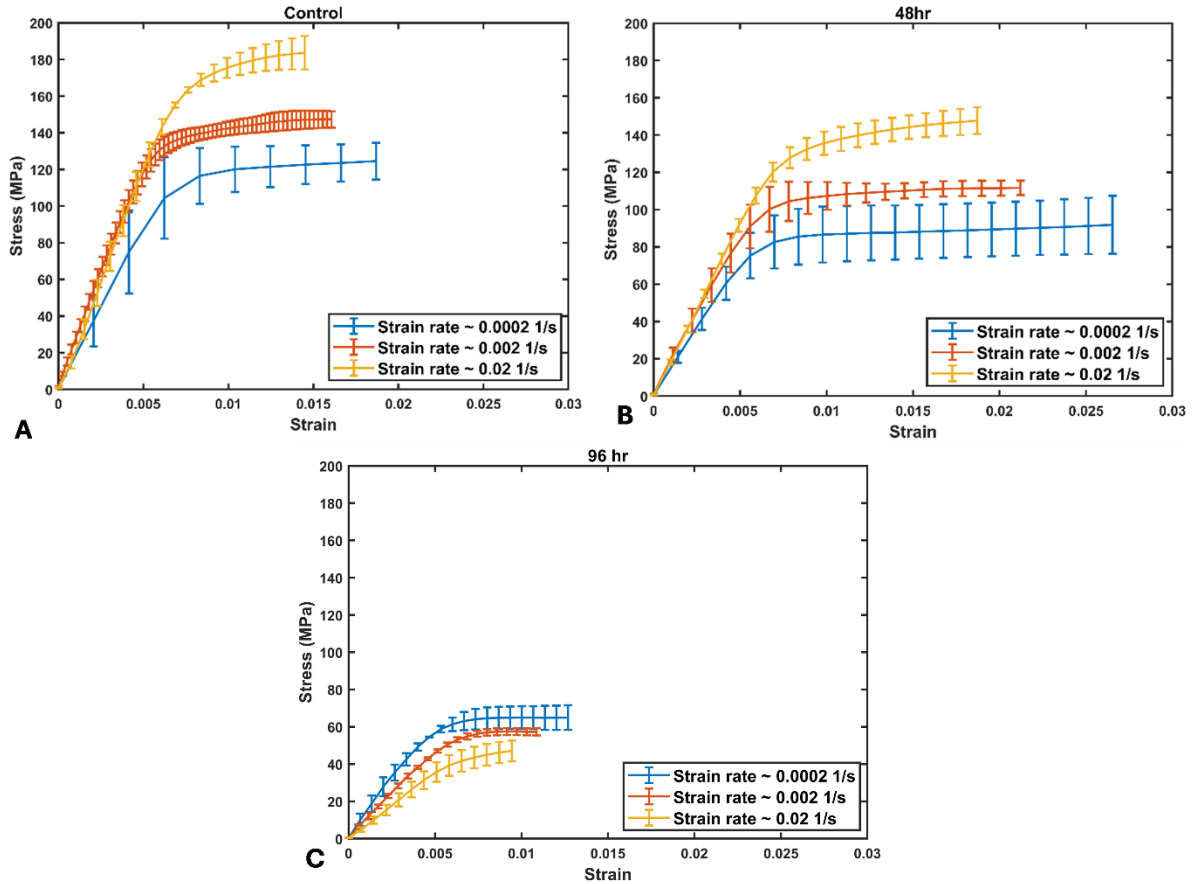


Figure 15. Stress-strain curves for A) Control B) 48 hr and C) 96 hr oxidative exposure specimens under different strain rates.

Key findings from the uniaxial tensile tests include:

- All mechanical properties, except ultimate strain, significantly declined with increased oxidation for all strain rates. This decrease in the mechanical properties where significant in yield properties at all three different rates including $\dot{\epsilon}_0 = 0.0002 \text{ s}^{-1}$ (p-values=0.021), $\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$ (p<0.001), and $\dot{\epsilon}_2=0.02 \text{ s}^{-1}$ (p <0.001) as well as the ultimate strength at all three tested rates $\dot{\epsilon}_0 = 0.0002 \text{ s}^{-1}$ (p-values=0.015), $\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$ (p<0.001), and $\dot{\epsilon}_2=0.02 \text{ s}^{-1}$ (p=0.011). However, the yield modulus only showed significant decrease at the two higher rates $\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$ (p<0.001), and $\dot{\epsilon}_2=0.02 \text{ s}^{-1}$ (p <0.001).
- Control and 48-hour samples showed increased yield and post-yield properties at higher strain rates, whereas 96-hour oxidized samples exhibited reduced mechanical properties as strain rate increased.
- Yield strength and ultimate strength significantly degraded with longer oxidation exposure across all strain rates (p-values: <0.001 to 0.015).

4.1.1.3 Uniaxial compression test

To assess how oxidation affects the compressive mechanical properties of cortical bone, compression tests were conducted on 12 compression specimens depicted in Figure 12 (n=4 per group) at a displacement rate of 13 $\mu\text{m/s}$, mirroring the rate used in quasi-static tensile tests. Custom compression platens from Psylotech Inc. were utilized for these tests. To minimize friction, the platens were lubricated with MOLYKOTE® high vacuum grease before each test. Additionally, a specially designed fixture ensured the precise and repeatable positioning of the small compression specimens (see Figure 16).

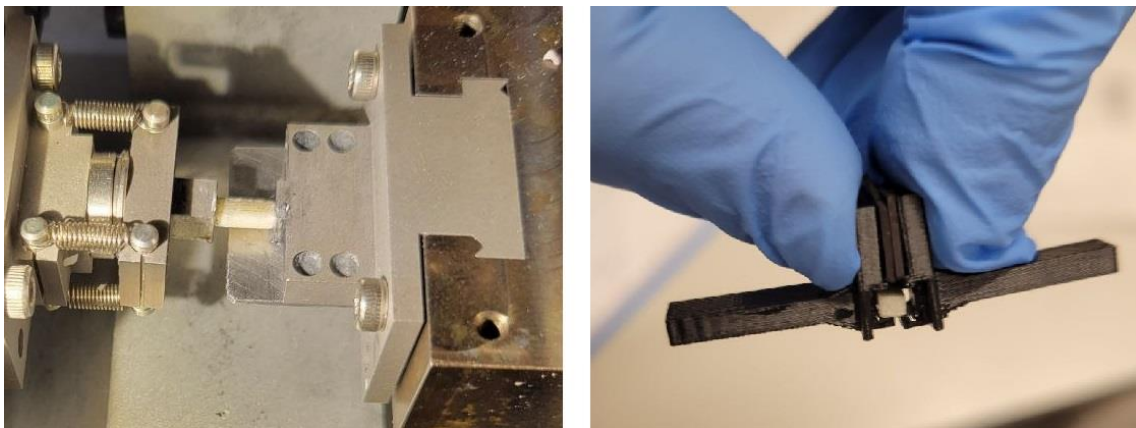


Figure 16. Compression test setup (left) and the fixture for holding and placing each specimen repeatably (right).

Representative stress-strain curves along with a typical fracture pattern from the uniaxial compression tests are shown in Figure 17. For control and 48-hour specimens, the material reaches maximum stress and then loses load-bearing capacity due to dominant damage mechanisms involving both local stress hardening and shear sliding, leading to gradual disintegration [197]. Increased oxidation exposure results in deteriorated yield properties, with failure occurring before ultimate stress due to the gradual propagation of cracks. Compressive mechanical properties for each time point are detailed in Table 2.

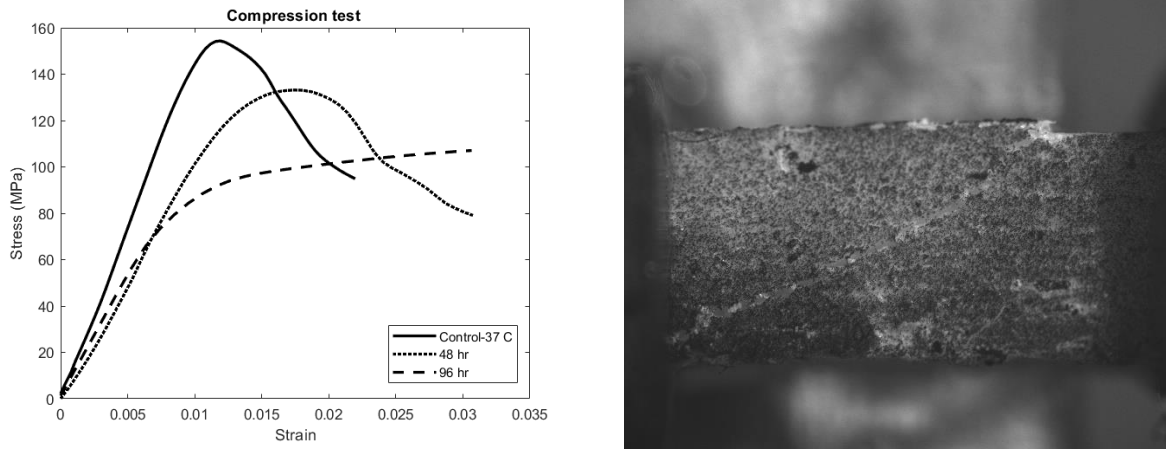


Figure 17. (left) Representative stress-strain curves from compression testing of specimens exposed to increasing levels of oxidation ($n=4$). (right) A picture of a specimen that failed under compression.

Table 2. Mechanical properties measured under compression for control and oxidised groups.

	Modulus (GPa)	Yield Strength (MPa)	Ultimate Strength (MPa)	Ultimate strain
Control	15.8 (± 2.2)	162 (± 15)	163 (± 15)	0.014 (± 0.003)
48 hr	10.2 (± 1.1)	131 (± 12)	136.7 (± 9.3)	0.018 (± 0.002)
96 hr	10.6 (± 2.1)	101 (± 15)	121 (± 17)	0.034 (± 0.007)
p-values (One way ANOVA)	0.106	0.002	0.043	0.002
p-values (RM One way ANOVA)	0.080	0.003	0.093	0.005

4.1.1.4 Load-unload tests

Load-unload tests were conducted to investigate how controlled oxidative stress impacts the damage behavior of bovine cortical bone. Nine flat dog-bone shaped specimens (n=3 per group) were used, with two groups undergoing oxidation. The specimens were tested using the same setup and grips as the tensile tests (see Figure 13). Tensile cycles of increasing amplitude were performed in displacement control mode, starting at 0.2 mm and increasing by 0.2 mm every cycle.

Figure 18 illustrates a representative load-unload curve, from which several key parameters are derived. These include the Secant modulus degradation E_5/E_4 (Secant modulus degradation between the last and second last cycle), capturing the modulus deterioration in the last cycle, and the permanent strain, ϵ_p , which denotes the residual strain post-anelastic deformation before reloading [278]. Another significant measure is the nonlinear strain, ϵ_{nl} , calculated by Equation 13 using the total axial strain, ϵ_{total} , stress, σ_{total} , and initial elastic modulus values, E_0 from the cycle, all specific to the last loading cycle. It should be noted that all these measures have been calculated for the last loading cycle [279]. Additionally, the load-unload test results provide the elastic release strain energy, W_{er} [278], representing the area under the unloading curve minus the elastic recovery energy. The cumulative areas between successive loading curves, W_{cr} , minus the elastic release strain energy, W_{er} , yield the permanent strain energy, W_p [278]. Finally, the hysteresis energy (W_h) quantifies the area between the loading and unloading curves [278].

$$\epsilon_{nl} = \epsilon_{total} - \frac{\sigma_{total}}{E_0} \quad (13)$$

Figure 19 presents a representative load-unload curve for each level of oxidation exposure. The key measures such as secant modulus degradation (E_5/E_4) between the last and second-to-last cycles, indicated modulus deterioration in the final cycle. Both permanent strain (ϵ_p) and nonlinear strain (ϵ_{nl}) decreased with higher oxidation [279]. These measures were calculated based on the last loading cycle. Energy dissipation measured through elastic release strain energy, permanent strain energy, and hysteresis energy also decreased with increased oxidation [279]. Detailed measures for each time point are reported in Table 3. These various measures were derived from the load-unload curves (See Figure 18).

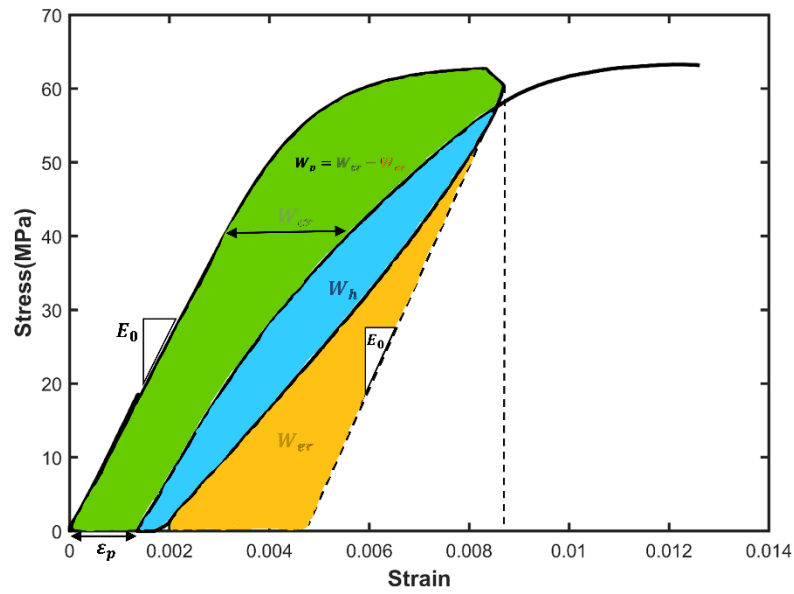


Figure 18 . Measures obtained from load-unload test data.

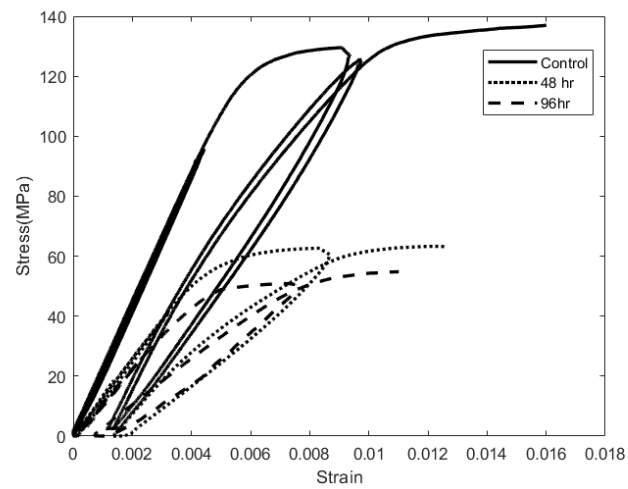


Figure 19. Representative stress-strain curves from load-unload tests for each oxidation exposure level.

Table 3. Characteristics of load-unload behavior at different oxidation exposures.

Property	Control	48 hr	96 hr	p-value
E5/E4	0.700 (± 0.040)	0.588 (± 0.036)	0.498 (± 0.045)	0.008
Permanent strain (%)	0.136 (± 0.030)	0.136 (± 0.036)	0.070 (± 0.011)	0.09
Nonlinear strain (%)	0.40 (± 0.14)	0.40 (± 0.05)	0.29 (± 0.13)	0.5
Elastic release strain energy (MJ/m ³)	0.121 (± 0.022)	0.045 (± 0.006)	0.020 (± 0.003)	<0.001
Permanent strain energy (MJ/m ³)	0.155 (± 0.016)	0.092 (± 0.012)	0.061 (± 0.012)	0.001
Hysteresis energy (MJ/m ³)	0.113 (± 0.025)	0.059 (0.009)	0.043 (0.007)	0.012

4.1.2 Sub-task 1-2: Chemical assay analysis

Oxidized and control bone specimens (n=51) were demineralized using 0.5 M ethylenediaminetetraacetic acid (EDTA) at pH 7.5-8.0 for approximately two months [280]. The solution was refreshed weekly. After demineralization, a small portion of the specimens dedicated for chemical assay analyses, were digested using 1 mg/ml trypsin in Hank's buffer [281][282] to prepare for chemical assay analysis.

Carbonyls were quantified as stable markers of protein oxidation using a modified fluorescence assay. Briefly, after trypsin digestion, 50 μ l aliquots were mixed with fluorescein-5-thiosemicarbazide (FTC) and incubated overnight. The protein was precipitated with trichloroacetic acid (TCA), washed with acetone, and dissolved in guanidine hydrochloride (GuHCl). Fluorescence was measured using a microplate reader with a standard curve of known FTC concentrations. Collagen content was measured via hydroxyproline colorimetry. Digested samples underwent acid hydrolysis and neutralization, followed by conversion of hydroxyproline to pyrrole-2-carboxylate using Chloramine-T and p-Dimethylaminobenzaldehyde (DMAB). Absorbance was measured at 560 nm. Carbonyl content was normalized to collagen content to determine carbonyl concentration per μ mol of collagen. The carbonyl fluorescence assay method employed in this study is an adaptation of previous methodologies [179].

The carbonyl contents are presented as medians and Inter-Quartile Ranges (IQR) because the data was not normally distributed [283] (see Figure 20). Data were analyzed using the Kruskal-Wallis test and Dunn's comparison post-test, with significance at $p < 0.05$ (see Table 4). All statistical analyses were performed using SigmaStat (Version 4.0, Systat Software Inc., CA, USA).

Figure 20 includes carbonyl contents from various mechanical test specimens for 19 controls, 15 specimens oxidized for 48 hours, and 15 specimens oxidized for 96 hours (median $\pm$ IQR). Additionally, to demonstrate that the oxidative damage induced in the bovine specimens is at levels comparable to those observed in aging and inflammatory diseases like T2D and CKD, the carbonyl content in bovine bone samples was contrasted with human data. This data was acquired from an unpublished study conducted within our laboratory, involving 13 young healthy donors (10 male, 3 female, average age=47.4) and 16 older donors with inflammatory diseases (13 male, 3 female, average age=77.1, all exhibiting T2D and/or CKD histories).

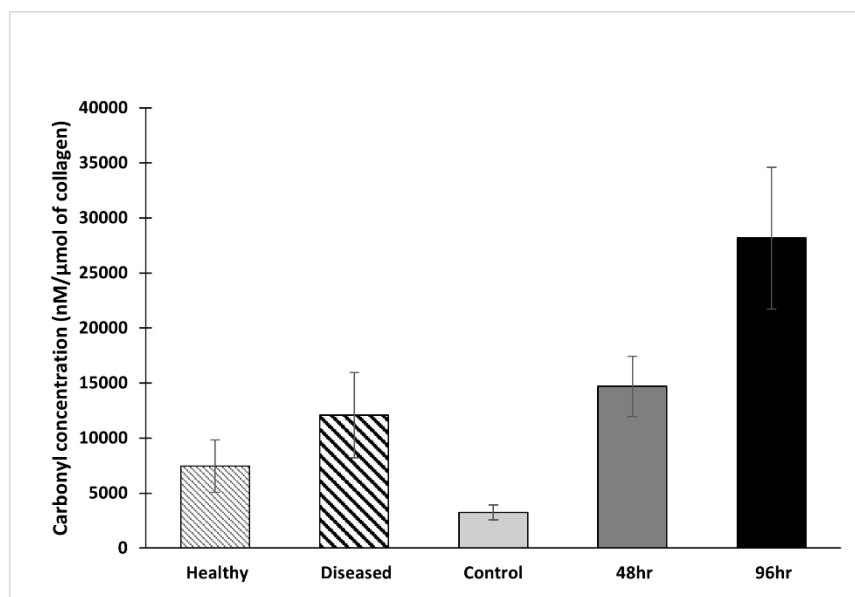


Figure 20. Carbonyl contents for control and oxidized groups compared with human bone collagen from nominally Healthy and Diseased donors. Data is presented as median $\pm$ IQR.

Table 4. Statistical comparison of control and oxidised groups.

Comparison	Control vs. 48 hr	Control vs. 96 hr	48 hr vs. 96 hr
P-value	0.002	<0.001	0.013

4.1.3 Sub-task 1-3: Thermo-mechanical Analyses

To investigate how the oxidative damage affected the bone collagen in terms of collagen nativity and connectivity, thermomechanical techniques were used. Demineralized bone specimens, part of which

was used for chemical assay analyses, underwent thermomechanical analysis using Differential Scanning Calorimetry (DSC) and Hydrothermal Isometric Tension (HIT).

I followed the established methods [284][192] for running differential scanning calorimetry (DSC) test using a TA Instruments DSC Q-2000 system. During DSC, the heat flow difference between a specimen in a sealed pan and an empty reference pan is measured as both are heated at a constant rate. This differential heat flow, which increases at the specimen's melting point (or denaturation for collagen), is recorded as an endotherm on a heat flow vs. temperature plot, indicating the collagen's thermal properties. Here, the specimens were sealed in aluminum pans and heated from 25°C to 95°C at a rate of 1.4°C/min. The resulting heat flow vs. temperature curves were analyzed to determine key metrics: onset temperature (T_{onset}), peak heat flow temperature (T_{peak}), enthalpy of denaturation (ΔH), and full width at half maximum (FWHM) of the denaturation peak (Figure 21-A). Post-test, specimens were lyophilized and weighed to calculate collagen density by dividing the dry mass by specimen volume.

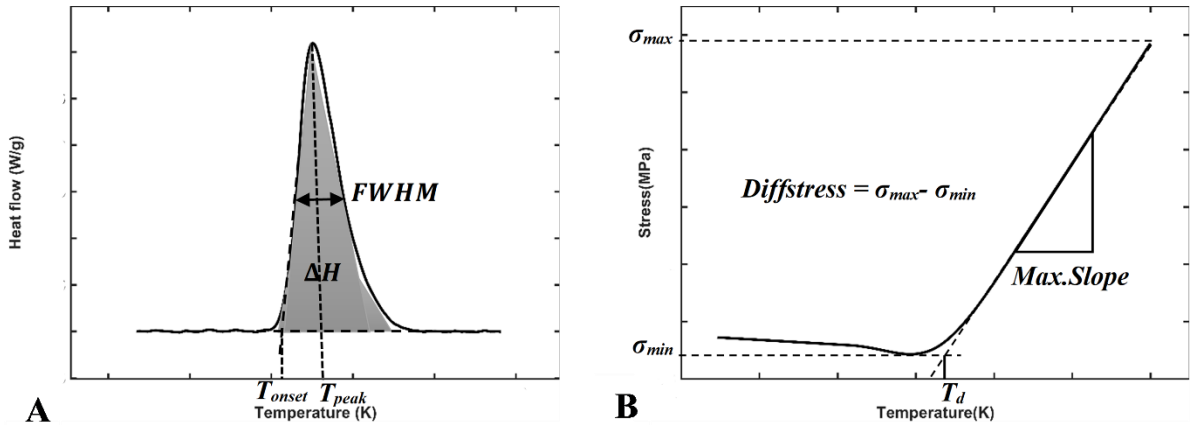


Figure 21. The various empirical measures taken from A) a typical Hydrothermal Isometric Tension (HIT) curve and B) a typical differential scanning calorimetry (DSC) curve

For the HIT test, a custom-built system, which can test six specimens simultaneously, was utilized. The HIT specimens, constrained under isometric conditions, were heated in a 4L water bath from room temperature to 90°C at an average rate of 1.4°C/min (equal to the heating rate of DSC). In an HIT test, as heating progresses, the collagen network denatures, transitioning from a triple helix to a random amorphous coil structure. The temperature at which this occurs is called the denaturation temperature (T_d in Figure 21-B). This transition causes the collagenous tissue to shrink, but due to the isometric constraint, tensile stress is generated instead. During the test, temperature and tensile load are recorded

and the tensile load is converted to stress by dividing it by the specimen's initial cross-sectional area. From the stress versus temperature curve, the maximum rate of change in isometric tensile stress is denoted as Max.Slope (refer to Figure 21-B), reflecting the overall connectivity of the collagen network [16][192]. The difference between the minimum and maximum stress during the test is computed and referred to as Diffstress (see Figure 21-B).

For HIT, specimens were cut into dimensions of 20mm×2mm×1.5mm, while circular DSC specimens (4-mm diameter) were prepared using a biopsy punch. The height of each DSC specimen was measured with digital calipers to estimate volume.

From the two sets of test data, six measures were derived: three from HIT (Max.Slope, T_d, Diffstress) and three from DSC (ΔH , T_{peak}, FWHM). Pearson correlations between these measures and carbonyl concentration were investigated (See Table 5). Notably, Max.Slope ($r=-0.46$, $p=0.0038$) and its covariate Diffstress ($r=-0.48$, $p=0.0024$) as well as FWHM ($r=-0.42$, $p=0.007$) had a significant negative correlation with carbonyl content. Also, T_d ($r=0.36$, $p=0.03$) showed a positive correlation with carbonyl content.

Table 5. Pearson correlation results for carbonyl concentration versus other measures of DSC and HIT for the control and oxidised groups.

<i>Carbonyls vs.</i>	<i>r</i>	<i>p-value</i>
Max.Slope	-0.46	0.0038
T_d	0.36	0.03
Diffstress	-0.48	0.0024
ΔH^*	ns	0.47
T_{peak}	ns	0.8
FWHM	0.36	0.029

*Enthalpy of denaturation.

ns = not statistically significant

Bold r values are indicative of significant correlation

4.2 Task 2: Develop a model of HIT-DSC testing to aid the interpretation of HIT data during bone collagen quality assessment

This section presents a relatively simple linear systems model of HIT testing which was proposed to combine the denaturation kinetics from DSC measurements with a new model for isometric tension generation. The model outputs two parameters, α and β , that have physical meaning and aid in the assessment of collagenous tissue quality in terms of connectivity and nativity.

To validate the model, it was applied to two sets of human bone collagen data, totaling 134 data points, obtained from previous studies [285]. The first study examined human bone collagen with and without a history of type 2 diabetes (T2D) and/or chronic kidney disease (CKD). The second study involved human bone collagen specimens, half of which underwent ribose treatment, while the other half did not. It should be noted that ribose treatment of bone generally involves soaking the bone in a solution that includes ribose, a naturally occurring crosslinking agent known for its biocompatibility [286][287]. Ribose is a naturally occurring sugar in the body and the ribose treatment of bone can induce new cross linking such as accumulation of AGEs in bone [285] which can affect the mechanical properties of bone [286][288][90][289].

To validate the model parameters, correlation analyses were conducted between α and β and various HIT and DSC measures, including Max. Slope, Td, ΔH , age, duration of type 2 diabetes, among others. Note that a more detailed presentation of the presented results in this section can be found in Appendices B-C, each of which includes a published peer-reviewed paper.

4.2.1 Sub-task 2-1: Propose the linear systems theory model to bridge DSC and HIT

An early investigation conducted by Le Lous et al. [186] sheds light on the connection between paired HIT and DSC test results. While informative, their research lacked a quantitative assessment of the relationship between HIT and DSC tests, prompting the question of why the final segment of the HIT curve couldn't be anticipated by DSC curves despite the tight connection in kinetics between these tests. This gap motivated the development of our mathematical framework to bridge DSC and HIT analyses. Consequently, a combination of convolution integrals and optimization techniques was employed to establish connections between DSC and HIT test results. Prior to this study, a quantitative, physics-based model was missing.

Convolution is a mathematical operation that processes an input signal and generates an output signal using the impulse response unit of the system. If the system's input is represented as $x_{in}(t)$, and the system's impulse response is denoted as $J(t)$, the system output, $x_{out}(t)$, can be predicted through the convolution integral [290]:

$$x_{out}(t) = \int_0^t J(t-\tau)x_{in}(\tau)d\tau = J(t) * x_{in}(t) \quad (14)$$

The impulse response of a system, $J(t)$, is the system's output when the input signal is the impulse function $\delta(t)$. Please refer to Figure 22.

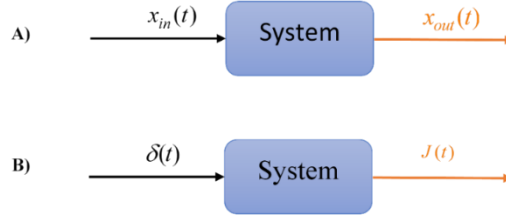


Figure 22. Black-box schematic of the convolution operation. A) Response of the system to a typical input signal, $x_{in}(t)$ B) Response of the system to impulse function, $\delta(t)$

In this context, the DSC and HIT signals are considered as the input and output signals, respectively. The impulse response is regarded as a function of absolute temperature rather than time since, in both the HIT and DSC tests, time correlates linearly with temperature via the heating rate. The proposed impulse response is as follows:

$$J(T) = \alpha + \beta \times (T - T_{onset}) \times u(T - T_{onset}) \quad (15)$$

Where $u()$ is the unit step function, and α and β are two parameters developed to provide a physics-based model. The nature of the DSC and HIT tests provide the reason for this proposed function. The isometric tension in the first part of the HIT curve generates depending on the total number of denatured collagen molecules. Thus the first part of HIT is directly affected by the DSC signal because the DSC signal (the rate of heat flow into the sample) is proportional to the rate of collagen denaturation [186]. This proportionality is captured using the multiplier α . Therefore, α represents the amount of instantaneous isometric tension developed per unit of collagen denaturation.

In the final part of the HIT curve, the additional evolved isometric tension is proportional to the absolute temperature as in rubber elasticity. Therefore, after onset of denaturation (which is captured by step function to make sure that the denaturation has occurred), the proportionality between T and the generated isometric tension is captured with the coefficient β . The parameter, β , measures the connectivity of the denatured collagen network. In eq. (15), T_{onset} marks the onset of denaturation in the DSC endotherm.

By this decomposition, the total isometric tension developed by each collagen packet during HIT testing (σ_{HIT}) is the sum of tension generated by the denaturation process (σ_{DSC}), and the tension developed due to rubber thermo-elasticity ($\sigma_{rubber-elasticity}$). Therefore,

$$\sigma_{HIT} = \sigma_{DSC} + \sigma_{rubber-elasticity} \quad (16)$$

Based on dimensional analysis, it is necessary to incorporate collagen density in the tissue to enable comparison of model parameters, α and β , across various tissues and be able to run correlation analysis on them [291]. With the inclusion of collagen density, parameter α becomes dimensionless, while parameter β is expressed in units of K^{-1} .

In the modified version of this model, a new term was added to the optimization parameters to account for the initial stress relaxation that happens in some of the HIT tests. Thus, the equation for the output signal would have the following form.

$$x_{out}(T) = \rho_{collagen} \cdot [\alpha + \beta \cdot (T - T_{onset})u(T - T_{onset})] * x_{in}(T) + AT + B \quad (17)$$

A schematic of the algorithm used to implement the model is depicted in Figure 23.

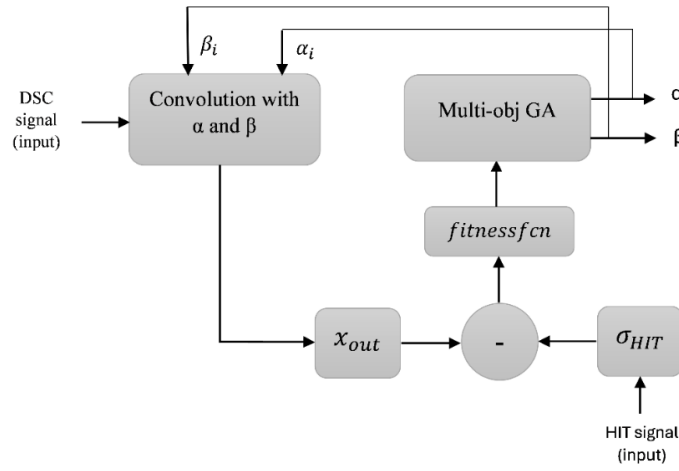


Figure 23. Schematic of the algorithm used to implement the model. After baseline correction of the DSC curves, the DSC signal was convolved with $J(T)$ (Eq.(15)) to form the output signal, x_{out} . The genetic algorithm (GA) optimization technique was then used to find α and β . The fitness function was chosen to be the absolute difference between the real HIT curve and the model output, $x_{out}(T)$, ($fitnessfcn = |\sigma_{HIT} - x_{out}|$).

Figure 24 showcases sample plots that overlay the model output, x_{out} , onto the paired DSC (x_{in}) and HIT data.

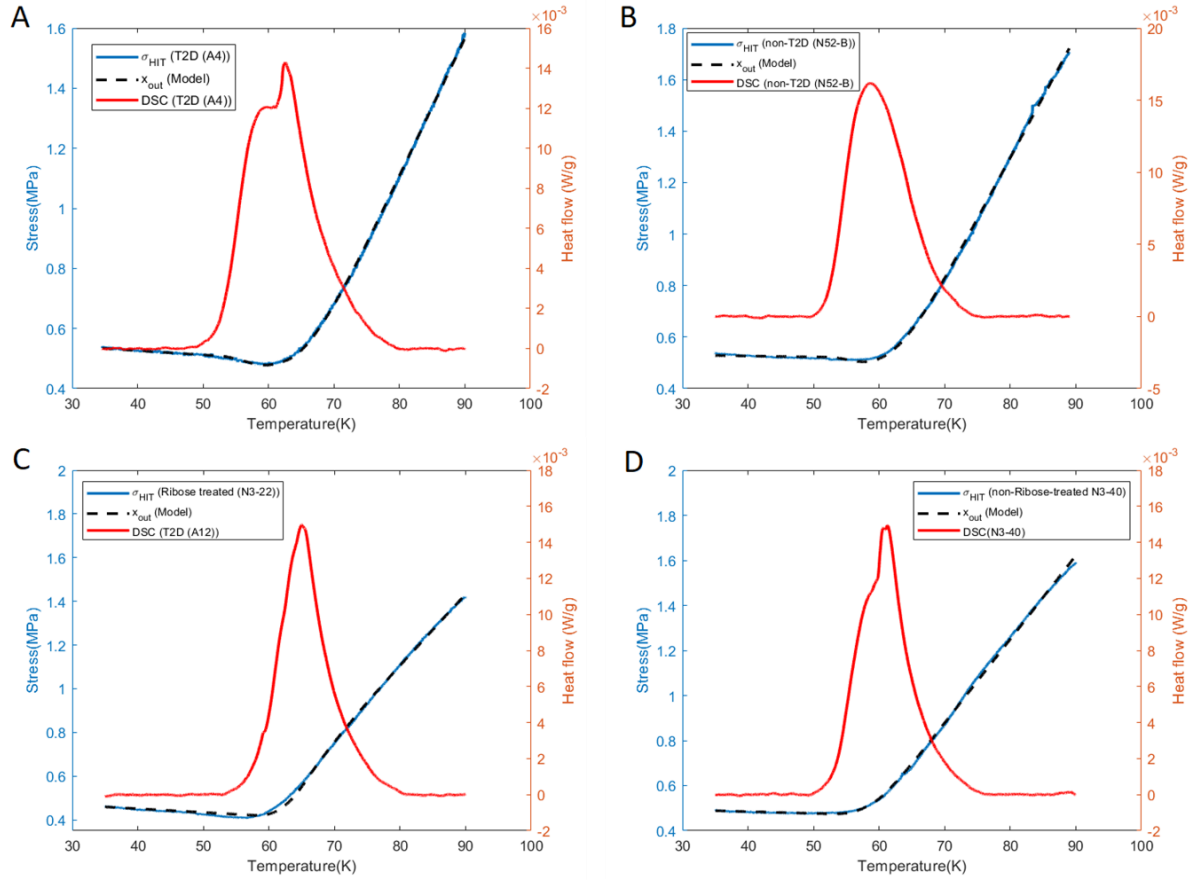


Figure 24. DSC and HIT data and the resulting model curve (x_{out}) for four example specimens.

4.2.2 Sub-task 2-2: Verification of the model using two human bone collagen datasets

In the preliminary study, the model has been applied to limited amount of data including two native bovine bone collagen specimen, one ribose-treated bovine bone collagen specimen, and one irradiated bovine bone collagen specimen [291]. However, due to the limited data used in the model's application, questions remained regarding its capability and applicability across various datasets, specifically human specimens.

To address this concern, a complementary study expanded the application of the model to include a wider array of HIT-DSC data. This dataset consisted of human bone collagen specimens from both healthy and diseased groups, as well as ribose-treated cases. In addition to the application of the model to a broader dataset, Pearson correlation analyses were conducted to examine the relationship between the model's two parameters, α and β , and various other metrics. These metrics encompassed empirical

measures from the HIT test, such as Max. Slope (maximum slope of the HIT curve) , T_d (temperature at which the collagen denaturation starts), and diffstress (refer to Figure 21-A), as well as measures from the DSC test, such as enthalpy of denaturation (ΔH) and full width at half maximum (FWHM) (refer to Figure 21-B). Additionally, demographic factors such as age, and duration of type 2 diabetes were included in the analysis.

The results of these correlation analyses provided valuable insights into the validity of the model's parameters, α and β . For instance, certain measures like T_d and Max.Slope are widely regarded as independent indicators of collagen network stability and connectivity, respectively [16]. Moreover, previous researches have shown a positive correlation between Max. Slope and the fracture resistance of human cortical bone [15][16].

This broader analysis aimed to verify and validate the proposed model by assessing its performance across a wider human dataset. Specifically, the study aimed to investigate whether the two model parameters, α and β , proposed to reflect bone collagen quality, exhibited correlations with experimentally determined measures such as Max. Slope, T_d , age, and disease duration. This process included further validation of Max. Slope as an independent indicator of collagen network connectivity.

The main hypothesis of this study was that α would correlate strongly with HIT denaturation temperature, T_d , and that β would correlate positively and strongly with Max.Slope.

4.2.2.1 Application of the model to human diseased and control groups

The first application of the model targeted a group comprising HIT and DSC test results obtained from 74 donors. Among these, 38 donors (15 males; mean age= 74 ± 12 years old and 23 females; mean age= 68 ± 12 years old) had a history of type 2 diabetes (T2D) with or without CKD, while the remaining 36 donors (17 males; mean age= 72 ± 14 years old and 19 females; mean age= 68 ± 10 years old) had no history of T2D and/or CKD.

In this specific cohort, Pearson correlations were examined between α and β and seven predictors listed in Table 6. Notably, the duration of T2D was relevant only to the T2D donor subset. Also, due to missing data (T2D duration), four specimens from this subset were excluded from this analysis.

The results reported in Table 6 revealed that the two HIT measures, Max. Slope ($r=0.58$, $p < 0.0001$, Figure 25), along with its covariate Diffstress ($r=0.52$, $p < 0.0001$), exhibited strong correlations with parameter β . Additionally, a weak negative correlation ($r= -0.23$, $p=0.04$) was observed between the

DSC measure, FWHM, and β . Moreover, the denaturation temperature, T_d , indicative of collagen network thermal stability, demonstrated a notable association with α ($r=-0.58$, $p<0.0001$), while a weak correlation was intriguingly noted between the duration of type 2 diabetes (T2D duration) and parameter α ($p=0.036$).

Table 6. Pearson correlation results for α and β versus experimental measures for the Type 2 diabetic (T2D) specimens.

α vs.	r	p-value	β vs.	r	p-value
Max.Slope	na	0.42	Max.Slope	0.58	<0.0001
Age	na	0.85	Age	na	0.83
T2D duration	0.36	0.036	T2D duration	na	0.52
T_d	-0.58	<0.0001	T_d	na	0.2
ΔH^*	0.40	0.0004	ΔH^*	na	0.07
Diffstress	na	0.07	Diffstress	0.52	<0.0001
FWHM	na	0.57	FWHM	-0.23	0.04

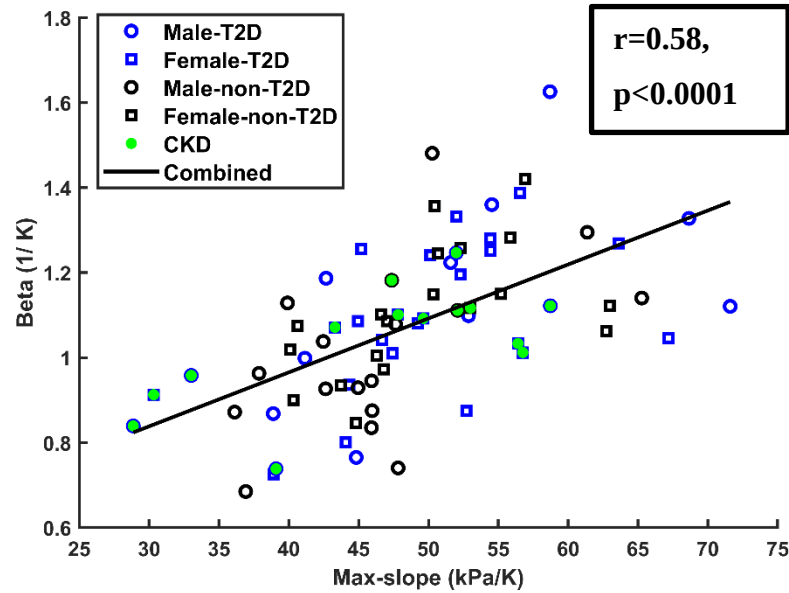


Figure 25. β versus the collagen connectivity measure, Max.Slope. A relatively strong correlation was found over a wide range of specimens and diseased groups.

4.2.2.2 Application of the model to human ribated and control groups

In the second phase of the model's verification, specimens from 15 donors (comprising 8 males with an average age of 56 ± 5 years and 7 females with an average age of 54 ± 5 years) were utilized, including HIT and DSC specimens. From each femur in the study, four cortical bone samples were

procured from the lateral mid-shaft. Among these samples, two were randomly designated to the control group (without ribose incubation), while the remaining two were assigned to the ribation group. The specimens were then subjected to a 4-week incubation in 0.1M ribose at 37°C [285].

The examination of the ribation study specimens was directed towards five predictors (Max.Slope, T_d , diffstress, ΔH , and FWHM), as age and T2D duration were not applicable (refer to Table 7). The analysis revealed parallels with the initial assessment on diseased group (refer to Table 6), demonstrating a positive correlation between both Max.Slope ($r=0.55$, $p<0.0001$) and diffstress ($r=0.53$, $p<0.0001$) with β . Furthermore, a negative correlation was observed between T_d , an indicator of thermal stability, and α ($r=-0.5$, $p<0.0001$), consistent with previous findings with the diseased group. Interestingly, two new correlations between α and diffstress as well as β and enthalpy of denaturation emerged in this investigation.

To compare α parameter values between the two groups (0.0 M ribose and 0.1 M ribose), a student t-test was employed. The findings, depicted in Figure 26, signify a significant difference ($p<0.05$) in the α parameter for bone samples incubated in 0.1 M ribose. This outcome corresponds with the findings of [285], where a significant distinction was observed in the T_d values between the two ribation groups.

Table 7. Pearson correlation results for α and β versus other measures from the ribation study.

α vs.	r	p-value	β vs.	r	p-value
Max.Slope	na	0.57	Max.Slope	0.55	<0.0001
T_d	-0.495	0.0001	T_d	na	0.41
ΔH^*	na	0.87	ΔH^*	-0.47	0.0002
Diffstress	0.44	0.0004	Diffstress	0.53	<0.0001
FWHM	na	0.14	FWHM	na	0.06

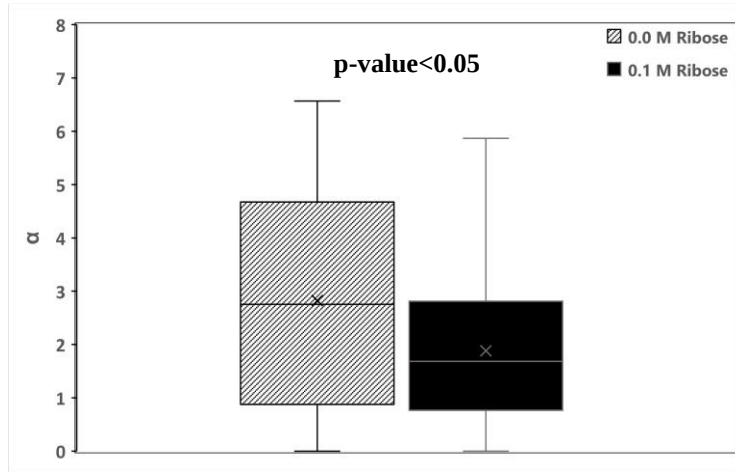


Figure 26. The effect of ribose incubation of human bone collagen on the model parameter α .

4.2.2.3 Application of the model to bovine oxidized bone specimen

The model was applied to the test results from HIT-DSC tests on bovine oxidized specimens. The thermo-analytical test details were addressed in section 4.1.3. Similar to previous datasets, the correlation between the model parameters, α and β , and the six measures from HIT and DSC, namely Max.Slope, T_d , diffstress, ΔH , and FWHM was explored. According to the results reported in Table 8, similar to the previous investigations, there was a positive correlation between Max.Slope ($r=0.47$, $p=0.0036$) and Diffstress with β ($r=0.48$, $p=0.0029$), as well as a negative correlation between T_d and α ($r=-0.61$, $p<0.0001$).

Table 8. Pearson correlation results for α , β and carbonyl concentration versus other measures of DSC and HIT testing for the control and oxidised groups.

α vs.	r	p-value	β vs.	r	p-value
Max.Slope	na	0.46	Max.Slope	0.47	0.0036
T_d	-0.61	<0.0001	T_d	na	0.21
Diffstress	na	0.91	Diffstress	0.48	0.0029
ΔH^*	na	0.28	ΔH^*	-0.51	0.001
FWHM	na	0.24	FWHM	na	0.41

*Enthalpy of denaturation

4.3 Task 3: Constitutive Modeling and Size Effect Analysis of Oxidatively Damaged Cortical Bone with Application to aging and disease states

This chapter explores the generation of a constitutive model of oxidatively damaged cortical bone. The model incorporates elastoviscoplastic deformation mechanisms coupled with damage accumulation, aiming to simulate the mechanical behavior of bone under various levels of oxidative stress and implementing this model into a user material subroutine (UMAT) implemented in commercial finite element software Abaqus. As discussed in section 2.6.2.2, the major elements of the constitutive model (including yield/damage criteria, evolution functions, and flow rule) are based on the model developed by Schwiedrzik and Zysset [247], which is one of the most robust models developed for bone tissue to date. The details of this model, including the constitutive equations, failure criterion, etc., were discussed in section 2.6.2.2. This model has been adapted and fine-tuned to incorporate the effects of oxidation using the experimental results reported in section 4.1 and the model presented in section 4.2.

This chapter also investigates the size effect on the microdamage process zone (MDPZ), a critical toughening mechanism in bone, using the model. It is noted that a more detailed presentation of the presented results in this section can be found in Appendix D, which includes a manuscript currently under preparation.

4.3.1 Sub-task 3-1: Integration of Oxidative Stress Effects into the Constitutive Model

In this research, due to the in vitro nature of the oxidation method employed, oxidative damage does not evolve over time, unlike in vivo conditions. Although oxidative damage influences mechanical damage in bone—and while oxidation might accelerate or decelerate the rate at which mechanical damage occurs—the fundamental mechanisms underlying mechanical damage in diseased bone are presumed to remain consistent with those in healthy bone. To integrate the impact of oxidative damage into the constitutive model for bone material, we adopted the methodology proposed by Murakami and Mizuno [275], for incorporating the effects of irradiation into a creep-hardening surface model. With a similar approach, the impact of oxidative damage was incorporated into the constitutive model by modifying the coefficients of the constitutive and evolution equations (as outlined in Section 2.6.2.2). Hence, the coefficients of the constitutive equations are substituted with corresponding material functions of variables that measure oxidative damage. These measures include carbonyl content (CC) derived from chemical assays (detailed in Section 5.2.4.1.2) and the model parameters α and β obtained

from the use of the HIT-DSC model (refer to Section 4.2). These biomarkers are crucial because they quantify the extent of oxidative damage in the bone collagen.

The coefficients of the constitutive equations (as reviewed in Section 2.6.2.2) can be classified into several categories. These parameters, which are enumerated in Table 9, include the elastic constants (constants of the elasticity tensor $\mathbb{E}$ for transverse isotropy), parameters related to the cortical bone yield criterion (Y_t^+, Y_t^-), hardening parameters, (y_r, k_s), the parameter of the damage law (k_p), and viscous parameters (η, m).

Table 9. Coefficients of the constitutive model

	Coefficients of the oxidized bone model
Elastic constants	$E_t, E_l, \nu_t, \nu_{tl}, \mu_{lt}$
Compressive yield strength	$Y_t^-, Y_l^-(CC, \alpha, \beta)$
Tensile yield strength	$Y_t^+, Y_l^+(CC, \alpha, \beta)$
Tensile ultimate strength	$U_t^+, U_l^+(CC, \alpha, \beta)$
Hardening law parameters	$y_r(CC, \alpha, \beta, \dots), k_s(CC, \alpha, \beta)$
Viscosity parameters	$\eta(CC, \alpha, \beta, \dots), m(CC, \alpha, \beta)$
Parameter of the damage evolution law	$k_p(CC, \alpha, \beta)$

Based on the experimental results presented in Section 4.1.1.2, the elastic properties of bone, determined from quasi-static tensile tests, do not exhibit significant changes due to oxidation. This is because alterations in the organic phase of bone predominantly influence the yield and post-yield behavior without substantially impacting the elastic properties. Consequently, the elastic parameters are considered constant in this study.

Moreover, prior research [292][293] indicates that the average collagen orientation in lamellar bone is within 30° of the bone's longitudinal axis, suggesting that collagen fibers are predominantly aligned with this axis. This alignment of collagen is crucial for the longitudinal mechanical properties of cortical bone. Given this context, our analysis is concentrated on the changes in longitudinal properties due to oxidation, as detailed in the experimental section (Section 4.1).

Here, since the constitutive model is furnished to account for the effect of oxidative stress, the fourth-order tensor $\mathbb{A}$, and the second-order tensor $\mathbf{A}$ in eq. (10) change as follows:

$$\begin{aligned}
\mathbb{A} = & \sum_{i=1}^2 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \mathbf{M}_i \otimes \mathbf{M}_i \\
& + \frac{1}{4} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} + \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right)^2 \mathbf{M}_3 \otimes \mathbf{M}_3 \\
& - \sum_{i,j=1; i \neq j}^2 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j \\
& - \frac{1}{4} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} + \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right)^2 \left(\sum_{i=1}^2 \zeta_{i3} \mathbf{M}_i \otimes \mathbf{M}_3 \right. \\
& \left. + \sum_{i=1}^2 \zeta_{3i} \mathbf{M}_3 \otimes \mathbf{M}_i \right) + \sum_{i,j=1; i \neq j}^3 \frac{1}{2T_{ij}^2} \mathbf{M}_i \bar{\otimes} \mathbf{M}_j \\
\mathbf{A} = & \sum_{i=1}^2 \frac{1}{2} \left(\frac{1}{Y_i^+} - \frac{1}{Y_i^-} \right) \mathbf{M}_i + \frac{1}{2} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} - \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right) \mathbf{M}_3
\end{aligned} \tag{18}$$

Based on the above changes in the fourth-order tensor $\mathbb{A}$, and the second-order tensor $\mathbf{A}$ in eq. (10), the yield function defined in eq. (8)(24) changes accordingly.

Also, the hardening function in eq.(9) transforms into the following form:

$$\begin{aligned}
r(\kappa) &= 1 + (1 - y_r(CC, \alpha, \beta, \dots))g(\kappa) \\
g(\kappa) &= 1 - e^{-k_s(CC, \alpha, \beta, \dots)\kappa} \\
y_r(CC, \alpha, \beta, \dots) &= \frac{Y_3^+(CC, \alpha, \beta, \dots)}{U_3^+(CC, \alpha, \beta, \dots)}
\end{aligned} \tag{19}$$

Where U_3^+ is the ultimate tensile strength in the longitudinal direction.

The rate-dependent yield function can be defined by modifying eq. (12), as follows:

$$\bar{Y} = Y(CC, \alpha, \beta, \dots) + \frac{m(CC, \alpha, \beta, \dots)}{2} - \left(\frac{m(CC, \alpha, \beta, \dots)^2}{4} + \frac{\eta(CC, \alpha, \beta, \dots)}{\Delta t} \frac{\Delta \kappa}{h} \right)^{\frac{1}{2}} = 0 \tag{20}$$

4.3.1.1 Implementation of the constitutive model for the oxidized bone

4.3.1.1.1 Numerical algorithm implementation

The numerical algorithm is composed of two iterative processes. The first process, which operates on a local level, incorporates the elasto-viscoplastic behavior of the material over a given time step using a return mapping algorithm (iterating over index i). The second process functions on a global level (iterating over increment number m , integrating the results from the local level into the equilibrium equations and assessing the equilibrium error by calculating the residual. Figure 27 provides a flow chart illustrating this numerical algorithm.

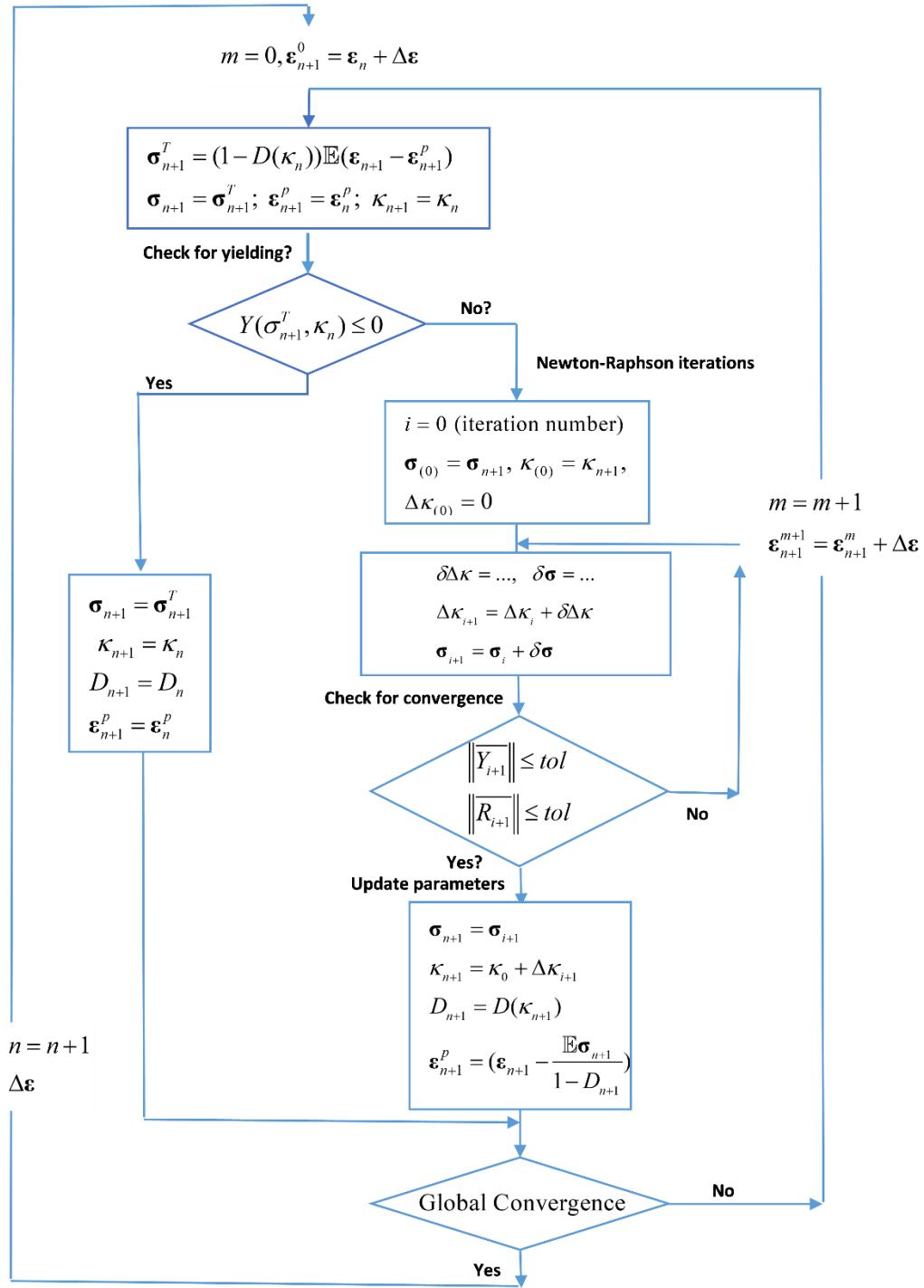


Figure 27. Flow chart of the updating stress-strain tensors and the state variable procedure

For the return mapping algorithm, the residual error of elastic strains and the yield function are linearized with respect to their variables σ and $\Delta\kappa$. This linearization results in a system of equations that is solved using the Newton-Raphson algorithm. By solving this system, the variable increments ($\delta\sigma$ and $\delta\Delta\kappa$) are determined, which are then used to update the values of σ and $\Delta\kappa$ during the return mapping process [247].

4.3.1.1.2 Identification and illustration

To implement the constitutive model for oxidized bone, it is crucial to first define the coefficients listed in Table 9 as functions of oxidative stress biomarkers derived from experiments outlined in Section 4.1. Parameters related to the cortical bone yield criterion ($Y_i^+(CC, \alpha, \beta, \dots), Y_i^-(CC, \alpha, \beta, \dots)$) were determined through uniaxial quasi-static tension and compression tests performed under varying levels of oxidative stress. Additionally, uniaxial tensile tests were utilized to define the hardening parameters ($\gamma_r(CC, \alpha, \beta, \dots), k_s(CC, \alpha, \beta, \dots)$). The damage law parameter $k_p(CC, \alpha, \beta, \dots)$ was identified using load-unload tests conducted across different oxidative stress levels. For viscous parameters ($\eta(CC, \alpha, \beta, \dots), m(CC, \alpha, \beta, \dots)$), uniaxial tensile tests carried out at various strain rates and oxidative stress levels were utilized.

To identify these parameters, mechanical test simulations were conducted to model the uniaxial tensile tests. The geometry and dimensions of the tensile specimens, as detailed in Figure 12, were used in Abaqus FEA software. The model was meshed with 7,007 four-node tetrahedral solid elements. Boundary conditions replicated the experimental setup, with one shoulder of the specimen subjected to a prescribed displacement while the other shoulder was fixed to prevent translational movement. The numerical algorithm, as described in Figure 27, was implemented via a user-defined material subroutine (UMAT) in Abaqus Standard.

To correlate the oxidative damage biomarkers with changes in model parameters, multivariate linear regression was employed. This approach allowed the prediction of bone behavior under oxidative damage by linking mechanical degradation of bone tissue to measurable levels of oxidative damage. Table 10 presents the regression coefficients and the derived linear relationships. These relationships provide the basis for integrating oxidative stress effects into the constitutive model, allowing accurate predictions of bone mechanical behavior under oxidative stress conditions.

Table 10. Multivariate linear regression relationships between coefficients of the constitutive model and oxidation biomarkers.

	Equation	R^2	$p - value$
Longitudinal tensile yield strength	$Y_t^+(CC, \alpha, \beta) = 129.25 - 0.003 CC - 2 \alpha - 13.44 \beta$	0.87	0.00
Longitudinal Tensile ultimate strength	$U_t^+(CC, \alpha, \beta) = 148.41 - 0.0003 CC - 2.2 \alpha - 17.7 \beta$	0.88	0.001
Compressive yield strength	$Y_t^-(CC) = 159.24 - 0.0017 CC$	0.56	0.004
Hardening law parameters	$y_r = 0.88 - 4.099e^{-6}CC - 0.00044\alpha + 0.01\beta$	0.75	0.01
	$k_s(CC, \alpha, \beta) = 41.45 - 0.001CC + 1.8 \alpha + 2.9 \beta$	0.72	0.02
Parameter of the damage evolution law	$k_p(CC, \alpha, \beta) = 18.78 + 0.0018CC + 4.3\alpha + 9.2\beta$	0.86	0.03
Viscosity parameters	$\eta(CC, \alpha, \beta, ...) = 0.56349 - 1.69e^{-5}CC - 0.0049\alpha - 0.057\beta$	0.87	<0.0001
	$m(CC, \alpha, \beta) = 20.72 - 0.0006CC - 0.47 \alpha - 3.76 \beta$	0.87	<0.0001

It's important to highlight that due to the small size of the compression test specimens, as depicted in Figure 12, there was insufficient material for conducting thermo-analytical tests. As a result, only the carbonyl content was measured for these specimens, and the compressive yield strength was determined solely based on the level of carbonylation.

According to the constitutive equations outlined in Section 2.6.2.2, besides the previously discussed coefficients, additional material constants must be defined. These include elastic and yield properties in the transverse direction, as well as interaction coefficients (Y_t^+ , Y_t^- , γ_{t3} , ζ_{tt} , ζ_{t3} , ...). Given that the material studied is bovine cortical bone, the mechanical properties of bovine cortical bone were utilized for these constants (Table 11) [195], [197], [294], [295].

Table 11. Overview of the material constants

Elastic properties	E_t (MPa)	E_l (MPa)	ν_t	ν_{t3}	μ_{3t}
	10000	20000	0.46	0.35	4300

Material constants	Y_t^+ (MPa)	Y_t^- (MPa)	γ_{t3} (MPa)	ζ_{tt}	ζ_{t3}
	48	125	54	0.3	0.5

As an illustration of the calibration process, Figure 28 shows the results of the calibrated finite element model, which uses identified parameters alongside experimental uniaxial tensile tests conducted at various strain rates. The data is presented for three oxidation levels, indicated by oxidation hours. For each oxidation duration, the calibration utilized three critical regions from the experimental stress-strain curves: the linear regime, the yield point, and the post-yield region. Additionally, Figure 29 depicts a typical longitudinal stress field, revealing the expected uniform distribution of uniaxial stress along the gauge length.

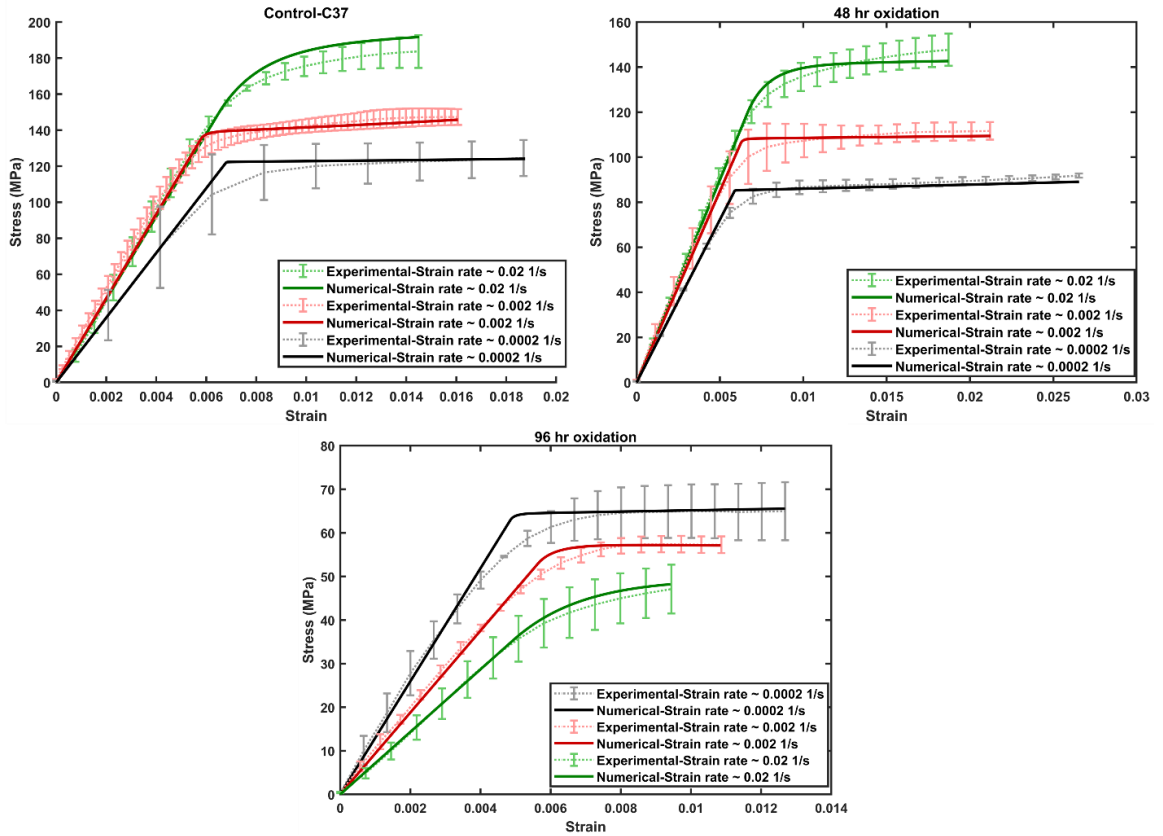


Figure 28. Experimental vs. computational uniaxial tensile curves for different incubation time points

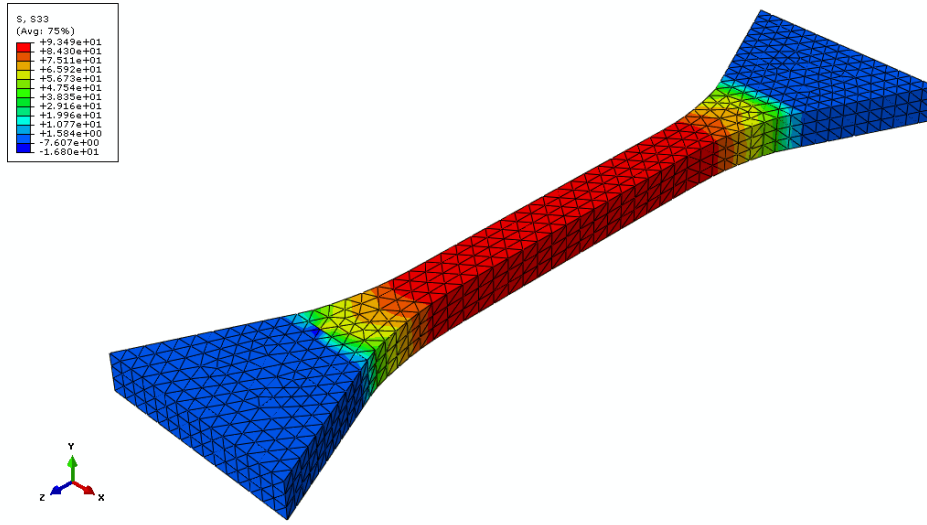


Figure 29. The stress field in the longitudinal direction (z) in the finite element model of a tensile test specimen.

4.3.2 Sub-task 3-2: Experimental validation of the model

Before validation simulations, single element verifications were conducted in both longitudinal and transverse directions for tension and compression cases (Appendix E). Three-point single edge notched bending (SENB) tests was modeled using the developed user-material subroutine. The specimen ($50 \text{ mm} \times 4 \text{ mm} \times 4 \text{ mm}$) had a pre-notch with a prescribed length (2mm), and only half of the specimen was modeled due to symmetry (refer to Figure 30). The model's length aligned with the bone's longitudinal direction (Z direction). The mesh included 57,607 unstructured tetrahedral elements, with a finer mesh around the notch tip. Boundary conditions simulated supports and restricted translational movement. A prescribed displacement was applied along the specimen's symmetric line, with one pinned support fixed in the vertical direction, and nodes on the symmetric face constrained horizontally and rotationally. To mimic experimental conditions at the supports, movement perpendicular to the page was restricted to ensure stability.

The bone was modeled in two states: a control state without oxidation and a γ -irradiated state to simulate extreme oxidative stress.

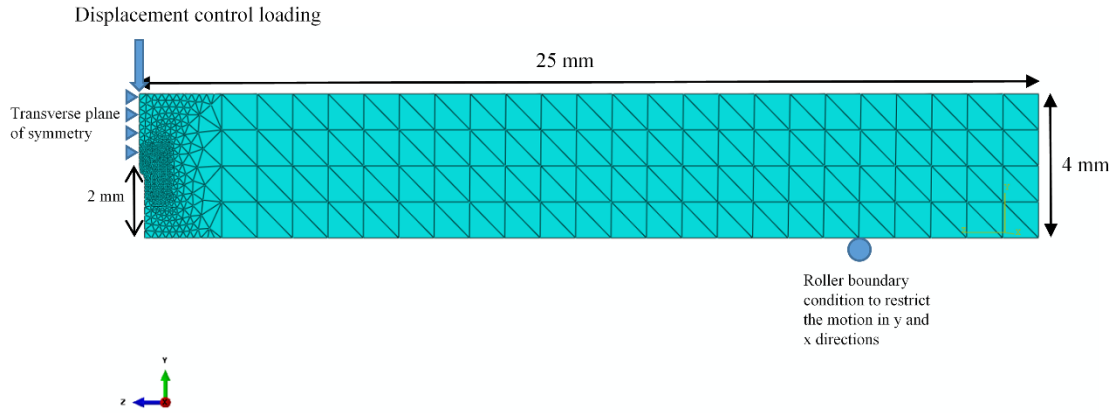


Figure 30. Mesh pattern and boundary conditions as well as the dimensions of the SENB model.

Figure 31 presents the load-deflection curves for the SENB model up to the peak load, at which significant crack growth initiates during the test. Both experimental results from a previous study [296] and computational results from the generated model are shown. The agreement between the experiment and model is strong, with a mean absolute error of 7%. A mesh sensitivity analysis confirmed the adequacy of the mesh size (Appendix F).

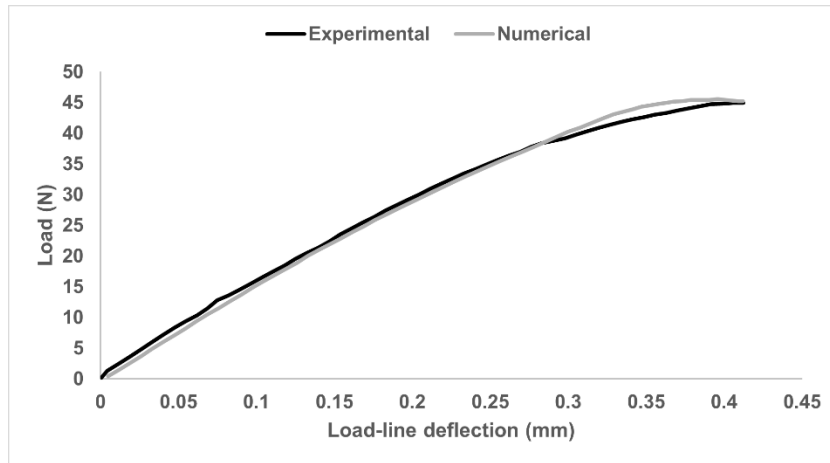


Figure 31. Experimental and computational Load vs. load-line deflection curves for SENB testing of bovine cortical bone

Figure 32 displays, on the left side, the DIC strain fields in the longitudinal direction of the SENB sample at peak load. The red regions indicate strain exceeding 0.5%, the strain corresponding to the onset of micro-damage process zone [297]. This threshold strain aligns with the values reported in the literature at which non-linearity (micro-damage) initiates in cortical bone during uniaxial tensile testing

[298][299]. The figure also compares strain fields from the model (grey, on the right) and experimental data (red, on the left), highlighting both the morphology and size of the micro-damage process zone where strains surpass 0.5%.

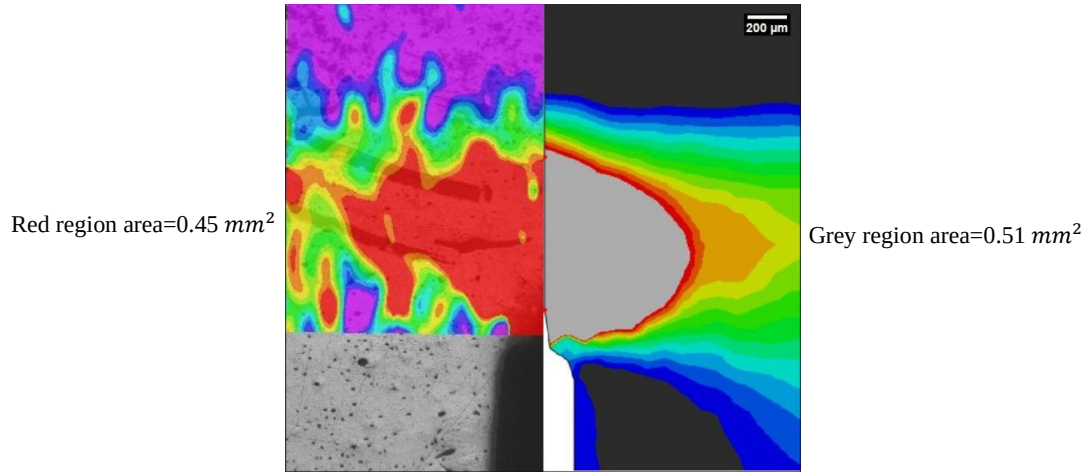


Figure 32. Comparison between the micro-damage process zones from the model (gray color area in the right) and from experimental DIC strain measurements (red color area in the left) for a SENB test of bovine cortical bone at peak load in Figure 10). Note the model does not account for tissue heterogeneity.

Next, the model was applied to simulate micro-damage zone formation in γ -irradiated bovine cortical bone samples. Despite different methods—gamma radiation and chemical oxidation—both induce oxidative damage, leading to comparable damage mechanisms. In a prior study [286], bovine cortical bone was subjected to gamma irradiation with a 33 kGy dose, followed by conducting 3-point bending tests on the SENB specimens. We used the experimental data from the aforementioned study. Given that the method used to generate oxidation in our study is a different method based on oxidation time, and [286] used gamma-irradiation, we determined the equivalent oxidation time to be approximately 50 hours for similar amount of oxidative stress, based on load-deflection curves (see Figure 33).

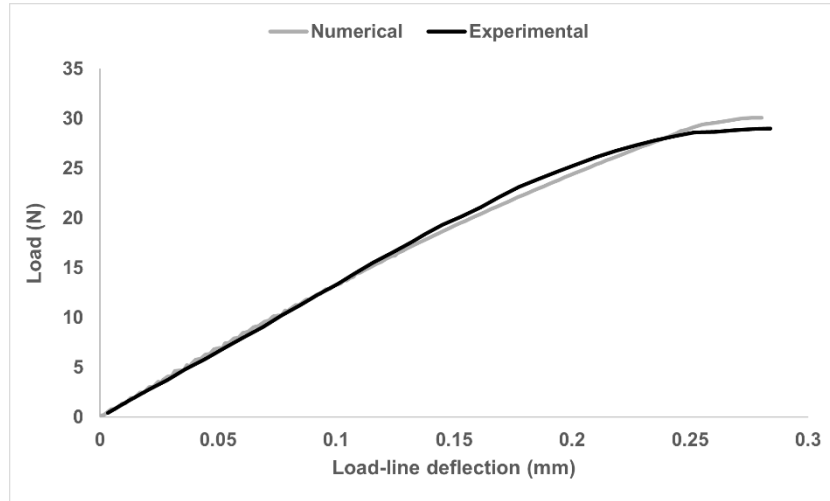


Figure 33. Experimental and computational (calibrated) Load vs. load-line deflection curves for SENB testing of irradiated bovine cortical bone.

The micro-damage zone in the specimen irradiated with a 33 kGy dose was modeled and compared to experimental data from [192]. That study reported a 49% reduction in the micro-damage process zone volume due to irradiation, measured as the decrease in damage volume (DV) relative to the region of interest (TV). In our model (see Figure 34), the micro-damage area decreased from 0.51 mm² to 0.21 mm², reflecting a 41% reduction.

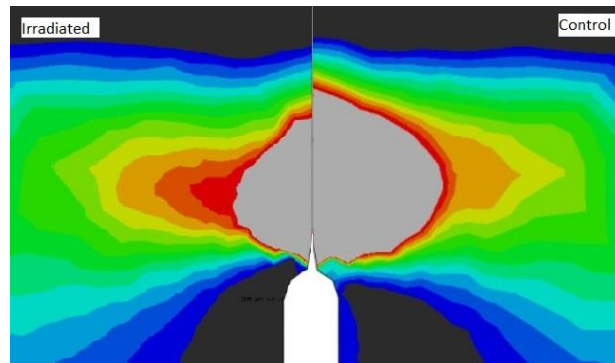


Figure 34. The micro-damage process zone models for control vs. irradiated SENB bovine specimens. Grey indicates the MDPZ.

4.3.3 Sub-task 3-3: Exploring the size effect on the microdamage process zone (MDPZ) using the established constitutive model

One critical aspect that has emerged from the investigations of MDPZ in bone tissue is the observed size effect. This phenomenon, where certain material behaviors vary with specimen size, has been notably evident in bone fracture toughness [300][301]. The size effect carries significant implications

for predicting bone fracture behavior, highlighting the necessity for a thorough understanding of bone mechanics that goes beyond traditional fracture mechanics analyses.

Central to the size effect in bone is the concept of fracture toughness, which represents the material's resistance to crack propagation [302]. Although fracture toughness is an intrinsic property of bone, its variation with specimen size has drawn increasing attention in recent years [300], [301], [303], [304]. Research has shown that as the size of bone specimens changes, fracture toughness markedly changes as well, indicating a size-dependent enhancement in resistance to crack growth [300], [301], [303], [304]. This observation challenges conventional assumptions about bone mechanics and underscores the need to account for size effects in bone fracture analyses.

It seems that the role that the MDPZ plays in enhancing the toughness of bone material caused the size effect to extend beyond fracture toughness to encompass the size-dependency of the MDPZ in bone material. Understanding the size-dependent behavior of the MDPZ, which has received very little attention so far, is essential for clarifying the mechanisms governing crack initiation and fracture processes in bone.

To investigate the specimen size effect on the MDZP size, simulations were conducted using four geometrically proportional SENB models with a size scaling factor of $(1:\sqrt{6}:6:10)$, as described in references [301] and [300], while also incorporating different oxidation levels. The test specimens had widths (W) of 3 mm, 7.35 mm, and 18 mm, and 30 mm while their thickness (B) remained constant at 4 mm. The span-to-width (S/W) ratio was consistently maintained at 4 and the crack length to width ratio (a/W) was set to the fixed value of 0.2. Detailed specimen dimensions are listed in Table 12.

The simulations were performed with three boundary conditions. The two supporting rigid cylinders were fully constrained, allowing no translational or rotational movement at their reference points. The loading cylinder was fixed in all directions except for the Y direction, where displacement loading was applied. The models were meshed with 8-node linear brick elements. To simulate interaction between the beam and cylinders, "frictionless" tangential behavior and "hard" normal behavior were employed (see Figure 35).

Table 12. The dimensions of specimens to investigate size effect [301]

Specimen size	$W(\text{mm})$	$S(\text{mm})$	$a(\text{mm})$	thickness(mm)
Extra-large	30	120	6	4
large	18	72	3.6	4
Medium	7.35	29.4	1.47	4
small	3	12	0.6	4

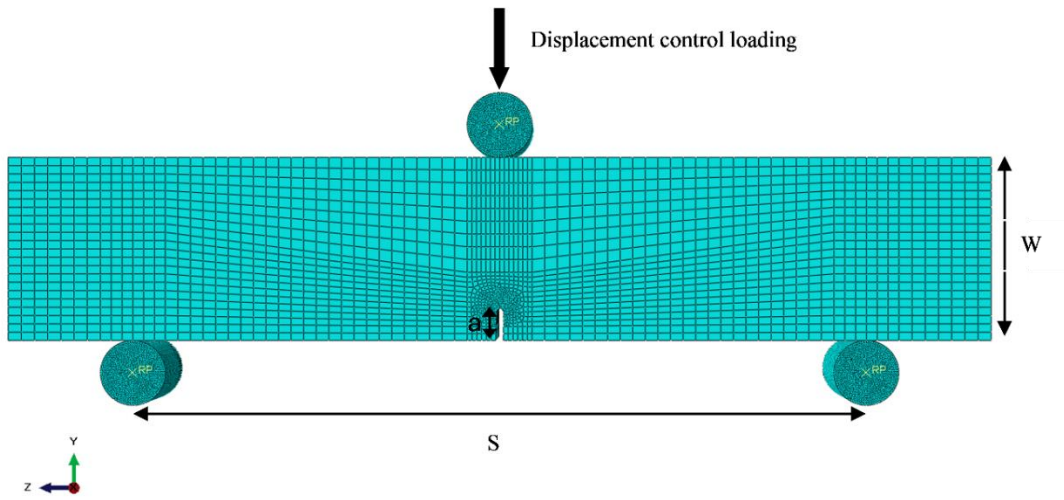


Figure 35. Mesh configuration, boundary conditions, and dimensions of the SENB model for studying size effects.

Figure 36 shows the MDZP area versus specimen width (W) for different oxidation levels. The results indicate that an increase in specimen size leads to a corresponding increase in MDPZ area. However, analysis of the fitted curves suggests that this size-dependent growth follows an exponential trend reaching a saturation point beyond which further increases in specimen size do not affect the MDPZ area although other functions may fit this trend too. Notably, this size dependency is also observed in oxidized specimens, though saturation occurs at larger sizes when compared to control specimens.

The load-deflection curves for various sizes of SENB specimens and oxidation levels are illustrated in two ways. Initially, changes in load-deflection curves due to oxidation for each size are shown by depicting different oxidation hours for each size (Figure 65). Subsequently, load-deflection curves for different specimen sizes are illustrated for each oxidation level (Figure 38). To facilitate comparison of the load-deflection curves given the significant variation in pick load across specimen sizes, the load

was normalized by the sample cross-sectional area ($W*t$) for each size, and the deflection was normalized by the span value. The results are presented in Figure 39. The findings from these figures suggest that as oxidation increases, nonlinearity also increases, while with increasing size the slope changes more smoothly, leading to a decrease in nonlinearity (see Figure 39, where the small size curve in black exhibits the highest nonlinearity around the peak load).

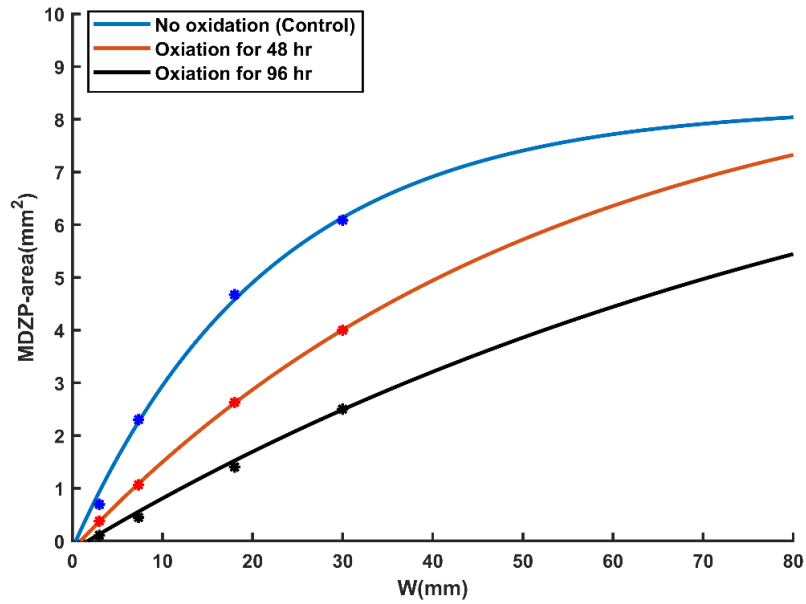


Figure 36. Effects of variation in size of the specimen on the area of the microdamage process zone.

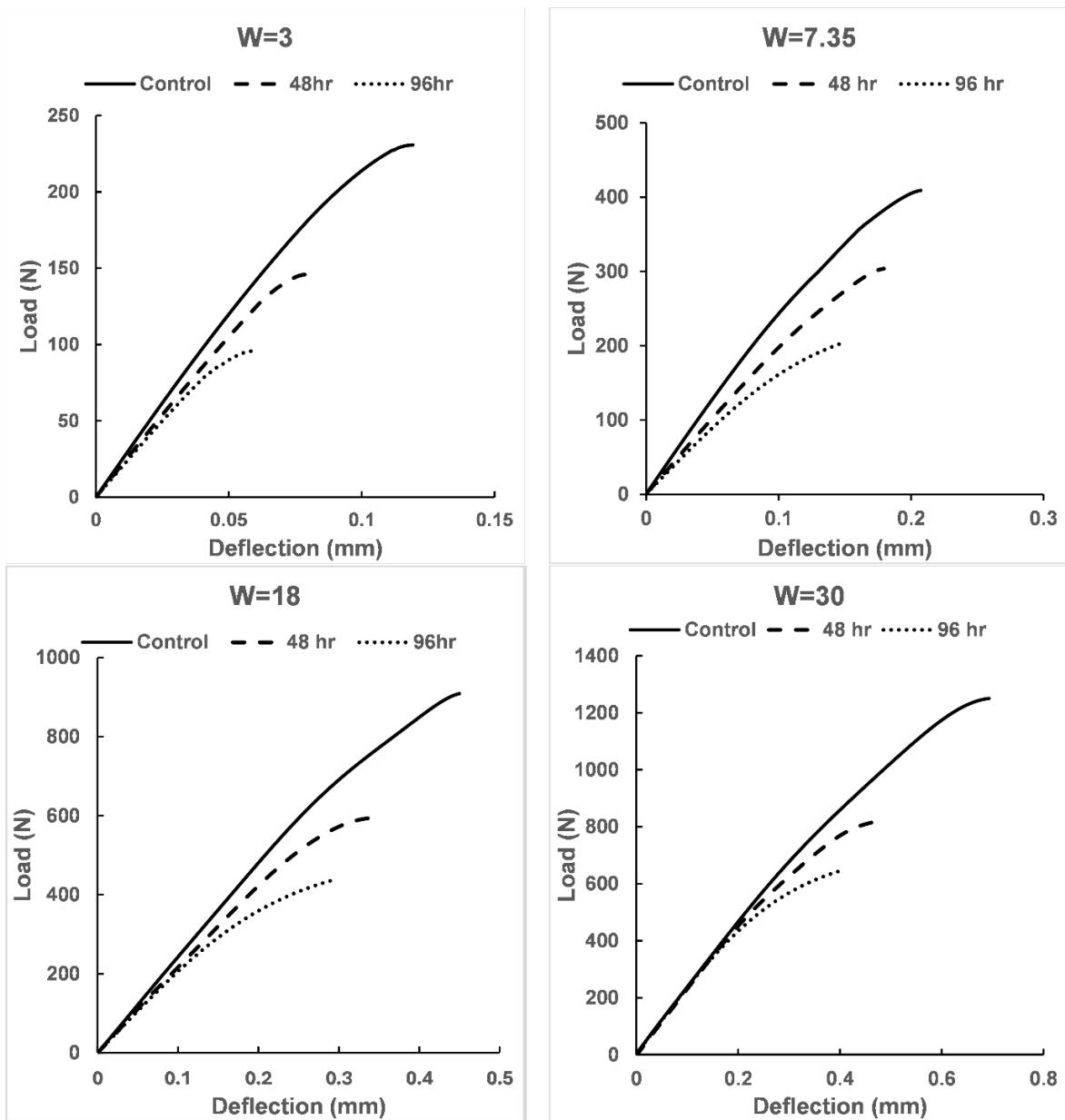


Figure 37. Load-deflection curves for different oxidation levels at each size.

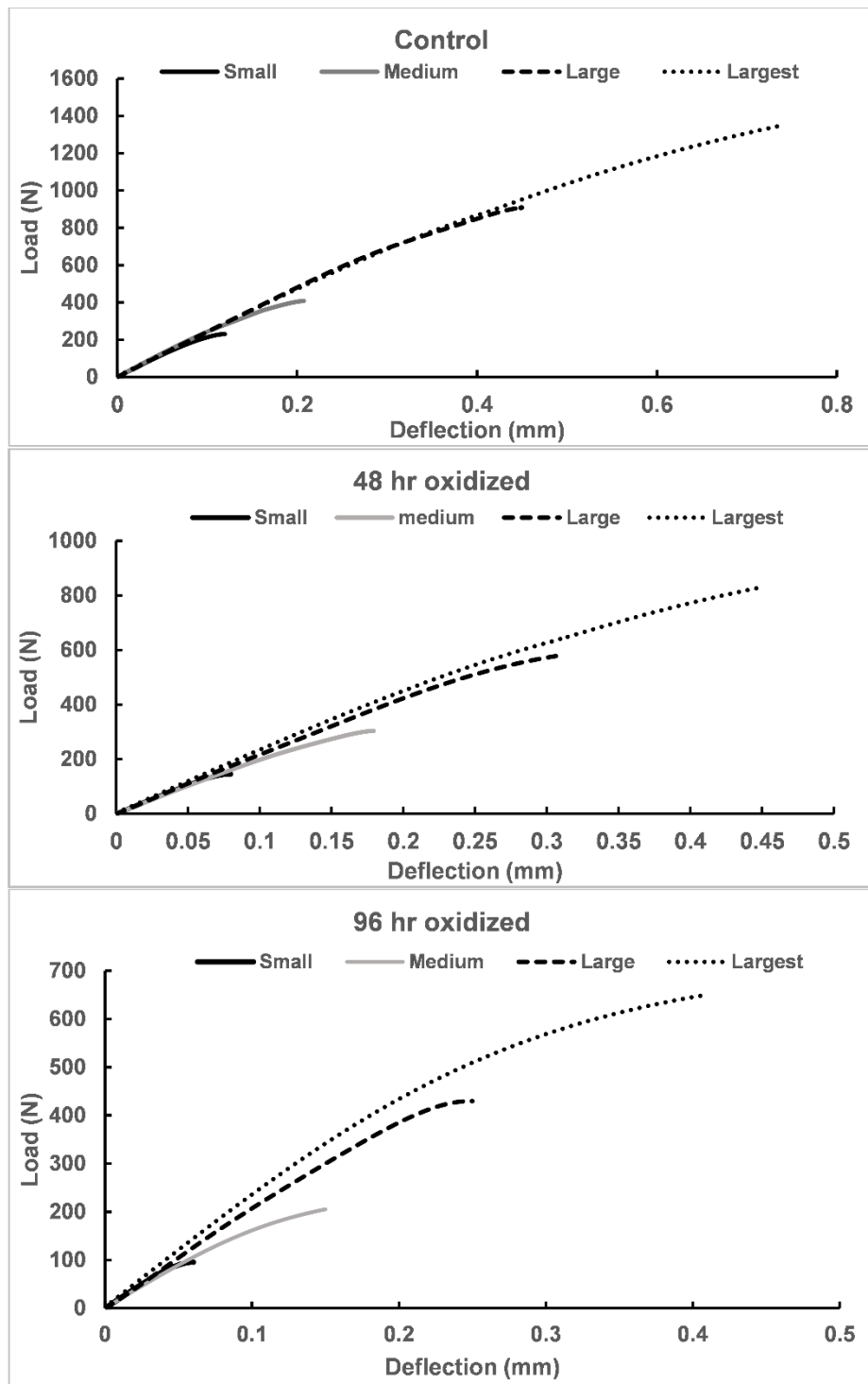


Figure 38. Load-deflection curves for different specimen sizes at each oxidation level.

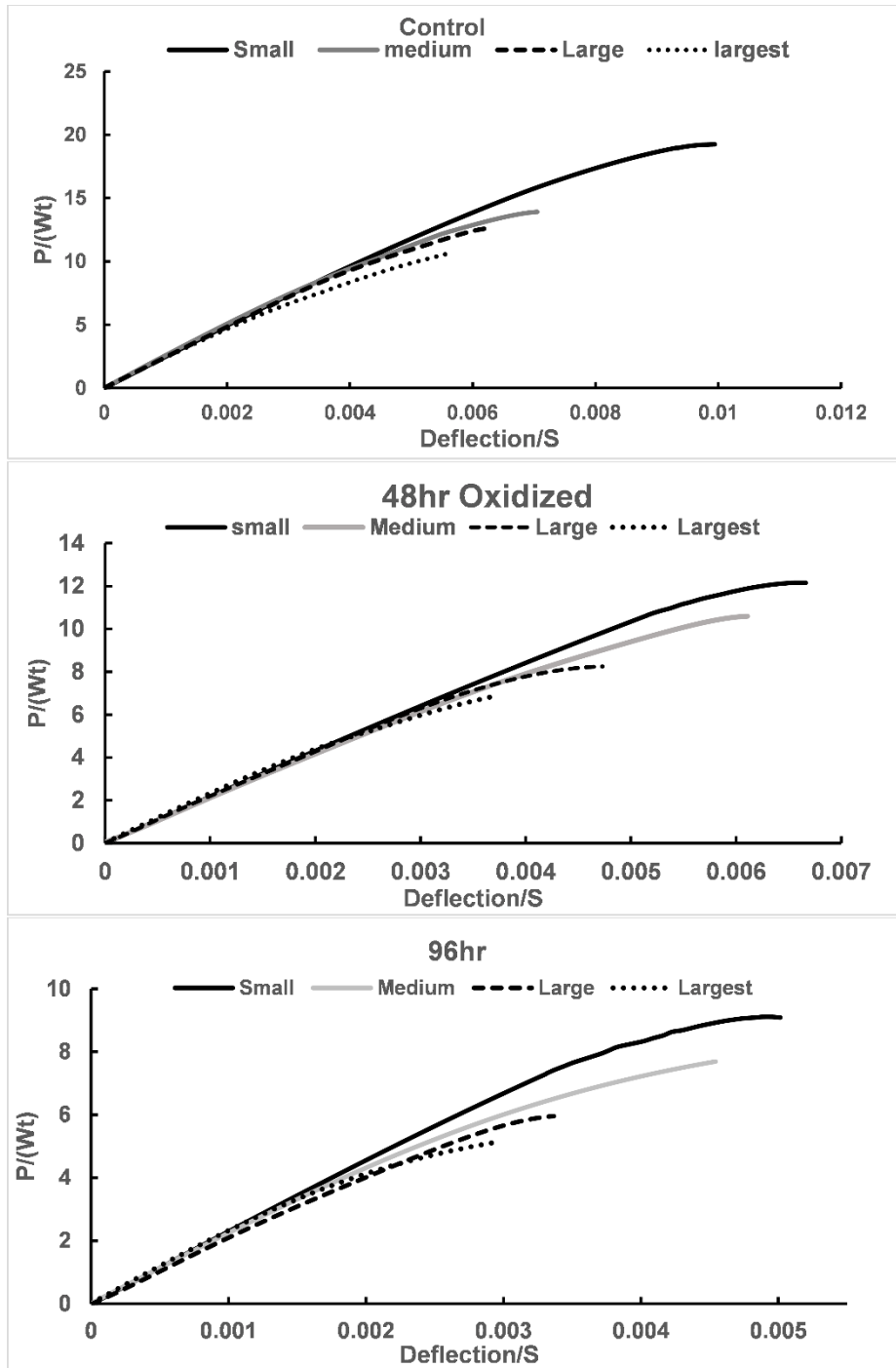


Figure 39. Normalized load-deflection curves for different specimen sizes at each oxidation level.

Chapter 5: Discussion, Conclusions & Recommendations for Future Work

5.1 Discussion

This study aimed to explore how oxidative damage, resulting from oxidative stress, which is a significant factor in the aging process and the development of diseases, impacts bone quality. The hypothesis was that physiological levels of oxidative damage, which occur during aging and inflammatory diseases, degrade bone quality by damaging bone collagen. To investigate this hypothesis, a variety of techniques were employed, ranging from experimental analysis to mathematical and computational modeling.

As outlined in section 4.1, a series of experiments including mechanical testing, biochemical assays and thermomechanical measurements were conducted to investigate different aspects of bone quality under the effects of oxidation. By applying neutral hypochlorous acid (HOCl) as a physiological oxidizing agent in vitro, oxidative stress conditions were simulated, allowing to explore direct consequences of the resulting oxidative damage on the mechanical and biochemical properties of bovine cortical bone.

The mechanical experiments encompassing four types of mechanical tests with the aim of examining different mechanical properties of bone clearly demonstrated that oxidative stress profoundly affects the quality of bone tissue. Across all three oxidative exposure periods (Control, 48 hours, 96 hours), there was a marked degradation in mechanical properties, specifically yield and post-yield properties such as tensile (29-30%, 17-46%, 30-40% respectively for increasing strain rates) and compressive (19-23%) yield strength, as well as tensile (26-34%, 25.5 - 45%, 25% respectively for increasing strain rates) and compressive (11-16%) ultimate strength. Additionally, there was an increased degradation of secant modulus (15-16%), an important measure for quantifying damage accumulation observed in load-unload testing [305] all reflecting a substantial decrease. Also, according to load-unload testing results, the dissipation of energy in all three pathways, namely permanent strain [278], elastic release energy [278] and hysteresis energy [278] decreased with increasing incubation time. These findings show that oxidative damage of bone tissue can lead to compromised load-bearing capacity and presumably increased fracture susceptibility.

Regarding elasticity, although the elastic properties did show degradation, this change was not detectable ($p > 0.05$), especially at the quasi-static rate in both tension ($p = 0.368$) and compression ($p = 0.106$). This finding suggests that the method primarily damages the collagen in the bone, which mainly contributes to the bone's yield and post-yield properties.

In the uniaxial tensile tests, both control and 48-hour oxidized samples exhibited an increase in yield and post-yield mechanical properties (excluding ultimate strain) with higher strain rates. However, an intriguing reversal was observed for the 96-hour oxidized specimens: as the strain rate increased, both pre- and post-yield mechanical properties decreased. Previous studies [306]–[309] have shown that the elastic modulus and yield properties of cortical bone generally increase with strain rate in both tension and compression. However, the effect of strain rate on post-yield mechanical properties is less clear. More recent research [307][310][311] suggests that bone behavior becomes more brittle as strain rate increases, contrasting with older studies [306][312][313] that did not find this relationship.

It is now well established that at low strain rates, bone tissue fails in a ductile manner. As the strain rate increases, the toughness decreases, making the bone more brittle [307][314][315]. A recent study by Zioupos et al. [315] investigated the combined effects of increasing strain rate and aging on bone behavior, finding that cortical bone has the highest fracture resistance at physiological strain rates (0.001 – 0.01 s^{-1}). The transition from ductile to brittle behavior occurs at strain rates of about 0.001 s^{-1} in young cortical bone, whereas in older cortical bone, this transition occurs at much lower rates, ranging from 10 to 40 times lower. In this study, as depicted in Figure 15-C, similar to the effects of aging, increased oxidation exposure time caused the bone to become more brittle, with the ductile-to-brittle transition occurring at a lower strain rate (around 0.002 s^{-1}). This suggests that the method used to induce oxidative damage in bone may replicate the physiological responses observed in vivo during aging and disease states and may partially explain the cases of bone fragility and aged and diseased bone where the heightened fracture risk is not associated with decreased BMD.

The inversion observed at 96 hours likely results from a combination of oxidation-induced molecular degradation, damage accumulation, and changes in the material's viscoelastic response. This has consequences for biological aging and disease processes, particularly in bone tissues subjected to oxidative stress. However, to make sure where this inversion exactly starts more time points need to be considered. Also tests at higher rates with cameras enable of capturing those higher rate tests are required to implement to see if this behavior is consistent.

Regarding the compression tests, as anticipated, the yield and ultimate strength and strain in tensile mode were significantly lower than in compression. In tensile testing, after reaching the ultimate strength, the material loses its load-bearing capacity due to prevailing damage mechanisms. In contrast, in compression testing, the stress-strain curve is shaped by the combined effects of local stress hardening and shear sliding. The steeper the stress-strain curve, the greater the local stress hardening and the lesser the shear sliding [197].

According to a previous study by Li et al. [197], compression tests yield three types of stress-strain curves based on the crack propagation pattern. The first type, observed in our control group's stress-strain curve, shows a sudden drop in stress at lower strain when a dominant crack spans the specimen (see Figure 17). The second type, seen in the 96-hour oxidized specimens, involves multiple fractures throughout the specimen, resulting in a flatter compressive stress-strain curve (Figure 17). The third type, occurring in the 48-hour oxidized specimens, represents an intermediate scenario with continuous stress reduction and multiple cracks progressively propagating (Figure 17). Li et al. [197] suggested that a sharper stress-strain curve corresponds to more pronounced local stress hardening and reduced shear sliding. Consequently, as oxidation levels increase, the ability to resist shear sliding diminishes, accompanied by a reduction in stress hardening.

The increase in protein carbonylation, a key marker of oxidative protein damage [150][181], obtained from the chemical assay analyses, further corroborates the mechanical testing results. The significant rise in carbonyl content with oxidation time verified that the utilized oxidation method has damaged the collagen phase of the bone. This result is particularly striking compared to the carbonyl content in human cortical bone from young healthy donors versus those aged donors with inflammatory diseases (T2D and/or CKD), suggesting that the oxidative damage induced in our in vitro model reflects clinically relevant oxidative stress levels seen in pathological conditions. The observed differences between the human and bovine control groups can be explained by several factors. Most notably, the bovine tissue used in this study was obtained from young, healthy cows (aged 12-30 months), while the human control group had an average age of 47.4 years. Although the humans did not have any diagnosed inflammatory diseases, they likely experienced some degree of oxidative stress and inflammation accumulated over their lifespan, leading to higher carbonyl levels compared to the bovine group. Additionally, species differences (human vs. bovine) may also contribute to the disparity. To improve the comparison, younger human samples could be used, though this may not fully resolve the issue, as even a young, seemingly healthy individual could have an unhealthy lifestyle that leads to

oxidative stress accumulation. Thermal analyses through Differential Scanning Calorimetry (DSC) and Hydrothermal Isometric Tension (HIT) provided additional evidence of the structural impairment of bone collagen. The negative correlations between carbonyl concentration and measures such as Max.Slope, and Diffstress, which are indicative of collagen network connectivity [192][16] highlight the detrimental effects of oxidative damage on the bone collagen's network connectivity. Also, the correlation found between the DSC measure FWHM and the carbonyl content, suggested that oxidative damage disrupts the organization of the collagen at the fibril level. This finding supports the notion that oxidative damage not only affects the biochemical composition of bone collagen but also alters its structural and functional properties.

In the second part of the work presented in section 4.2, a simple linear systems theory model was proposed to better utilize and interpret the results of both differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) testing. Due to the lack of a physics-based model for interpreting HIT results and challenges in linking HIT with DSC outcomes given their similar nature, this new model provides a much-needed quantitative framework. This model revolves around two parameters α and β , being candidate for assessing the collagen quality in a collagenous tissue in terms of its structural features such as network connectivity, nativity and stability. By quantifying collagen quality, α and β were subsequently incorporated into the proposed constitutive model to account for the effects of oxidative damage on bone collagen. The parameter α captures the proportionality between the integral of the DSC signal and the early part of the HIT signal, while β contributes to the most crucial part, which is the development of additional tension at temperatures above T_d .

After preliminary investigation of the model using limited data, to show the validity and applicability of the model to a broader range, the modified version of the model was applied to HIT-DSC datasets from human bone collagen specimens, including those from individuals with type-2 diabetes and chronic kidney disease, and specimens subjected to in vitro ribation [285]. The objective was to validate the model's parameters α and β , for assessing bone collagen quality, by investigating their correlation with some experimental measures, some of which such as Max. Slope and T_d are believed to be indicative of collagen network connectivity and stability [192][16]. Additionally, enthalpy of melting (ΔH) emerged as a significant predictor of β in the ribose study, suggesting its influence on collagen integrity. α showed correlations with T_d , establishing its role as a metric for collagen quality assessment. Differences between ribation groups further highlighted the model's sensitivity to collagen alterations.

The model was also applied to the results of our experiments obtained from the thermo analyses of the oxidized bovine bone specimens (section 4.1.3). Results confirmed associations between α and T_d , and between β and Max.Slope, supporting their roles in assessing collagen quality. Strong correlations between β and Max.Slope were consistent across different datasets, affirming the validity of Max.Slope as an independent measure of collagen network connectivity.

In the final stage of the study, to utilize the experimental results and the DSC-HIT mathematical model for building a predictive tool for bone behavior under oxidative stress conditions, often observed in aging and disease—a constitutive model for oxidatively damaged cortical bone was proposed. Utilizing the robust framework initially developed by Schwiedrzik and Zysset [247] this model was adapted to integrate the effects of oxidative damage on bone material behaviour, guided by experimental findings presented in section 4.1.

The constitutive model was fine-tuned to account for oxidative damage by modifying the coefficients of the constitutive and evolution equations, inspired by the methodology proposed by Murakami and Mizuno [275]. This adaptation was done using multivariate linear regression and defining the coefficients of the constitutive model in terms of oxidative damage biomarkers, such as carbonyl content, which directly correlate with changes in mechanical properties, and HIT model outputs, α and β . Through comprehensive assessment and adjustment of these parameters, this study contributes to a deeper understanding of the interplay between oxidative damage and mechanical integrity in cortical bone, advancing the field's knowledge of bone material modeling under oxidative stress conditions. This incorporation enables the model to simulate the mechanical behavior of bone under varying degrees of oxidative stress, providing critical insights into the effects of aging and disease on bone integrity.

The validation of the constitutive model involved simulating 3-point bending tests at two states: healthy and gamma-irradiated which causes extreme oxidative damage. Notably, the model showed a correlation with experimental results, achieving a mean absolute error of 7%. The reduction in the micro-damage process zone area in gamma-irradiated specimens, compared to healthy bone, further emphasizes the impact of oxidative stress on bone toughness and fracture resistance. This validation step confirms the model's reliability in capturing the complex mechanical behavior of bone under oxidative stress.

Utilizing this established model, the effects of variation in size on the micro-damage process zone (MDPZ) have been investigated. Simulations using geometrically proportional SENB models with various oxidation levels demonstrated a size dependency of the MDPZ size. As specimen size increased, so did the MDPZ size, until a saturation point was reached. Specifically, the idea of the MDPZ reaching saturation comes from extrapolation of the curve associated with the control group. This trend was consistent across both healthy and oxidized bone specimens, although saturation occurred at larger sizes in oxidized samples. This observation underscores the significance of size effects in bone fracture analyses, challenging traditional assumptions and highlighting the need for size-aware mechanical models in predicting bone behavior.

The study is not without the limitations. The study does have limitations. The experimental part was conducted on $n=3$ specimens for each oxidized group which may be limited for statistical analyses both in terms of capturing a reliable distribution and verifying normality assumptions for statistical analyses and generalizability of the results. Furthermore, the oxidation groups (Control, 48 hr, 96 hr) were limited and could have encompassed additional time points. However, given the extensive mechanical testing volume planned for this research, and considering the substantial number of preliminary trial and error experiments, executing all the experiments on a larger specimen pool alongside more oxidation groups exceeded the capacity of this study.

It is important to acknowledge that bone comprises additional components like the mineral-collagen interface, non-collagenous proteins, water content and distribution, and the effects of oxidation on these elements have not been explored in this study. Regarding the linear systems theory model of HIT-DSC, the two model parameters α and β requires further validation against tissue mechanical properties and potential model refinements to enhance accuracy. Nonetheless, the findings demonstrated the model's utility in evaluating collagenous tissue quality based on paired HIT and DSC data. Also, in the constitutive model of the oxidized bone, the oxidation parameters do not evolve with time which is a limitation of this model.

5.2 Conclusion

In conclusion, this research provides evidence in favour of the impact of physiological levels of oxidative damage on bone quality by the use of an in vitro method of oxidative stress. The measurements of mechanical properties, biochemical assays, and thermomechanical analyses consistently indicates that oxidative damage compromises bone collagen quality, leading to a

significant decline in bone strength and fracture resistance. The deterioration of mechanical properties and the increase in oxidative damage markers, similar to the observed levels in human diseased groups, suggest that oxidative damage may play a role in the loss of bone tissue quality in aging and disease. The accumulation of oxidative damage in bone collagen may contribute to the age-related decline in bone quality and increase fracture risk. These insights underscore the importance of addressing oxidative stress and damage in clinical strategies towards preventing and treating bone diseases.

The proposed computational model presents a step toward understanding and modeling the mechanical behavior of oxidatively damaged bone. By integrating oxidative damage effects into a constitutive model, it provides a framework for predicting bone mechanical behavior under various conditions.

5.3 Major contributions

This study contributes to understanding the effects of oxidative damage on cortical bone quality. The original and impactful contributions can be summarized as follows:

1. This study has contributed to our understanding of a potential cause of bone fragility in aging and certain inflammatory diseases by illustrating a physiologically plausible mechanism of damage. This mechanism, oxidative damage, serves as a measurable factor, in terms of carbonyl content, leading to bone deterioration.
2. The linear systems theory model of DSC-HIT enhanced the evaluation of collagenous tissue quality through a quantitative approach. HIT data were difficult to interpret and we put some physical meaning to it by having a model based on rubber elasticity theory.
3. This study engaged a multi-faceted approach, combining experimental techniques with computational models to explore how physiological levels of oxidative damage influence bone quality. The integration of oxidative damage effects into a constitutive model allows for a more complete understanding of bone behavior under varying levels of oxidative stress.
4. The development of a sophisticated computational model that incorporates oxidative damage measures such as carbonyl content and model parameters α and β obtained from HIT-DSC testing provides a tool for accurately predicting bone mechanical behavior under oxidative stress conditions, aiding in the assessment of bone behavior in healthy and diseased states.
5. Using the established computational model, we demonstrated that oxidative damage changes not only the tensile or compressive behavior of the bone, as evidenced in experimental findings, but

also affects the fracture behaviour. This was exemplified by the reduction in size of the micro-damage process zone (MDPZ) in simulated SENB specimens.

6. By exploring the size effect on the MDPZ using the developed model, in bone, the research showed something that was described using fracture toughness testing and fracture criterion. Understanding the dependency of MDPZ size on specimen dimensions and oxidation levels sheds light on the mechanisms influencing crack initiation and fracture processes in bone, offering valuable insights for future studies in bone mechanics and bone quality research.

These contributions collectively signify an advancement in the understanding of bone mechanics under the influence of oxidative damage, paving the way for enhanced assessments of bone quality in aging and disease states and possibly new targets for treatments to improve bone quality.

5.4 Future work

In an effort towards improving our understanding of the role of oxidative damage in bone fragility and modelling of the mechanical behavior of oxidized bone, the following recommendations for future work are provided:

- Use a greater number of time points and therefore levels of oxidative damage. This might provide a more accurate prediction of the effects of oxidative damage on bone tissue mechanical behaviour.
- Use an in vivo animal model to induce oxidative stress in bone tissue. This could be achieved by modifying the animal's diet to better simulate real-world causes of oxidative stress and damage.
- Confirm the results of the MDPZ simulations by conducting additional experiments in which the fracture behaviour and the development of the MDPZs are measured.
- Incorporate the oxidation parameter in the constitutive model as a variable that evolves over time or in response to other factors. This might enable better modeling of the time course of the development of poor bone quality with age or chronic disease.
- Refine the constitutive model to incorporate more detailed interactions between oxidative stress and bone mechanics. This includes exploring different methods of inducing oxidative damage and assessing their distinct impacts on bone properties. Additionally, extending the

model to account for other environmental and physiological factors influencing bone health will provide a more comprehensive tool for predicting and mitigating fracture risk.

- Study correlations between measures of bone quality and oxidative damage in human cortical bone specimens in aging and various disease states.

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Appendix A

Iranmanesh F, Dapaah DY, Tupy J, Montesano J, Willett TL. An in vitro study of the effects of physiological levels of oxidative damage on cortical bone quality with application to aging and disease states. Bone. (under review)

An in vitro study of the effects of physiological levels of oxidative damage on cortical bone quality with application to aging and disease states.

Faezeh Iranmanesh^a, Daniel Y. Dapaah^a, Jindra Tupy^a, John Montesano^b, Thomas L. Willett^a

^a Composite Biomaterials System Lab, System Design Engineering Department, University of Waterloo, Ontario, Canada

^b Mechanical and Mechatronics Engineering, University of Waterloo, Ontario, Canada

Abstract

This study examined the impact of in vitro oxidative damage on bone quality by a combination of methods including mechanical testing, chemical assays, and thermo-analytical methods. It was hypothesized that physiological levels of oxidative damage to the bone collagen, induced by soaking in neutral hypochlorous acid solution, would cause poor bone quality. The results demonstrate degradation of bone mechanical properties due to prolonged oxidation. Specifically, uniaxial tensile tests revealed a significant degradation in bone tensile mechanical properties, except ultimate strain, with increasing oxidation, showing distinct trends at different strain rates. Uniaxial compression tests revealed distinctive stress-strain curve patterns based on oxidation time, while load-unload tests highlight oxidative stress-induced damage through various measures. Chemical assays, including carbonylation and hydroxyproline assays, demonstrated elevated carbonyl content per collagen content due to prolonged oxidation, indicative of protein oxidative damage. Thermo-analytical tests including differential scanning calorimetry (DSC) and hydrothermal isothermal tension (HIT) tests providing measures of collagen nativity and connectivity revealed negative correlations with

increasing carbonyl content. This study has confirmed the hypothesis that physiological levels of oxidative damage deteriorate bone mechanical properties by damaging bone collagen, emphasizing the importance of considering oxidative damage in understanding bone quality.

Keywords

Cortical Bone; Oxidative stress/damage; Bone quality; Aging and disease; In vitro model

1 Introduction

Oxidative stress is characterized by an elevated concentration of reactive oxygen species (ROS), resulting in an imbalance between the production of ROS or oxidants and the cellular capacity to mount an effective antioxidant response [38]. ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\bullet$), and hypochlorous acid ($HOCl$) encompass both radical and non-radical oxygen species formed through the partial reduction of oxygen [38]. These species are natural by-products of normal aerobic cellular metabolism and play important physiological roles in intracellular cell activities including inflammatory responses [316]. They can also be produced by external sources such as UV light and ionizing radiation [125]. ROS may either have beneficial or diminishing effects on tissues according to their concentration [125]. To maintain proper physiological function, the human body is equipped with antioxidant systems; however, elevated ROS levels beyond a certain threshold, determined by the body's removal capacity, result in oxidative stress (OS). Excessive free radicals under OS conditions cause oxidative damage to proteins, lipids, and nucleic acids, compromising cellular health and fostering disease development [41]. Additionally, they exacerbate inflammatory responses, contributing to the development of various inflammatory conditions [316]. ROS have been implicated in aging and related complications [42], contributing to various chronic diseases with age, including cancers, cardiovascular diseases, ischemia-reperfusion injury, renal diseases (chronic kidney disease), diabetes, and neurological disorders, all stemming from oxidative stress [43]. The harmful consequences of oxidative stress are caused by damage to cellular structures and impairment due to lipid and protein oxidation [317].

Increasing evidence indicates that bone biology is especially affected by ROS [317]. Various experimental studies have revealed that OS causes an osteoblast/osteoclast imbalance leading to a disruption in the equilibrium between bone formation and bone resorption, favoring the latter and adversely affecting the mineralization of bone [143][318]. Several studies have given proof of a positive association between OS and osteoporotic status. Specifically, individuals with osteoporosis demonstrate a decline in bone mineral density (a hallmark of osteoporosis)

which was correlated with elevated levels of OS [319][320]. Studies on aging mice also demonstrated the role of ROS in age-related osteoporosis where the bone formation slows down with increasing ROS levels [321]. In addition, oxidative stress has been linked to the development of bone tumors [322][323], increased fracture risks in diabetic patients [115], and inflammatory conditions affecting the joints [324][325].

In type II diabetes (T2D), individuals with elevated or normal bone mineral density (BMD) have higher fracture rates [29]. The reasons for the increased susceptibility to bone fracture in T2D are complex and poorly understood. Nevertheless, numerous studies have emphasized a role for advanced glycation end products (AGEs) and OS as primary factors contributing to skeletal fragility in T2D [9]–[11], [24], [115]. These observations highlight the need for additional investigations to fully understand the role of oxidative stress and damage in bone fragility.

Collagen, the dominant and long-lived structural protein in bone tissue, is a good target for ROS and can be degraded by the combined actions of ROS and proteinases during the early phases of inflammation [37]. Collagen type I comprises 90% of the total protein in bone, and the remaining 10% consists of non-collagenous proteins [57]. According to previous studies, collagen mainly contributes to bone yield and post-yield mechanical behaviour by providing ductility and the ability to absorb energy before fracture [14], [15], [19], [326]. In healthy bone, enzymatic collagen crosslinks enhance bone strength and toughness by increasing the strength and stiffness of collagen fibrils [53][24]. However, with aging, and some chronic diseases, such as diabetes, non-enzymatic collagen crosslinking caused by glycation and/or oxidation may affect bone strength [24]. In addition, accumulation of AGEs, observed in type 2 diabetes, may compete with enzymatic collagen crosslinking [24][33].

Various ROS can affect collagen. For instance, collagen can be attacked by the superoxide anion, leading to fragmentation within the triple helical structure of the molecule. This is evidenced by the liberation of small peptides containing 4-hydroxyproline, serving as an indicator of collagen fragmentation [37]. Furthermore, in oxygen-rich environments, hydroxyl radicals are capable of breaking down collagen into smaller peptides, with the cleavage process

appearing to target proline or 4-hydroxyproline residues specifically [37]. Also, when collagen comes into contact with hypochlorous acid (HClO), a prominent oxidizing agent produced at inflammation sites by neutrophils and monocytes, and also produced by osteoclasts, significant alterations occur in the structural characteristics of the collagen molecule [37]. Studies by Monboisse *et al.* [37] claimed a substantial reduction in the content of α_1 and α_2 chains in hypochlorous acid-treated collagen [37]. Additionally, it appears to stimulate the formation of new cross-links, the nature of which is unclear [327][22]. Hence, ROS can alter collagen in different ways; they have the capacity to cause either collagen fragmentation or cross-linking. These differing outcomes may indicate distinct underlying pathophysiological conditions, with collagen fragmentation linked to inflammatory degradation and collagen cross-linking potentially associated with the aging process and related complications [37].

Surprisingly, while OS has been shown to damage collagen molecules through various mechanism[44][45], any resulting degradation of bone collagen nativity and connectivity and subsequent degradation of bone mechanical properties due to the oxidative damage has received little attention in the literature. While some previous in vitro models have attempted to simulate the accumulation of AGEs in bone, these models have produced inconsistent results and do not align with the observations when testing aged and diseased human bone. Instead of causing collagen degradation and subsequent deterioration of bone mechanical properties, these models have actually increased collagen crosslinking, stability, stiffness, and strength of the bone collagen [328][284].

To quantify the production and activity of ROS, one approach involves evaluating stable biomarkers that signify systemic or tissue-specific oxidative stress. Lipids, proteins and DNA undergo modifications due to their interaction with ROS in the surrounding microenvironment [329]. While some of these alterations—like the suppression of an enzyme function—are recognized for their direct impact on the function of target molecules, other modifications only serve to indicate the local level of oxidative stress [150].

Proteins, as a wide target for ROS generated under normal or oxidative stress conditions, can be considered as general scavengers of ROS. Protein oxidation products are generated as a

result of oxidative changes to proteins, occurring when amino acid residues react with ROS [150][152]. These modifications mainly include protein carbonylation, and formation of protein nitrotyrosin, s-nitrosylation, s-glutathionylation, sulfur-containing residue oxidation, and aromatic and aliphatic group hydroxylation [155][153]. Additionally, oxidation can result in the scission of the polypeptide chains and the formation of covalent crosslinks of unknown nature [37]. These changes can either be reversible or permanent [152], [153], [155]. Carbonyl formation and protein nitrotyrosine formation, as well as cleavage of the histidine and tryptophan rings, and hydrolysis of the peptide bond in the vicinity of proline are examples of permanent modifications of proteins [150].

Carbonylated proteins are well-known indicators for evaluating the oxidative damage of proteins associated with aging and disease states [158]. The formation of carbonyl groups stands out due to their early occurrence, irreversibility, and stability, setting them apart from other markers of protein oxidation [162][163]. Protein carbonylation occurs through several mechanisms such as oxidative breakage of the protein backbone, particularly impacting glutamyl side chains, and oxidative removal of lysine [158]. This modification can also form on several amino acid residues, like proline, lysine, histidine, threonine, and arginine [155], [158]–[161]. Since this modification impacts numerous amino acids [46]–[48], it demonstrates a substantially larger impact compared to modifications limited to certain amino acid residues [159][160], making it notably more detectable.

In this study, we aimed to investigate the effects of oxidative damage on cortical bone collagen and, consequently, on cortical bone quality by using a new in vitro oxidative damage model. We hypothesized that exposing bone to oxidation using neutral HClO solution would degrade bone mechanical properties, particularly yield and post-yield properties, because of degraded bone collagen nativity and connectivity. This oxidative damage would be confirmed using measures of collagen carbonylation with these measures compared to levels in nominally healthy and diabetic human cortical bone. To test this hypothesis, bovine cortical bone specimens were oxidized using neutral hypochlorous acid solution, a potent endogenous oxidizing agent. The oxidized bone samples underwent comprehensive characterization

through distinct experimental approaches: 1) multiple sets of mechanical testing were conducted to measure bone property degradation as a result of oxidation 2) carbonyl assay analyses quantified protein carbonylation, a key marker of oxidative protein damage 3) thermo-analytical techniques (DSC and HIT testing) assessed oxidative damage effects on the bone collagen integrity and connectivity.

Figure 40 offers a graphical overview of the study.

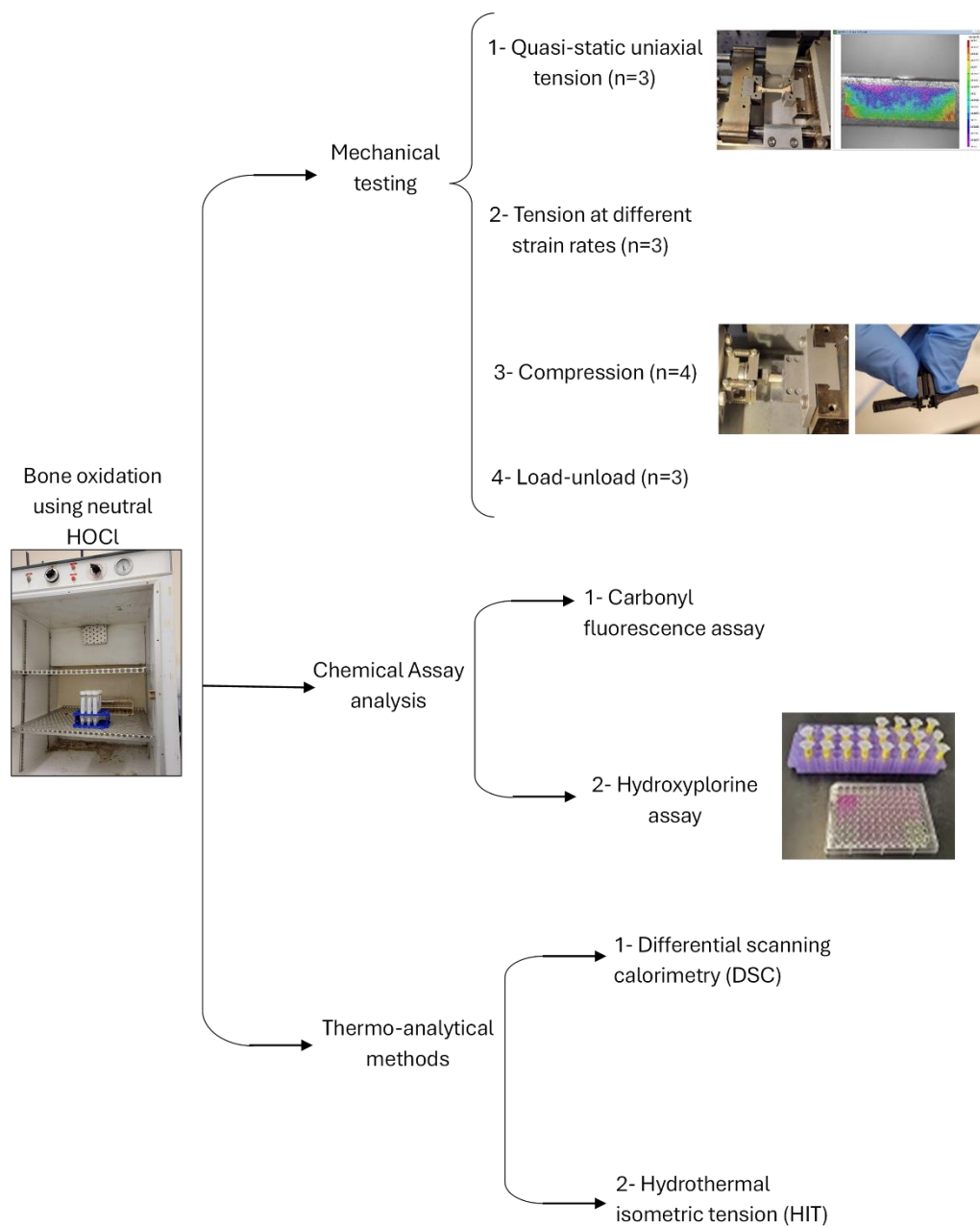


Figure 40 . A graphical depiction of the study depicting the different experimental methods used.

2 Materials and Methods

2.1 Sample preparation and oxidation

The specimens were obtained from bovine steer tibiae sourced from a local farm. The steer were aged between 18 and 24 months. To prepare the specimens, the flesh was meticulously removed from each tibia using a combination of a razor blade and scalpel. Subsequently, a wood band saw (14" Craftex CX104, Busy Bee tools, Canada) was employed to isolate the posterior section of the diaphysis.

Two types of samples were created to accommodate various mechanical tests. Tensile dog-bone-shaped specimens, aligned with the bone's longitudinal axis were cut using a vertical mini-CNC mill (Sherline, California, USA) for the tensile and load-unload tests (see Figure 41). The dimensions of the gauge section for the tensile specimens ($12\text{ mm} \times 2.4\text{ mm} \times 3\text{ mm}$) were chosen in accordance with [314]. Three to four pairs of tensile specimens were prepared from each tibia's posterior diaphysis, corresponding to the required number of incubation time points. Consequently, as all time points originated from a single specimen, variations arising from different animals among specimens could be managed.

Small cuboids, again oriented parallel to the bone's longitudinal axis and with a cross-section like the tensile specimens ($2.4\text{ mm} \times 3\text{ mm}$), were prepared (Figure 41) for compression testing. The length of these compression specimens was set at 7 mm, as per [314]. All compression specimens were taken from a single tibia due to their small size.

To eliminate surface imperfections and microcracks, the top and bottom surfaces of the specimens were polished using a series of diamond discs and polycrystalline diamond suspension ($45\text{ }\mu\text{m}$, $15\text{ }\mu\text{m}$, $9\text{ }\mu\text{m}$, $0.5\text{ }\mu\text{m}$) (MetaDi Supreme, Buehler, IL, USA). The sides were also polished using sandpaper with grit sizes ranging from 600 to 2000, in addition to the polycrystalline diamond suspension ($0.5\text{ }\mu\text{m}$). All specimens were stored in saline soaked gauze at -20°C .

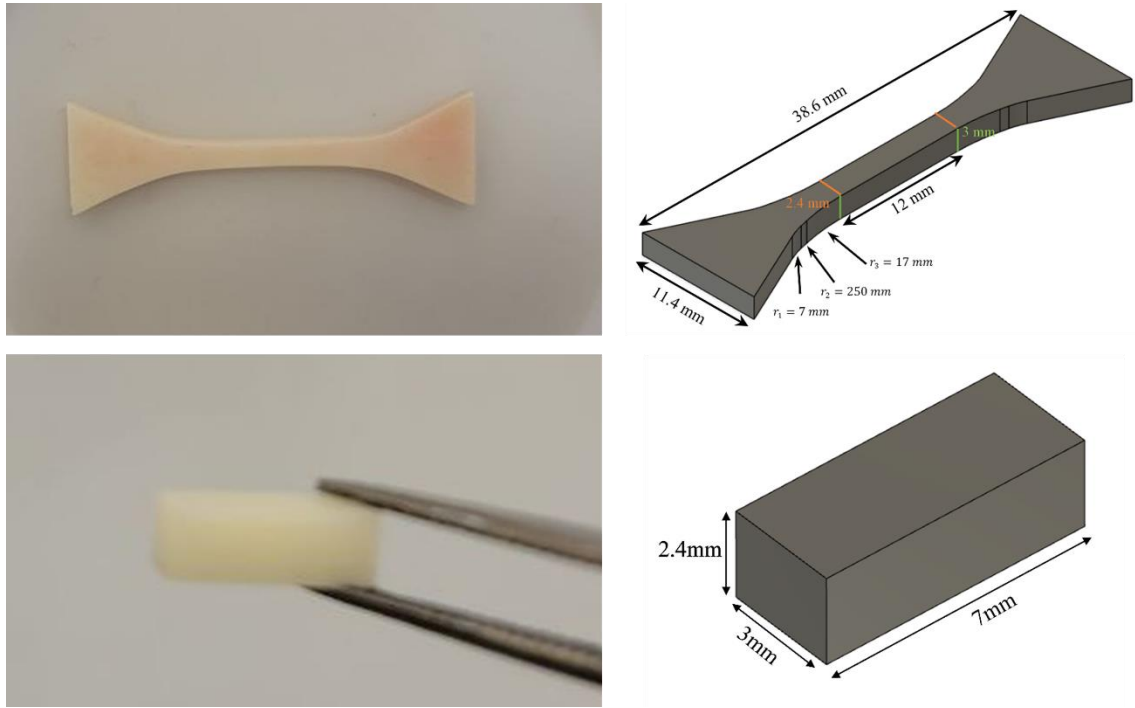


Figure 41 . Geometry of the tensile and compression testing specimens.

The bone specimens were subjected to oxidation using a neutral hypochlorous acid (HOCl) solution, a strong physiological oxidizing agent. The HOCl solution was prepared in the lab fume hood by mixing 5% Acetic acid (HOAc) and 5.5% Sodium Hypochlorite (NaClO), resulting in a stable solution with a pH of approximately 7 lasting for up to 24 hours.

The specimens were incubated in 10 ml of the neutral HOCl solution in 15ml Falcon tubes in a 37°C oven for two time periods: 48 hours and 96 hours. For each exposure period, three samples were incubated. The solutions were refreshed every 24 hours. Prior to mechanical testing, the samples were rinsed and submerged in PBS for three hours. A control group of three samples was immersed in PBS at 37°C was also included in each trial.

2.2 Analysis of the distribution of oxidation using confocal imaging

Prior to commencing in-depth characterization of the oxidized specimens, it was imperative to investigate the distribution of oxidative damage throughout the interior of the specimens to

ensure uniformity throughout the entire cross-section, rather than solely occurring on the surface.

Therefore, two specimens were prepared: one served as a control specimen, while the other was oxidized for 24 hours. Subsequently, the specimens were sectioned and stained using a 5 μ M fluorescein-5-thiosemicarbazide (FTC) solution dissolved in 50% (v/v) DMSO and deionized water [330]. FTC reacts irreversibly with carbonyls.

The stained surfaces were imaged using a laser scanning confocal microscope (Leica TSC SP5, Leica Microsystems, Concord ON) equipped with an argon laser. The excitation light had a wavelength of 488 nm, with the emission filter configured to the preset settings for isolating Fluorescein isothiocyanate (FITC) signals [331]. A 10x objective lens was utilized, and each sample was imaged as a Z-stack, with scans approximately 10 μ m apart. These stacks were then projected onto a 2D composite image using ImageJ's "z project" function, employing "max intensity" to ensure focus across the imaged region [331].

To establish a threshold value for identifying oxidatively damaged collagen on the surfaces, each image underwent thresholding using ImageJ's "threshold" function. This process binarizes the image, with pixels assigned values of 1 (white) or 0 (black). The threshold was set to exclude 99.5% of the control specimen's pixel intensity, defining pixels brighter than the threshold level as indicative of oxidized collagen. A threshold value of 99.5% was chosen to ensure accurate rejection of background staining during surface analysis, necessitating the removal of all signals from pixels with intensity values below 110 in the control specimen. This threshold was then applied to other images to standardize pixel intensity values relative to the control, ensuring consistency in isolating damage-specific signals while minimizing background noise [331].

2.3 Mechanical testing

As presented in Figure 40, this study involved four sets of mechanical tests. The initial set of tests, involving quasi-static uniaxial tensile tests, was conducted on nine specimens that had been oxidized for three different time periods (Control, 48 hr, 96 hr). These tests were carried

out at the lowest rate equivalent to 13 $\mu\text{m/s}$ (with an average strain rate of $\dot{\epsilon}_0 = 2 \times 10^{-4} \text{s}^{-1}$). The results from these tests were utilized for determining the effects of oxidation on tensile mechanical properties, including elastic modulus, yield, and ultimate strengths, as well as ultimate strain.

To investigate the rate-dependent behavior of cortical bone exposed to different levels of oxidation, uniaxial tensile tests were conducted on an additional 18 samples ($n=3$ per group) at two elevated strain rates ($\dot{\epsilon}_1 = 2 \times 10^{-3} \text{s}^{-1}$, $\dot{\epsilon}_2 = 2 \times 10^{-2} \text{s}^{-1}$) in addition to the quasi-static rate given above.

All mechanical experiments were conducted using a mechanical testing system (μTS , Psylotech, IL, USA) equipped with a 1.6 kN windowing load cell. Throughout the testing process, a microscope (Olympus Corp., Center Valley, PA, US) fitted with a 5-megapixel camera (Point Grey, 5 MP, 2/3" detector) was positioned above the system to capture high-resolution images of the specimens. These images were then analyzed using VIC 2D digital image correlation (DIC) analysis software (v6, Correlated solutions, Irmo, SC, US) to determine the strain field. To enable DIC strain measurements, high-resolution toner powder (Xerox Phaser 6000) was applied to the specimens to create speckle patterns. These particles, measuring 4-5 μm in diameter, were evenly distributed using an atomizer jar. Despite the absence of any irrigation during the test, primarily due to concerns of potential damage to the testing equipment, the short duration of the test (less than 1-2 minutes per sample) prevented the specimens from experiencing dehydration [296].

For DIC strain measurement, a region of interest with approximate dimensions of 6816 pixels in length and 2235 pixels in width was selected on the reference image. The virtual line extensometer tool was employed to calculate both longitudinal and transverse logarithmic (true) strains. In all tests, the subset window and step size were consistently set at 125 and 7 pixels, respectively (with 1 μm corresponding to 0.358 pixel), and a uniform weighted Gaussian filter was applied to smooth the displacement fields before strain calculations.

Flat dog-bone-shaped specimens were securely held by shoulder grips, and displacement-controlled tests were carried out at room temperature. Data for the tests with $\dot{\epsilon}_0$ (quasi-static) and $\dot{\epsilon}_1$ rates were collected at a rate of 10 Hz, while the high-rate test data were collected at the maximum rate of the camera, which was 40 Hz. Figure 42 provides an illustration of the tensile test setup, including the grips, the specimens held within the grips, and a typical strain field of the gauge section analyzed using digital image correlation (DIC).

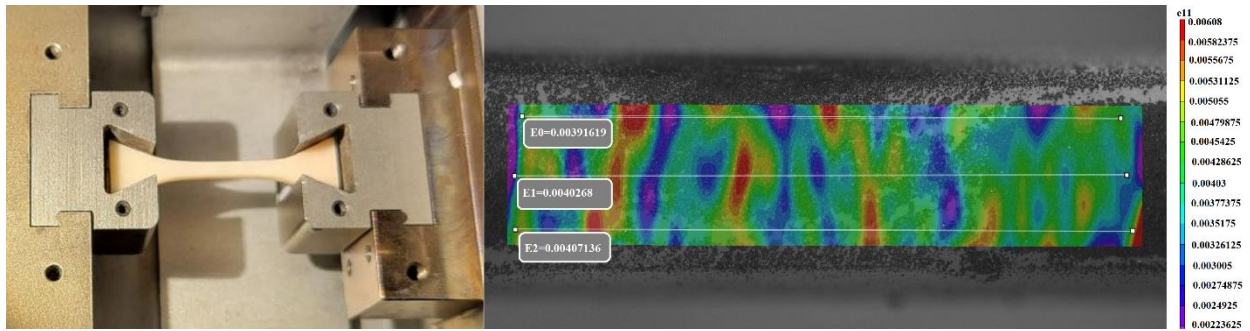


Figure 42 . Tensile testing setup and DIC analysed bone specimen surface with the strain field of the gauge section (average length of the virtual extensometer $\approx$ 2400 pixels).

To evaluate the changes in compressive mechanical properties of the cortical bone specimens in response to oxidation, compression tests were conducted on a total of 12 specimens (with $n=4$ in each group). These tests were carried out at a displacement rate of 13 $\mu\text{m/s}$, which corresponds to the rate used in quasi-static tensile tests. To perform these tests, custom-made compression platens from Psylotech Inc. were used.

Prior to each test, the platens were lubricated with MOLYKOTE® high vacuum grease to prevent friction between the platens and the specimens. Due to the small size of the compression specimens, a special fixture was designed and employed to securely hold and repeatably position the specimens within the testing fixtures (see Figure 43). The strain was also measured using a procedure like that described for the tensile test above.

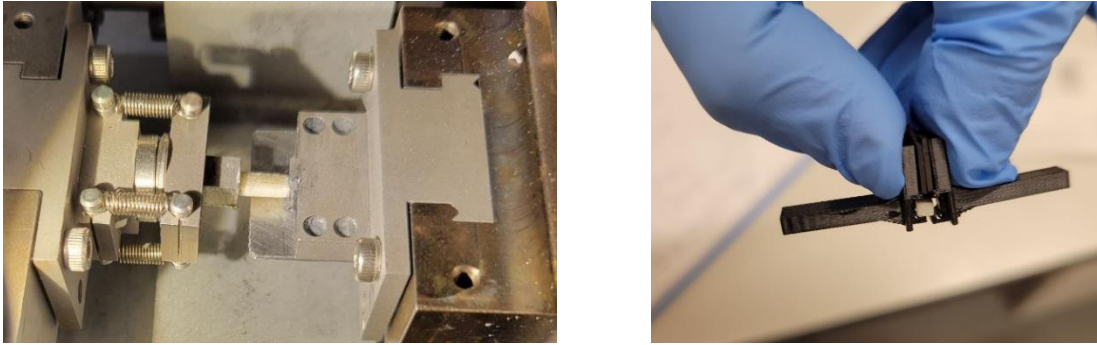


Figure 43. Compression test setup (left) and the fixture for holding and placing each specimen repeatably (right).

Load-unload tests were also conducted to explore the damage behavior of bovine cortical bone as a function of controlled levels of oxidative stress. For this series of tests, nine flat-dog-bone shaped specimens ($n=3$ per group), like the tensile test specimens, were tested. Two groups of specimens underwent oxidation like the other tests. The test setup and grips were the same as those used in the tensile tests (Figure 42). Tensile cycles with increasing amplitude were conducted in displacement control mode with an initial displacement of 0.2 mm . The displacement increased by 0.2 mm every other cycle (Figure44).

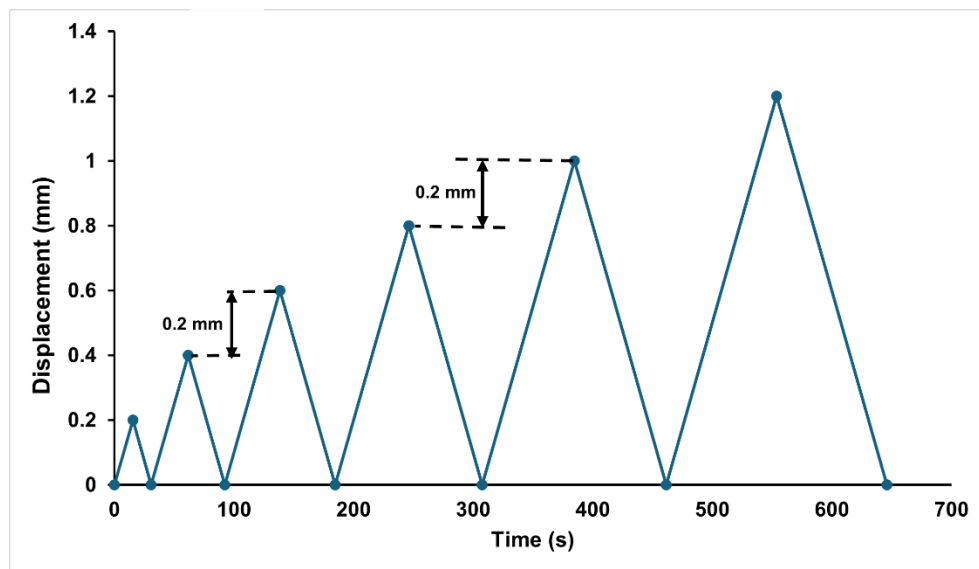


Figure44 . Load-unload test schedule.

Various measures were derived from the load-unload curves (See Figure45), including E_5/E_4 (Secant modulus degradation between the last and second last cycle) which represents the deterioration in modulus in the last cycle. Another parameter is ε_p , or permanent strain, which is the residual strain after anelastic deformation and before reloading [278]. The next measure is ε_{nl} or nonlinear strain. This value is determined according to equation 1, where ε_{total} represents the total measured axial strain in the cycle, σ_{total} is the corresponding stress at the same point, and E_0 is the initial elastic modulus. It should be noted that all these measures have been calculated for the last loading cycle [279]. Another measure, obtained from the load-unload test results is the elastic release strain energy, W_{er} [278] which is the area under the unloading curve minus the elastic recovery energy. Permanent strain energy, W_p [278] is the cumulative areas between successive loading curves, W_{cr} , minus the elastic release strain energy, W_{er} , and finally the hysteresis energy, W_h [278] is the area between the loading and unloading curves.

$$\varepsilon_{nl} = \varepsilon_{total} - \frac{\sigma_{total}}{E_0} \quad (21)$$

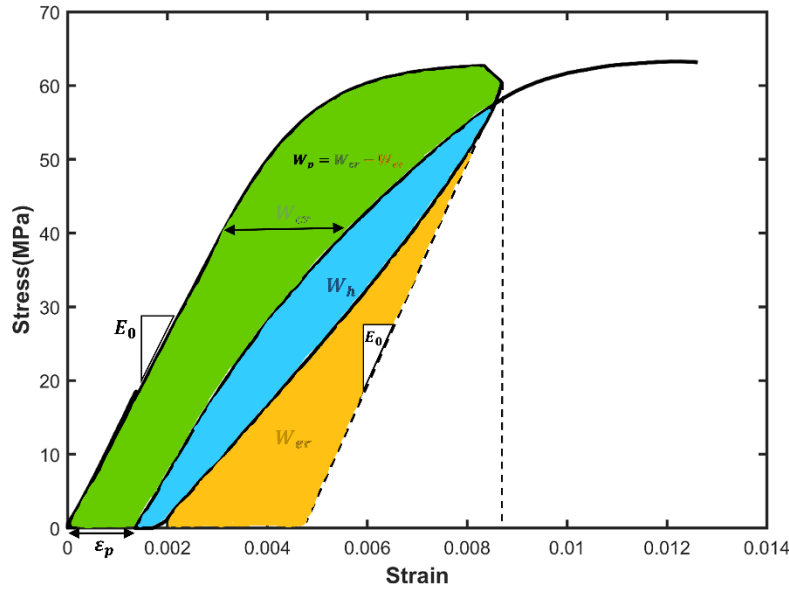


Figure45 . Measures obtained from load-unload test data.

2.4 Preparation for biochemical assays

Oxidized and control group specimens which underwent mechanical testing were demineralized before starting the chemical assay process. To demineralize the bone specimens (n=51), 0.5 M ethylenediaminetetraacetic acid (EDTA) with a pH of ~ 7.5-8.0 was used to gently decalcify the bone. EDTA yields the highest degree of tissue preservation but requires significantly more time than other methods [280]. Consequently, each previously tested bovine bone specimen was immersed in 10 ml of 0.5 M EDTA for approximately two months to ensure thorough decalcification. The solution was changed weekly to prevent saturation and to ensure that the demineralization process continues uninterrupted.

Following demineralization, the specimens were digested to be ready for the two chemical assays, namely carbonyl fluorescence assay and hydroxyproline colorimetric assay. The digestion was carried out using the enzyme trypsin. The trypsin was dissolved in Hank's buffer to make a 1 mg/ml trypsin in Hank's buffer solution. The trypsin digest allows the collagen to be broken down into smaller chains to facilitate carbonyl binding with FTC. From each demineralized bone specimen, a 15-25 mg piece was cut into smaller pieces, increasing the available surface area to enhance digestion. These fragments of each sample were initially denatured in 100 μ l of Hank's buffer in a micro-centrifuge tube at 85°C for one hour using an oven. Subsequently, 100 μ l of 1mg/ml trypsin in Hank's buffer solution was added to each tube and incubated at 37 °C for an additional 16 hours. Following this, the tubes with the samples and trypsin solutions were reheated at 85 °C for an hour, after which 50 μ l of 1mg/ml trypsin solution was added and incubated for 3 hours at 37°C. Subsequently, an additional 50 μ L of trypsin solution was added, followed by another 16-hour incubation at 37°C. Because there was still particulate matter of the sample visible, the last steps of heating at 85°C for one hour, then adding a couple of 50 μ l of trypsin solution while incubating for 3 hours and 16 hours at 37 °C was repeated to complete the digestion process [281][282].

2.4.1 Carbonyl Fluorescence Assay

To quantify the carbonyls, stable markers of protein oxidation, a fluorescence-based assay was used. This assay is a modification of a method initially reported by Ahn *et al.* [179]. Following digestion, a 50 μ l aliquot from each digest was transferred to a centrifuge tube. The 50 μ l extract was mixed with an equal volume of 0.2 mM fluorescein-5-thiosemicarbazide (FTC), previously diluted with [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] (HEPES) and incubated at room-temperature in the dark overnight.

The digested proteins were then precipitated with 400 μ l of chilled 30% trichloroacetic acid (TCA) (v/v) and centrifuged at 15000 RPM for 10 minutes at room temperature. Subsequently, the pellets were resuspended and washed three times with cold 100% acetone to eliminate unbound FTC. The final pellets were dissolved in 50 μ l of 6M Guanidine hydrochloride (GuHCl). Finally, 450 μ l of 0.1 M NaH_2PO_4 with pH 7.0 was added to each sample.

A standard curve was generated from the fluorescence intensity values of known concentrations of FTC using the following procedure. A 10 mM stock concentration of FTC (dissolved in dimethyl sulfoxide (DMSO)) was prepared and then diluted with 0.1 M NaH_2PO_4 to a final concentration of 100 μ M. The 100 μ M fluorophore was further diluted with 0.1 M NaH_2PO_4 through a series of dilutions to create 10 standard solutions.

100 μ l of each specimen and prepared standard was then transferred in duplicate to a 96-well black opaque plate and then read on a Synergy™ 4 microplate reader (BioTek Instruments, USA) (480 nm excitation filter, 530 nm emission filter, sensitivity 100%).

2.4.2 Hydroxyproline Colorimetric Assay

The hydroxyproline colorimetric assay, which is a modification of previous methodologies reported in [332]–[337], was used to quantify the amount of collagen in each digested specimen. This step is essential for normalizing the carbonyl content, as measured by the carbonyl fluorescent assay, to the collagen content. Collagen content can be indirectly assessed

through hydroxyproline levels, given that this amino acid is predominantly present only in collagen [332].

To establish a Hydroxyproline standard curve, serial dilutions were prepared from a 200 µg/ml standard working stock by dissolving and diluting L-hydroxyproline in deionized water (diode H₂O).

Since collagen contains approximately 8-10% hydroxyproline, this assay can be used to estimate the amount of collagen in a sample. Hydroxyproline is released from proteins and peptides through acid hydrolysis. For acid hydrolysis of protein, 25 µl of each digested sample was mixed with an equal volume of concentrated 12 N HCl in a glass screw-capped tube and heated at 110°C for 4 hours. The resulting hydrolysate was neutralized using 12N sodium hydroxide (NaOH) and then diluted by 1:40 with deionized water.

Subsequently, triplicate aliquots of 80 µl from each specimen, standards, and H₂O were transferred to a clear 96-well plate. The hydroxyproline amino acids are converted to pyrrole-2-carboxylate through oxidation via the addition of 40 µl of 0.05 N Chloramine-T to each well using a parallel pipettor, followed by incubation at room temperature for 20 minutes. Finally, 40 µl of 15% w/v p-Dimethylaminobenzaldehyde (DMAB) in n-propanol: perchloric acid (2:1), also known as Ehrlich's solution, was added to each well. The plate was incubated at 60°C for 20 minutes, and the absorbance measurements were taken at a wavelength of 560 nm using a microplate reader (Molecular Devices SpectraMax Plus 384, Sunnyvale, CA).

2.5 Thermo-mechanical methods

The demineralized specimens, previously subjected to mechanical testing and part of which were utilized for chemical assays, underwent thermomechanical analysis. The primary goal of thermo-mechanical analyses is to investigate how oxidation affects the structure of collagen in terms of collagen network nativity and connectivity. This study employed two established methods: Differential Scanning Calorimetry (DSC) and Hydrothermal Isothermal Tension (HIT).

For the HIT test, specimens were cut into the following dimensions 20mm×2mm×1.5mm (length × width × thickness) using a razor blade. Circular disks for DSC specimens were prepared using a 4-mm diameter cylindrical biopsy punch (Integra, York, PA, USA). The height of each DSC specimen was measured using digital calipers (MasterCraft, Vonore, TN, USA), and this measurement was used to estimate the volume of each specimen.

All specimens underwent testing with HIT and DSC following the same protocol, as described below.

2.5.1 Differential scanning calorimetry (DSC)

The differential scanning calorimetry (DSC) was carried out following methods reported in [284][192]. Differential scanning calorimetry (DSC) involved heating sealed specimen and empty reference pans at a constant rate. Differences in heat flow were recorded over time or temperature to analyze denaturation, when testing collagen or other proteins [184]. An endotherm peak in the heat flow vs. temperature curve indicated specimen melting.

The tests were carried out using a TA Instruments DSC Q-2000 system (TA Instruments, New Castle, DE, USA). Each specimen was placed in a hermetically sealed aluminum pan (TA Instruments, New Castle, DE, USA) and heated from 25°C to 95°C at a constant rate of 1.4°C/min. From each test, a heat flow vs. temperature curve was generated, followed by baseline correction and analysis using Universal Analysis Software (TA Instruments, New Castle, DE, USA) to obtain the desired measurements. These measures include three measures (Figure 7-A), encompassing enthalpy of denaturation, ΔH , temperature at maximum heat flow, T_{peak} , and full width at half maximum of heat flow, FWHM.

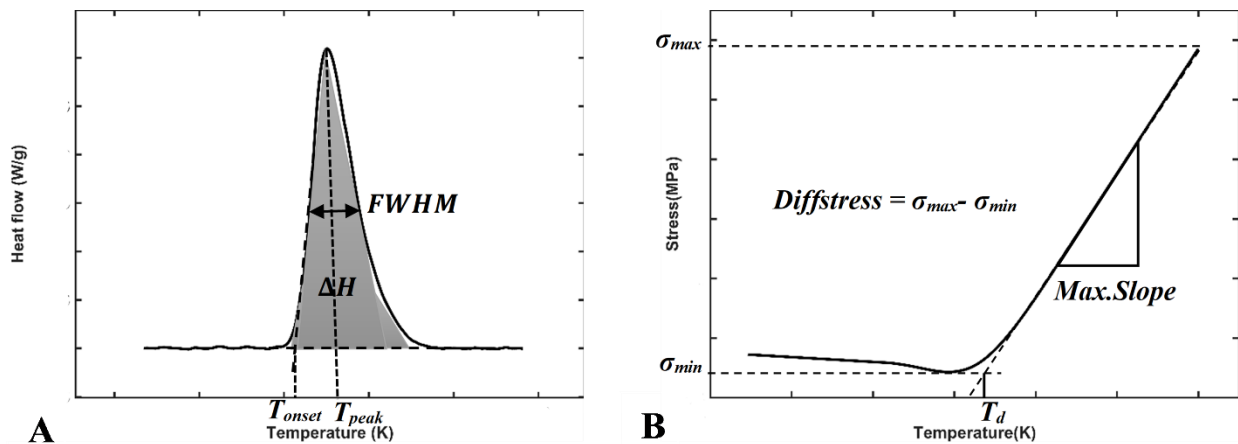


Figure46 . The various measures taken from A) a typical Differential Scanning Calorimetry (DSC) signal and B) a Hydrothermal Isometric Tension (HIT) signal.

After the test, the DSC specimens were freeze dried and then weighed to determine the dry mass of each. The collagen density of each specimen was calculated by dividing the dry mass of each specimen by the volume of each specimen.

2.5.2 Hydrothermal isometric tension (HIT)

Utilizing a specially designed HIT system capable of simultaneous testing of six specimens, the HIT samples underwent testing by securing them with custom-made clips approximately 3 mm away from each end [338]. These clips effectively maintained the specimens under isometric constraint. Following this, the constrained specimens were subjected to heating in a 4L water bath, transitioning from room temperature to 90°C at an average heating rate of 1.4°C/min, matching the heating rate of the DSC tests. Typically, at the initiation of heating, the specimens experience an initial stress relaxation owing to the viscoelastic properties inherent in the collagen network.

When collagen is gradually heated, at a specific temperature (the melting or "denaturation" temperature denoted here as T_d), a transition happens in the collagen structure that transforms collagen triple helices into random amorphous coils [15]. As a result of this transition, the collagen specimen would tend to contract in length, but since it is kept under constant length,

an isometric force will be generated instead. There are some characteristics of collagen network such as integrity (in terms of intermolecular network connectivity [192][16]) and crosslinking that are derived from an HIT test. The maximum rate of isometric tension generation denoted as Max. Slope [16], reflecting the overall connectivity of the collagen network and the denaturation temperature, T_d , identifying collagen's thermal stability are two common measures that have been reported for these measures (see Figure 46 -B).

Throughout the test, both the temperature and the tensile load generated by the specimen are measured and recorded by a Resistance Temperature Detector (RTD) and a load cell, respectively. The tensile load is converted to stress by dividing it by the specimen's initial cross-sectional area.

The HIT and DSC data from each specimen was finally used in the linear systems model previously proposed in [291] to generate α and β parameter values for each specimen. The model was used to make a connection between DSC and HIT test results and aid in the interpretation of these two test results using the two physics-based parameter α and β . These two parameters, being the model outputs, aid in assessing the quality of collagenous tissue. α reflects the amount of isometric tension generated per unit of collagen denaturation by capturing the relationship between the integral of the DSC signal and the early stages of isometric tension in HIT. Conversely, β represents the generation of additional tension after the denaturation stage starts, particularly at temperatures surpassing the denaturation (melting) temperature. β is formulated based on the relationship between temperature, and the developed isometric tension according to rubber (thermo)elasticity theory. It also correlates strongly with Max.Slope mentioned above.

2.5.3 Statistical analysis

Various statistical techniques were employed in this study, depending on the type and distribution of the data. The One-Way Analysis of Variance (ANOVA) test was used to determine if there were any statistically significant differences in the measured mechanical properties. In the case of the chemical assay data, the non-parametric Kruskal-Wallis test and

Dunn's comparison post-test were used as the data did not follow a normal distribution. These tests were used to assess differences in carbonyl concentration across different groups. Furthermore, Pearson correlation analysis was conducted to examine any potential correlations between the different parameters that were measured or obtained. Significant differences in means were indicated when $p\text{-value} < 0.05$ was detected. The statistical analyses were carried out using SigmaStat (Version 4.0, Systat Software Inc., CA, USA).

3 Results

3.1 Fluorescent image analysis

Prior to initiating the experiments, it was crucial to ensure that oxidative stress had uniformly permeated the entire specimen cross-section, not solely confined to the surface. This was achieved through the analysis of confocal images obtained from control and oxidized specimens using threshold analysis. Specimen cross-sections underwent analysis using the threshold value derived from the control specimen to identify staining attributable solely to oxidation-induced damage. The original and thresholded images are depicted in Figure 47. While the control specimen served to establish the threshold, the cross-section of the 24-hr oxidized specimen displayed distinct high pixel intensity regions seemingly corresponding to porosity and interfaces distributed relatively uniformly across its surface.

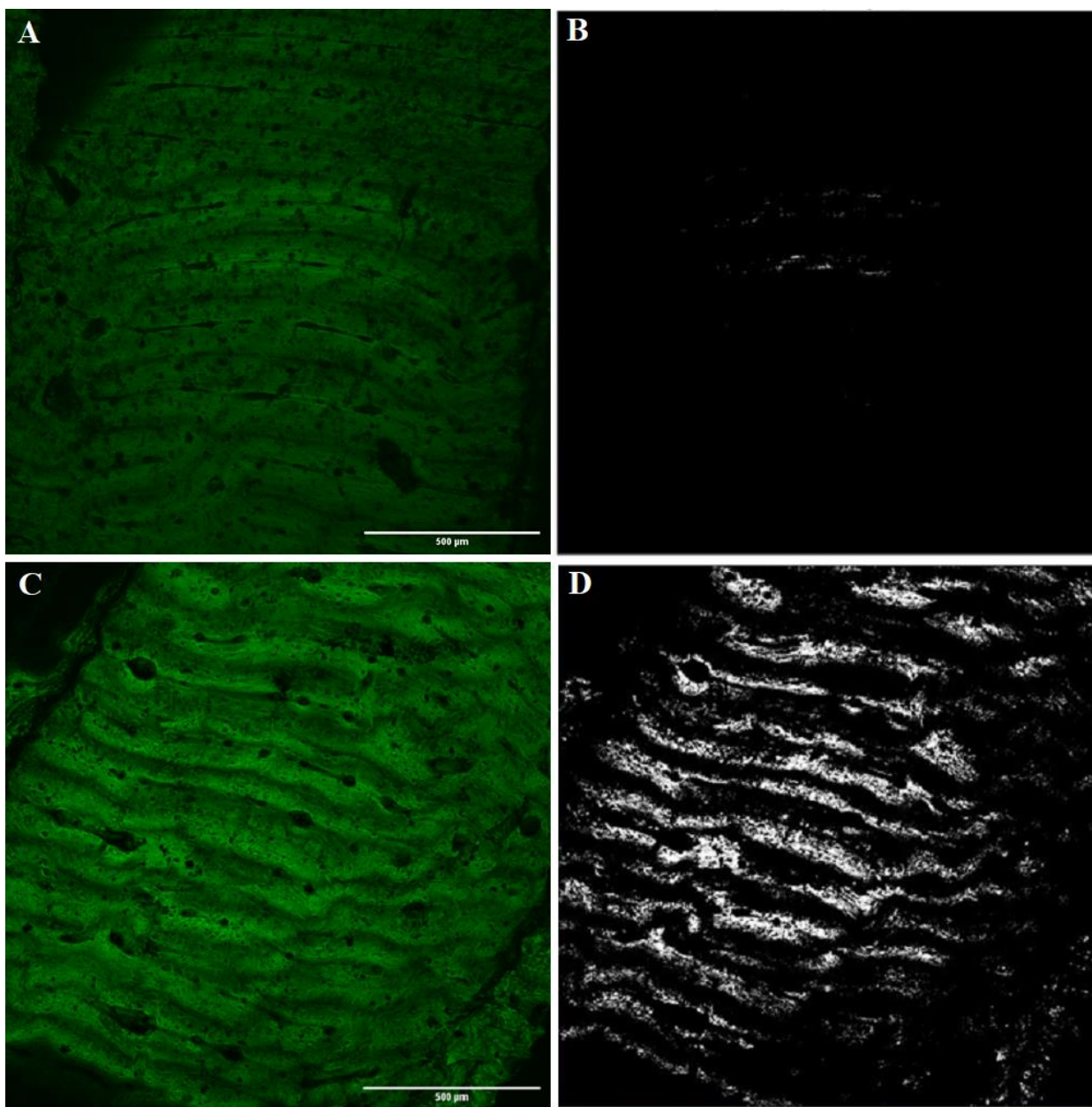


Figure 47. Confocal laser scanning microscopy images of A) control specimen cross-section and C) 24hr oxidized specimen cross-section. Threshold was set according to B) negative control (unoxidized) specimen to remove 99.5% of pixels. D) 24 hr oxidized specimen after thresholding demonstrating a notable increase in the detection of carbonyls and indicative of oxidative damage.

3.2 Mechanical testing

3.2.1 Uniaxial tensile tests at different strain rates

The results of the uniaxial tensile tests at three different strain rates, are presented below.

The results were investigated in two categories; Once, for each strain rate, the stress-strain curves for samples with different incubation hours were studied and compared and the statistically significant difference were also investigated between the groups. The stress-strain curves for $\dot{\epsilon}_0 = 0.0002 \text{ s}^{-1}$, $\dot{\epsilon}_1 = 0.002 \text{ s}^{-1}$ and $\dot{\epsilon}_2 = 0.02 \text{ s}^{-1}$ rate tests are depicted in

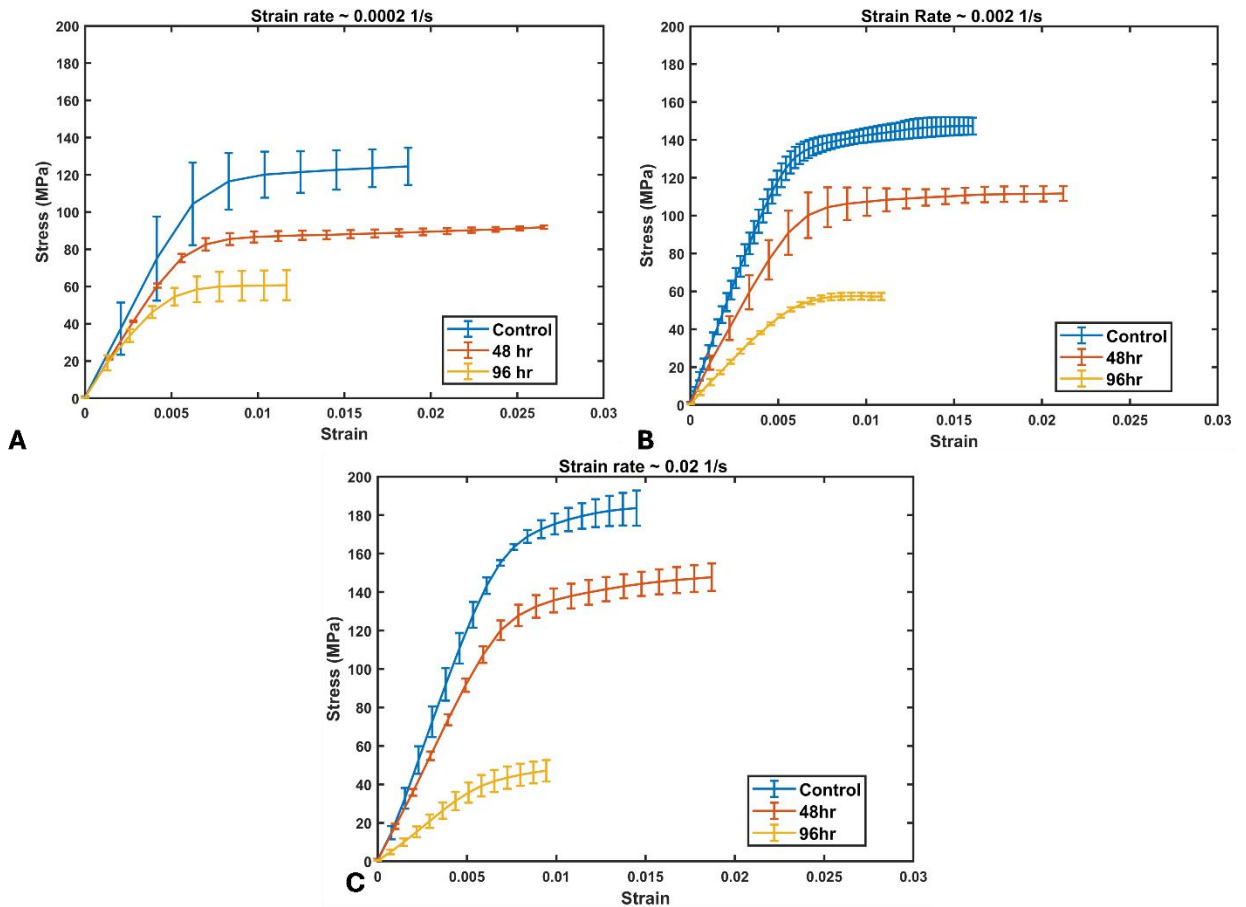


Figure 48.

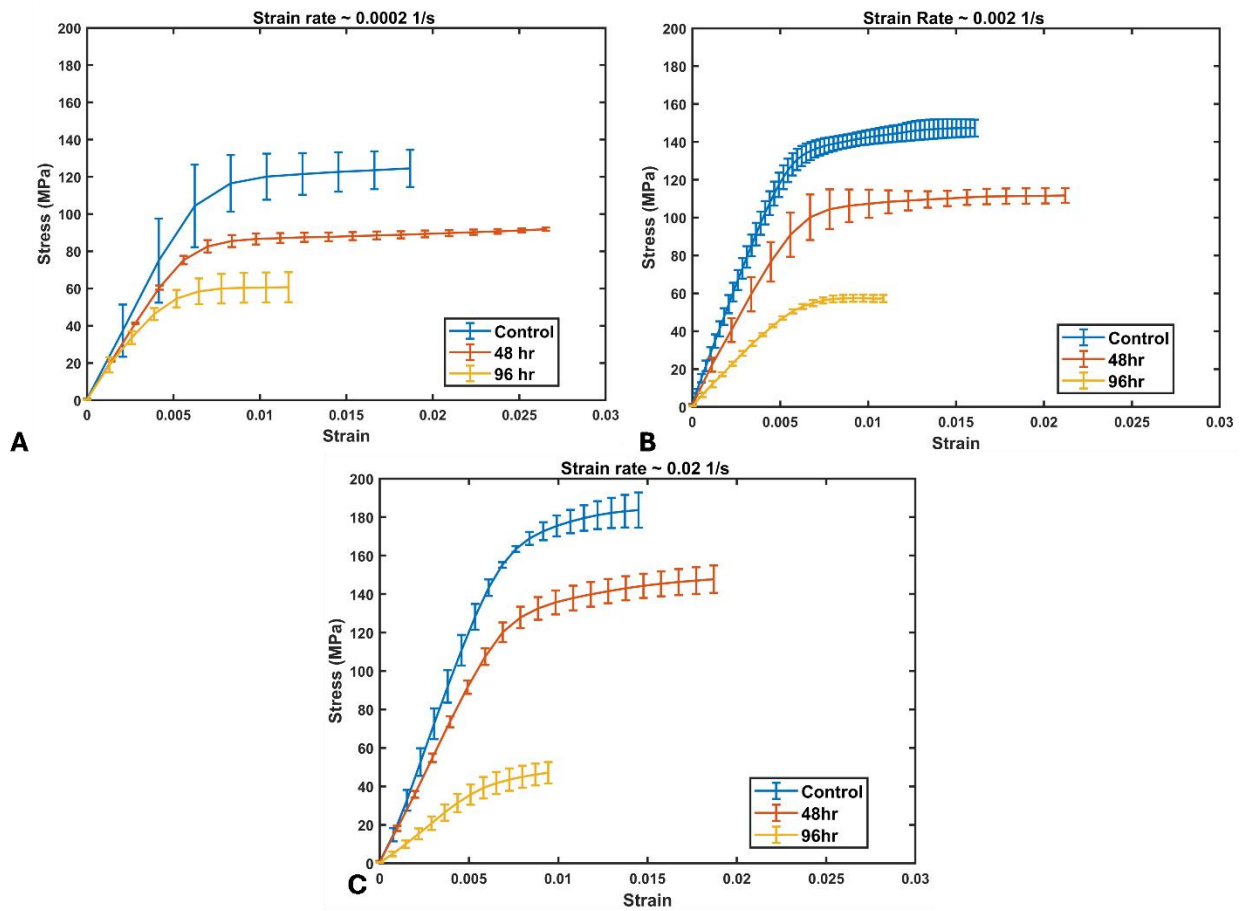


Figure 48. Representative stress-strain curves for the A) ϵ_0 strain rate, B) ϵ_1 strain rate C) and ϵ_2 strain rate tensile test specimens at different incubation time points.

Based on the stress-strain curves, at each strain rate, the mechanical properties (except ultimate strain), were increasingly degraded with increasing oxidation exposure. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test with 0.05 level of significance showed that there were significant meaningful differences between the mechanical properties (p-values in Table 13). The average mechanical properties measured at each strain rate are reported for the control and oxidised groups in Table 13.

Table 13. Tensile mechanical properties measured at each strain rate for the control and oxidised groups.

Strain rate		Modulus (GPa)	Yield Strength (MPa)	Ultimate Strength (MPa)	Ultimate strain ()
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$\dot{\varepsilon}_0 = 0.0002 \text{ s}^{-1}$	Control	18.0 (± 3.9)	119 (± 12)	125 (± 10)	0.0204 (± 0.0003)
	48 hr	14.5 (± 2.1)	85 (± 15)	92 (± 16)	0.0263 (± 0.0023)
	96 hr	13.0 (± 1.4)	59.4 (± 8.5)	60.5 (± 8.1)	0.0132 (± 0.0023)
	p-values	0.368	0.021	0.015	0.004
$\dot{\varepsilon}_1 = 0.002 \text{ s}^{-1}$	Control	23.4 (± 1.4)	128 (± 13)	150 (± 14)	0.016 (± 0.002)
	48 hr	17.0 (± 2.7)	106.1 (± 9.3)	111.7 (± 3.6)	0.021 (± 0.002)
	96 hr	9.39 (± 0.27)	56.9 (± 2.1)	57.2 (± 1.8)	0.011 (± 0.004)
	p-values	p < 0.001	p < 0.001	p < 0.001	p = 0.273
$\dot{\varepsilon}_2 = 0.02 \text{ s}^{-1}$	Control	24.6 (± 1.2)	169.1 (± 3.2)	186 (± 11)	0.015 (± 0.003)
	48 hr	15.8 (± 3.0)	118 (± 22)	139 (± 29)	0.019 (± 0.001)
	96 hr	7.2 (± 1.1)	45.1 (± 5.3)	49.9 (± 5.2)	0.010 (± 0.003)
	p-values	p < 0.001	p < 0.001	p = 0.011	p = 0.059

In

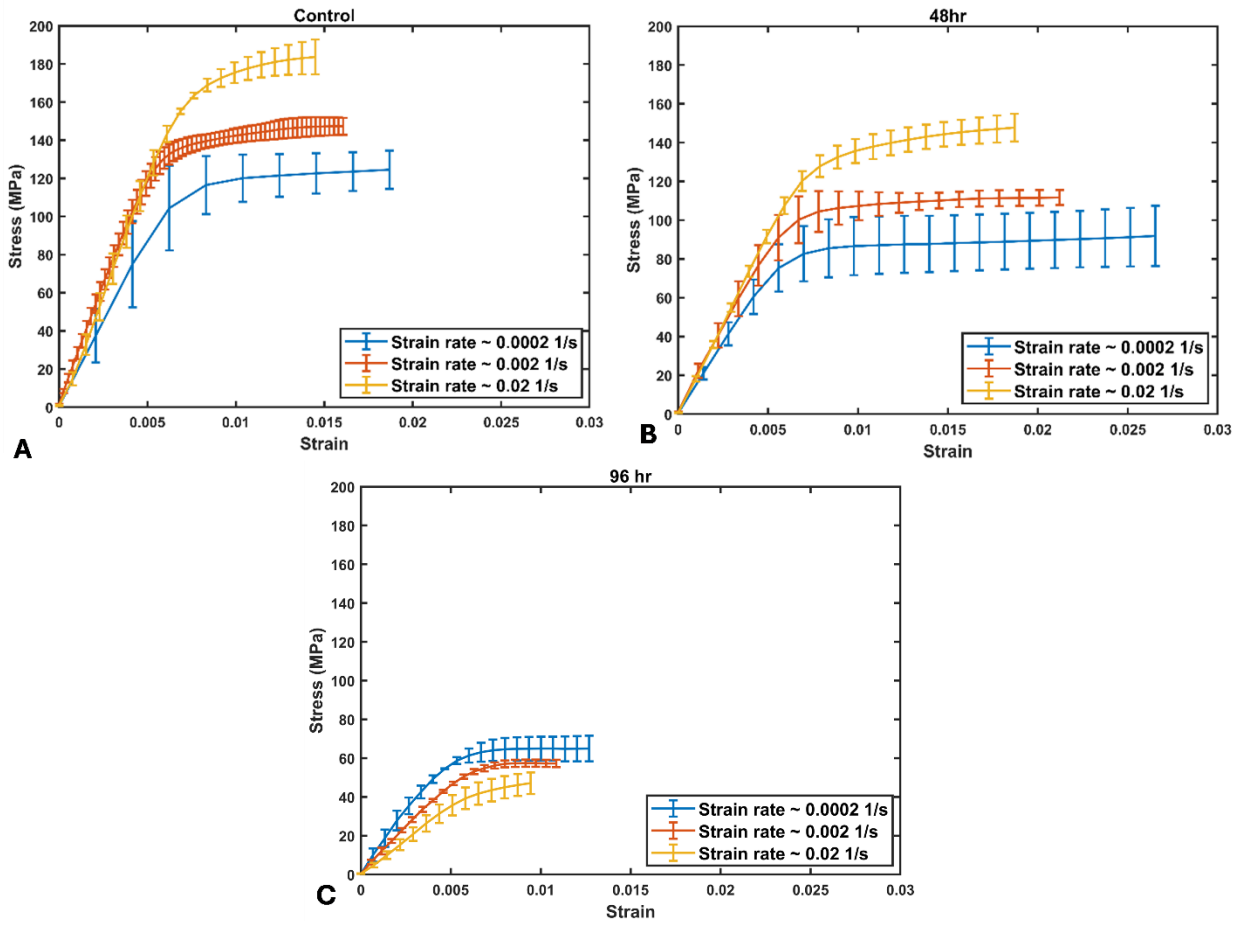


Figure 49 (A-C), the stress-strain curves for control, 48hr, and 96 hr specimens at different strain rates are presented.

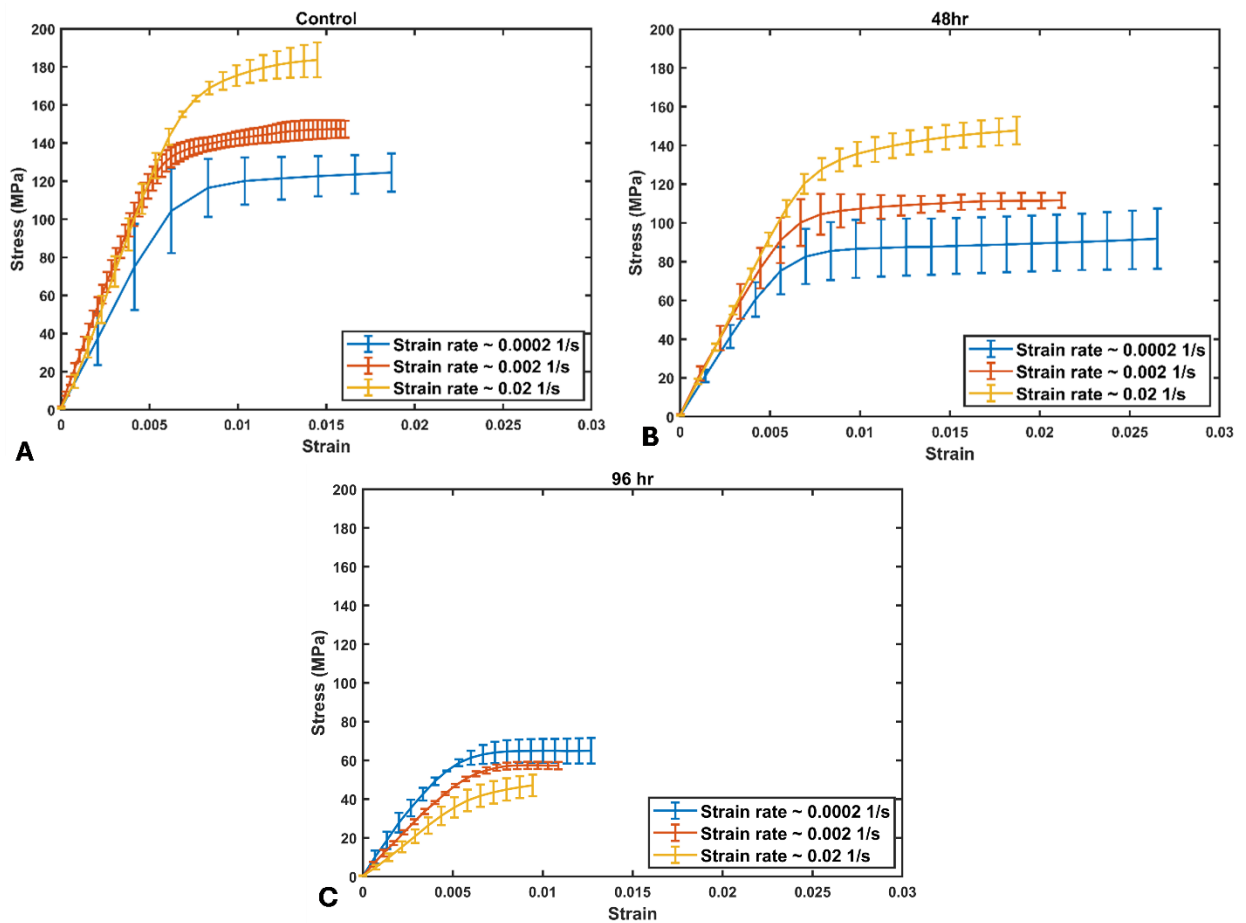


Figure 49. Stress-strain curves for A) Control B) 48 hr, and C) 96 hr oxidative exposure specimens tested under different strain rates.

For the control samples as well as the 48 hr oxidized specimens, as the strain rate increased the yield and post-yield mechanical properties (except the ultimate strain) increased, while the material became more brittle. However, a reverse trend is observed for the 96-hour oxidized samples, where an increase in strain rate led to a decrease in pre- and post-yield mechanical properties.

3.2.2 Uniaxial compression tests

The representative stress-strain curves for the uniaxial compression tests are depicted in **Figure 50**. According to these results, in control and 48 hr stress-strain curves, the material reaches its

maximum stress and subsequently loses its load-bearing capacity. This occurs due to the emergence of dominant damage mechanisms which are a combination of local stress hardening and shear sliding leading to gradual disintegration of the material [197]. With an increase in oxidation exposure, there is a deterioration in yield properties, however, due to the gradual propagation of cracks, a relatively slow process, a great deal of deformation occurs well before reaching the ultimate stress in the 96-hour group. The compressive mechanical properties for each time point are reported in Figure 50.

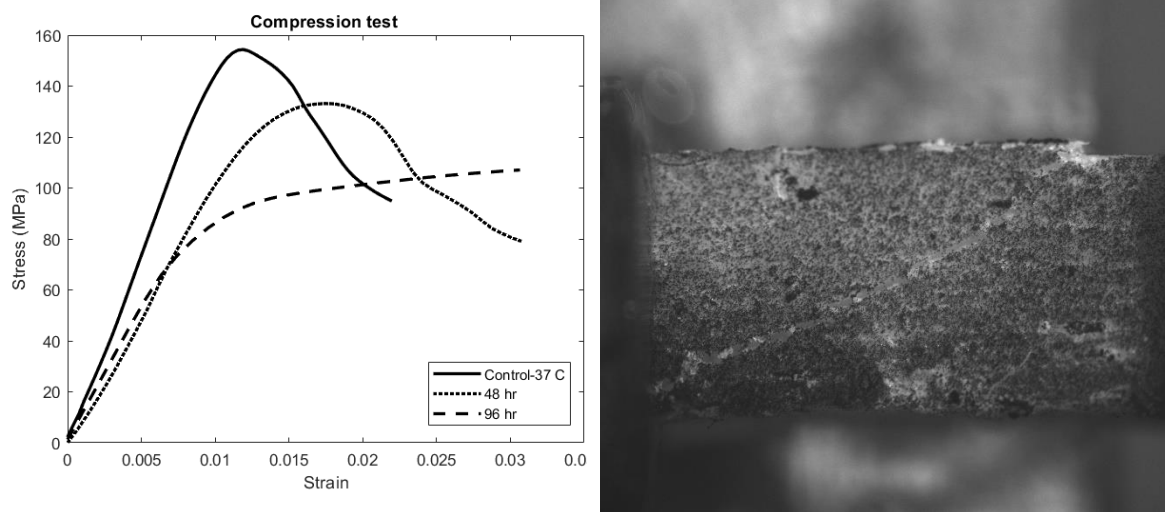


Figure 50. (left) Representative stress-strain curves from compression testing of specimens exposed to increasing levels of oxidation (n=4), (right) A picture of a specimen that failed under compression.

Table 14. Mechanical properties measured under compression for control and oxidised groups.

	Modulus (GPa)	Yield Strength (MPa)	Ultimate Strength (MPa)	Ultimate strain
Control	15.8 (± 2.2)	162 (± 15)	163 (± 15)	0.014 (± 0.003)
48 hr	10.2 (± 1.1)	131 (± 12)	136.7 (± 9.3)	0.018 (± 0.002)
96 hr	10.6 (± 2.1)	101 (± 15)	121 (± 17)	0.034 (± 0.007)
p-values	0.106	0.004	0.002	0.002

3.3 Load-unload tests

Figure 51 illustrates a representative load-unload curve for each level of oxidation exposure. Various measures have been derived from these curves, including E_5/E_4 (Secant modulus degradation between the last and second last cycle) which represents the deterioration in modulus in the last cycle. Also, both the permanent strain, ϵ_p , as well as the nonlinear strain ϵ_{nl} , decreased with increasing oxidation. It should be noted that all these measures have been calculated for the last loading cycle [279]. Energy dissipation in three different pathways, including the elastic release strain energy, permanent strain energy, and finally the hysteresis energy also showed decrease with increased oxidation. All these measures are reported in Table 15 for each time point.

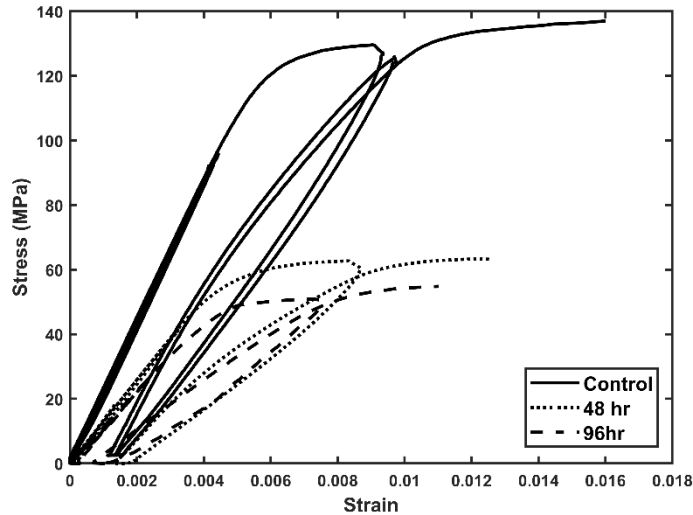


Figure 51. Representative stress-strain curves from load-unload tests for each specific oxidation exposure level.

Table 15. Characteristics of load-unload behavior at different oxidation exposures.

Property	Control	48 hr	96 hr	<i>p-value</i>
E5/E4	0.700 (± 0.040)	0.588 (± 0.036)	0.498 (± 0.045)	0.008
Permanent strain (%)	0.136 (± 0.030)	0.136 (± 0.036)	0.070 (± 0.011)	0.09
Nonlinear strain (%)	0.40 (± 0.14)	0.40 (± 0.05)	0.29 (± 0.13)	0.5
Elastic release strain energy (MJ/m ³)	0.121 (± 0.022)	0.045 (± 0.006)	0.020 (± 0.003)	<0.001
Permanent strain energy (MJ/m ³)	0.155 (± 0.016)	0.092 (± 0.012)	0.061 (± 0.012)	0.001
Hysteresis energy (MJ/m ³)	0.113 (± 0.025)	0.059 (0.009)	0.043 (0.007)	0.012

3.4 Chemical assay analysis

As described in section 2.4, the carbonyl content measured using the carbonyl assay was normalized using the collagen content measured by hydroxyproline assay to determine the carbonyl concentration per collagen concentration. The resulting concentrations of carbonyls per μmol of collagen for both the control and oxidized groups are presented in Figure 52. Since the data are not normally distributed, the median and Interquartile Range (IQR) [283] are used in Figure 52. It is important to note that specimens from various mechanical tests were combined, resulting in a total of 19 controls, 15 specimens oxidized for 48 hours, and 15 specimens oxidized for 96 hours. In each of the oxidized groups, one specimen was rejected due to its deviation from the group. A data point more than three standard deviations from the mean met the criterion for rejection. The p-values are presented in Table 16.

Additionally, to evaluate the presence of physiological levels of artificially induced oxidative stress in bovine cortical bone, human data from both healthy and diseased individuals were included for comparison. The diseased donors were diagnosed with inflammatory diseases, such as type 2 diabetes or chronic kidney disease, and had an average age of 77.1 years (all donors were above 60 age). The carbonyl content was measured by our group using the same methods as documented above. Figure 52 illustrates the medians obtained from 13 healthy

donors (10 male, 3 female), with an average age of 47.4, and 16 diseased donors (13 male, 3 female).

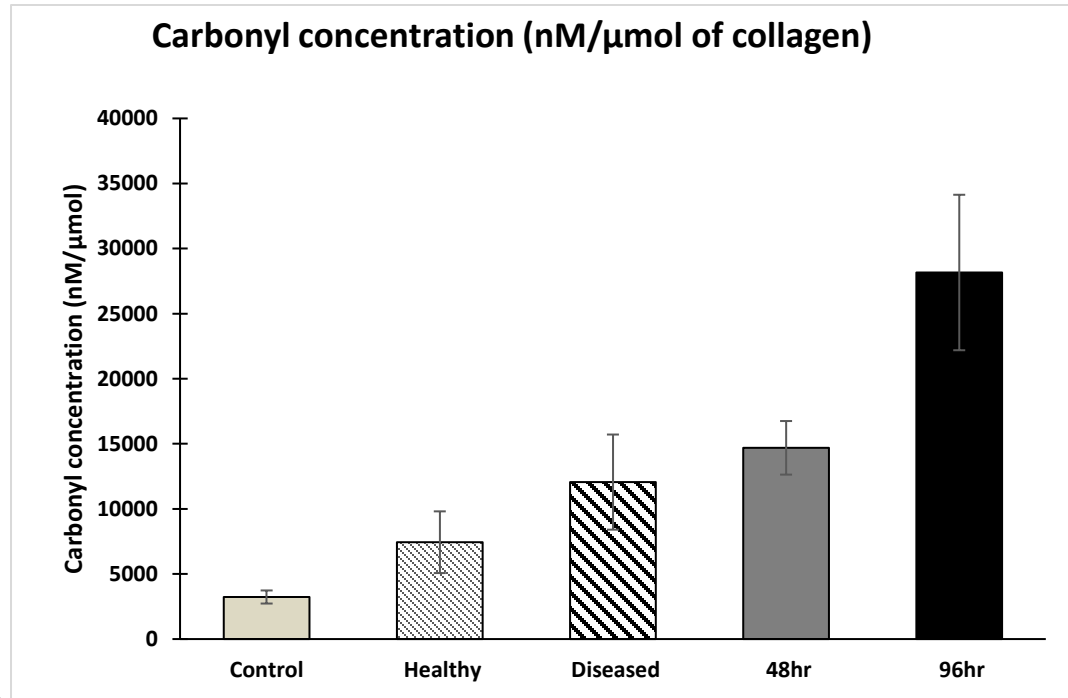


Figure 52. Carbonyl contents for control and oxidized groups compared with human bone collagen from nominally healthy and diseased donors.

Table 16. Comparison of control and oxidised groups

Comparison	Control vs. 48 hr	Control vs. 96 hr	48 hr vs. 96 hr
P-value	0.002	<0.001	0.013

3.5 Thermo-analytical methods

A total of six measures (refer to Figure46) were derived, with three from the HIT testing (Max.Slope, denaturation temperature or T_d , and Diffstress) and three from the DSC tests (enthalpy of denaturation, ΔH , temperature at maximum heat flow, T_{peak} , and full width at half maximum of heat flow, FWHM). Pearson correlations were conducted between the carbonyl

concentration and these measures, as presented in Table 17. Additionally, a model previously developed for DSC and HIT tests [291] was applied to the results to determine α and β parameter values for each specimen. The correlation between these parameters (α and β) and the six measures was also examined and reported in Table 17.

Table 17. Pearson correlation results for α , β and carbonyl concentration versus other measures of DSC and HIT testing for the control and oxidised groups.

α vs.	r	p-value	β vs.	r	p-value	Carbonyls vs.	r	p-value
Carbonyls	na	0.89	Carbonyls	-0.40	0.01	Max.Slope	-0.46	0.0038
Max.Slope	na	0.46	Max.Slope	0.47	0.0036	T _d	0.36	0.03
T _d	-0.61	<0.0001	T _d	na	0.21	Diffstress	-0.48	0.0024
Diffstress	na	0.91	Diffstress	0.48	0.0029	ΔH^*	na	0.47
ΔH^*	na	0.28	ΔH^*	-0.51	0.001	T _{peak}	na	0.8
T _{peak}	na	0.61	T _{peak}	na	0.11	FWHM	0.36	0.029
FWHM	na	0.24	FWHM	na	0.41			

***Enthalpy of denaturation**

As per the data presented in Table 17, both Max.Slope ($r=-0.46$, $p = 0.0038$) and its covariate Diffstress ($r=-0.48$, $p = 0.0024$) demonstrated significant negative correlations with the carbonyl content. Furthermore, a positive correlation was found between T_d ($r=0.36$, $p = 0.03$) and carbonyls and a notable negative correlation ($r=-0.42$, $p = 0.007$) was observed between the DSC measure, FWHM, and the carbonyls. Max.Slope is a measure of the connectivity of the bone collagen network and T_d, is a measure of collagen network stability. In addition, FWHM is a measure of the variation in thermal stability of the collagen, indicative of the organization of the collagen fibrils [16].

The model parameter, β , exhibited a negative correlation with the carbonyl concentration ($r=-0.4$, $p = 0.01$), while no significant correlation was identified between the other model parameter, α , and carbonyl concentration. Additionally, relatively strong correlations between

parameter β and DSC measure ΔH ($r=-0.51$, $p = 0.001$) was detected. Furthermore, a correlation between the model parameter α and denaturation temperature, T_d ($r=-0.61$, $p<0.0001$), was detected.

To assess the impact of oxidation on the mineral phase, we conducted thermal gravimetric analyses (TGAs) in our laboratory. These analyses were performed on oxidized and control specimens ($n=3$ for each group) to determine if there were any alterations in the mineral phase due to oxidation. The ash weight difference between the oxidized and control groups, originating from the same specimen, was 2% on average. This suggests that the oxidation method employed in this study had a minimal effect on the mineral phase.

4 Discussion

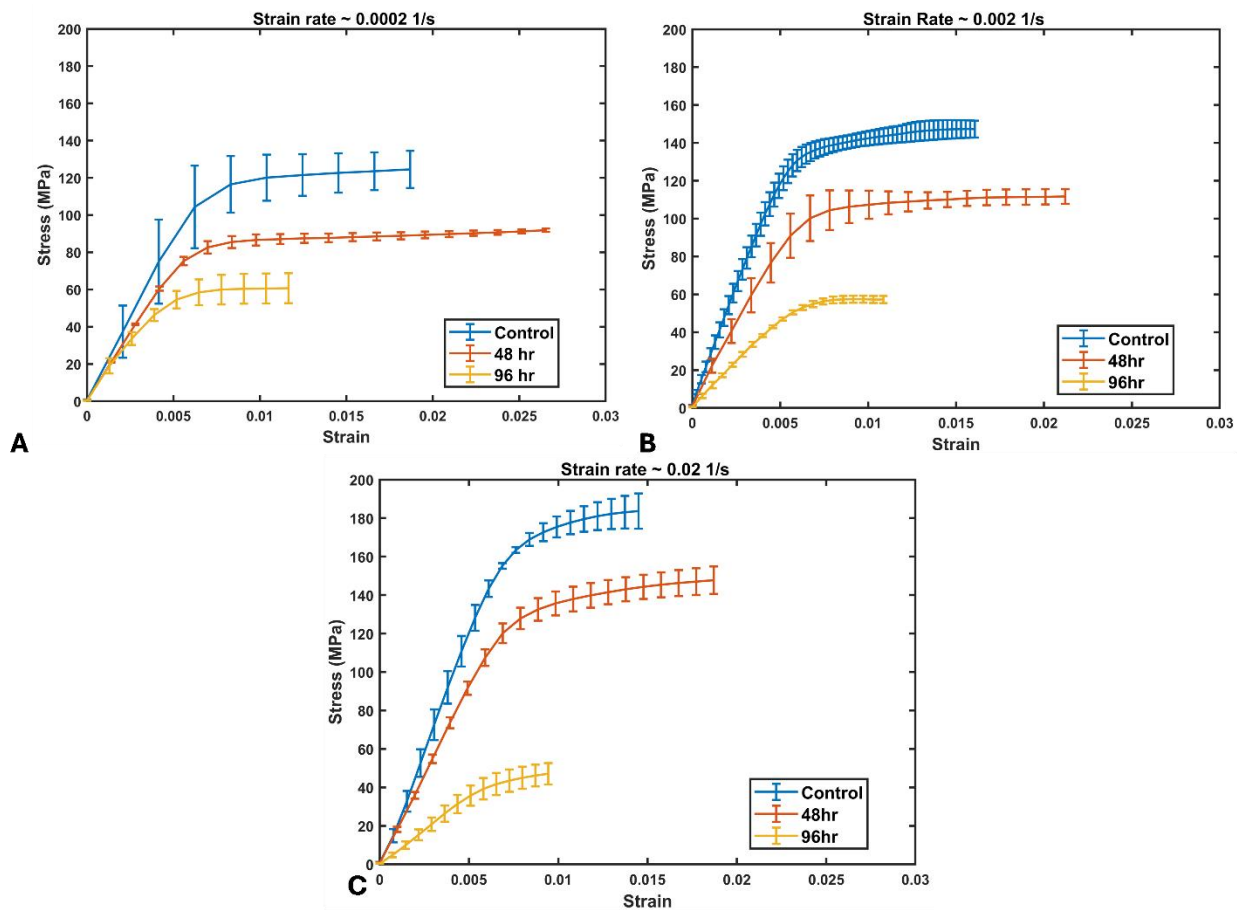
This study delves into the intricate relationship between oxidative damage, mechanical behavior, and thermo-mechanical properties in bovine cortical bone. Through meticulous experimental design and execution, the impact of controlled in vitro oxidation on bone quality has been probed, spanning from comprehensive mechanical testing to chemical assay analyses and thermo-mechanical methods.

The key hypothesis was that oxidative damage of bone collagen, induced by neutral hypochlorous acid in this context, would lead to a deterioration of the bone tissue mechanical properties. This results in degraded bone quality due to damage to bone collagen evident from increased carbonyl content and deteriorated collagen network organization and connectivity.

The carbonyl content, a stable marker of protein oxidative damage, significantly increased in the oxidized groups with incubation time (Figure 52). The normalization of carbonyl concentration with collagen content provided quantitative insights into the extent of protein oxidation in bone. It's noteworthy that doubling the incubation time from 48 hours to 96 hours resulted in nearly a twofold increase in protein carbonylation. For validation of the oxidation levels, the carbonyl levels in the oxidized and control bovine bone specimens were compared to carbonyl contents from human specimens from healthy and diseased donors. According to Figure 52, the mean value of the carbonyl content in the human diseased group (12000 ± 3900)

is close to the mean carbonylation value of the 48 hr oxidized group (14700 ± 2700) indicating that this amount of oxidation using HOCl in vitro creates a physiologically relevant level of oxidation similar to that found in inflammatory diseased conditions, such as type II diabetes with and without chronic kidney disease. However, the average amount of carbonylation in the healthy human group (7400 ± 2400) was nearly twice as high as that in our control bovine bone (3200 ± 680.3). This difference could be attributed to species differences between bovine and human samples, as well as the older age of the healthy individuals (mean age = 47.7), with 8 donors falling within the age range above 50. It is expected that the healthy cohort would have a higher amount of carbonylation than the bovine bone due to the biological age of the donors. Nonetheless, these values are significantly lower than those observed in our oxidized groups.

According to the results of the uniaxial tensile tests (



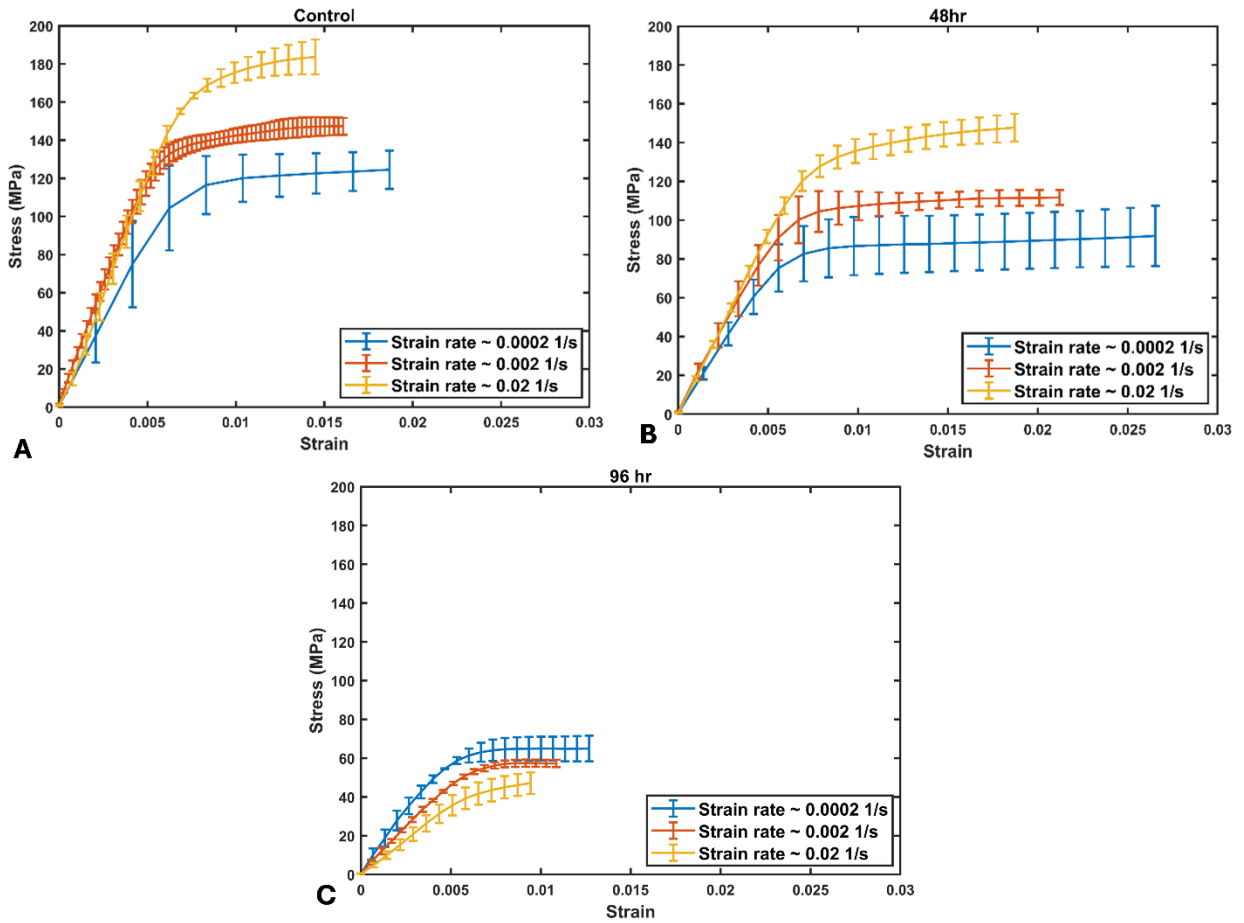


Figure 49), at each strain rate, the bone mechanical properties significantly degraded (except ultimate strain) upon increasing exposure to oxidation which underscores the susceptibility of bone collagen to oxidative stress and damage. In uniaxial tensile tests of both control and 48-hour incubated oxidized samples, there was an increase in yield and post-yield mechanical properties (excluding ultimate strain) with higher strain rates. However, for the 96-hour incubated oxidized specimens, an intriguing reversal occurred: an increase in strain rate resulted in a decrease in pre- and post-yield mechanical properties. According to previous studies [306]–[309], elastic modulus and yield properties of cortical bone increase with increasing strain rate in both tension and compression loading. However, the effect of strain rate on post-yield mechanical properties of cortical bone is conflicted, as more recent studies

[307][310][311] indicate that the bone behaviour becomes more brittle with increasing strain rate, whereas older studies did not [306][312][313]. Nowadays, it is well established that at low strain rates bone tissue fails in a ductile manner and as the strain rate increases, the energy absorbance reduces, and the bone becomes more brittle [307][314][315]. A recent study by Zioupos et al. [315], investigated the combined effect of increasing strain rate and aging on bone behavior. Their findings indicate that cortical bone exhibits the highest fracture resistance at physiological strain rates (0.001-0.01). The transition from ductile-to-brittle behavior occurs at strain rates of 0.001 in young cortical bone, while in older cortical bone, this transition occurs at lower rates, ranging from 10 to 40 times lower (all values specific to human bone). In our case, like aging, as the incubation time increased, the bone became more brittle, and the ductile-to-brittle transition happened at a lower strain rate (e.g. 0.002 1/s). This suggests that the method employed in this study to induce oxidative stress in bone may replicate the physiological responses observed in vivo during aging and in disease states.

The elastic modulus values obtained for the control group align with those documented in previous literature [197][339]. As expected, the yield and ultimate strength and strain in tensile mode are notably lower than those in compression. In tension, specimens experience prompt failure upon the initiation of damage, while compression involved a gradual damage progression impeded by the compressive state. This amalgamation captures both the influences of damage softening and strain hardening [197]. In tension, after the ultimate strength point, the material fails, losing its load-bearing capacity due to the prevalence of damage mechanisms, however, because of the shear damage mechanism in compression, the stress-strain curve's nature is determined through the combined effects of local stress hardening and shear sliding [197]. The steeper the stress-strain curve, the higher the local stress hardening and the lower the shear sliding, as per [197]. According to [197], three types of stress-strain curves manifest in a compression test based on the crack propagation pattern. The first type exhibits a sudden stress reduction at lower strain when a dominant crack spans the specimen, observed in the control group's stress-strain curve (refer to Figure 50). The second type involves multiple fractures throughout the specimen, resulting in a flat progression of the

compressive stress-strain curve, observed in the 96 hr oxidized specimens (Figure 50). The third type represents an intermediate scenario between the previous two types, featuring a continuous stress reduction alongside multiple cracks progressively propagating throughout the specimen, observed in the 48hr oxidized specimens (Figure 50). Li et al. [197] proposed that a more pronounced local stress hardening and a reduced degree of shear sliding result in a sharper stress-strain curve. Consequently, as oxidation levels rise, the ability to resist shear sliding diminishes, accompanied by a decrease in the extent of stress hardening as well.

Mechanical damage in bone is observed through the presence of inelastic stress-strain behavior [54]. However, it is not possible to differentiate the contributions of various constitutive behaviors, such as viscosity, plasticity, and damage, in the accumulation of inelastic strains during a monotonically applied test until failure. In order to address this, it is necessary to analyze the unloading and reloading characteristics [54]. The load-unload curves and associated measures reflect the damaging effects of oxidative stress. Progressive degradation of secant modulus, an important measure for quantifying damage accumulation, suggests a weakening of bone structure with prolonged incubation time. Also, the dissipation of energy in all three pathways, namely permanent strain, elastic release energy and hysteresis energy decreased with increasing incubation time. This implies that the bone material exhibits reduced post-yield deformation or ductility with greater incubation time.

Thermo-analytical tests, namely DSC and HIT, yielded six measures reflecting the thermo-mechanical properties of bone collagen. The negative correlations between carbonyl concentration and measures such as Max.Slope, and Diffstress, which are indicative of collagen network connectivity demonstrate the negative impact of oxidative damage on bone collagen. Also, there was a positive correlation between the denaturation temperature, T_d , a measure of collagen network stability and the carbonyl content which implies that because of oxidation the collagen network became more thermally stable. Furthermore, the application of a previously developed model [291] to the experimental data explored the parameters α and β . These parameters serve as good indicators for evaluating collagenous tissue quality in terms of collagen network connectivity and thermal stability, respectively. These correlations once

again suggest that oxidative damage negatively affects bone collagen molecular nativity and connectivity. In addition, a new correlation was found between the DSC measure FWHM and the carbonyl content, suggesting that oxidative damage disrupts the organization of the collagen at the fibril level. These results underscore the importance of considering oxidative damage as a critical factor in understanding bone quality, with potential implications for conditions where oxidative damage plays a role, such as aging or certain diseases like diabetes.

This work was not without limitations. Notably, during the mechanical testing phase, specimens weren't irrigated due to the limitations of the testing machine. However, as stated in [296], if the test duration is under 10 minutes, there is less than a 1% weight loss due to water evaporation, which applies to all our mechanical tests. This implies that the dehydration of specimens has minimal impact on the test results. Additionally, the study was confined to three specimens per time point. Increasing the number of specimens for each time point could bolster the robustness of the experimental design. In addition, it is important to note that while the effects of oxidation on mineral content were analyzed through TGA analyses and were found to have minimal impact, this study did not include an examination of any microstructural changes in bone that might be caused by oxidation.

5 Conclusion

This study has confirmed the hypothesis that physiological levels of oxidative damage deteriorate bone mechanical properties by damaging bone collagen, emphasizing the importance of considering oxidative damage in understanding bone quality. Various mechanical testing methods were adopted to investigate the degradation of bone mechanical properties due to oxidative damage. Additionally, chemical assay analyses were employed to measure protein carbonylation, a stable by-product of collagen oxidation, while thermo-mechanical techniques were applied to assess nativity and connectivity of bone collagen post-oxidation. The adoption of several investigative approaches ensured a thorough examination of changes in bone quality in response to oxidation. The choice of oxidation using a hypochlorous acid (HOCl) solution, with its strong physiological oxidizing properties, provided a controlled oxidative stress environment. The findings of this study open avenues

for further research into the specific mechanisms underlying oxidative stress-induced damage to bone tissue. Exploring the in-vivo effects of oxidative stress and its influence on bone properties could provide additional insights into bone fragility. Moreover, translating these findings to clinical contexts and investigating potential interventions to mitigate oxidative damage in bone could have implications for therapeutic strategies.

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Appendix B

Iranmanesh F, Willett TL. A linear systems model of the hydrothermal isometric tension test for assessing collagenous tissue quality. *Journal of the Mechanical Behavior of Biomedical Materials*. 2022 Jan 1;125:104916.

“Accessible through the link below as well as the University of Waterloo’s Institutional Repository (UW Space)”

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Appendix C

Iranmanesh F, Dapaah DY, Nyman JS, Willett TL. An improved linear systems model of hydrothermal isometric tension testing to aid in assessing bone collagen quality: Effects of ribation and type-2 diabetes. Bone. 2024 May 31:117139.

“Accessible through the link below as well as the University of Waterloo’s Institutional Repository (UW Space)”

<https://www.sciencedirect.com/science/article/pii/S8756328224001285>

Appendix D

Appendix D: Iranmanesh F, Montesano J, Zysset Ph, Willett TL. A constitutive model for the cortical bone that can capture the effects of relevant levels of oxidative damage observed in aging and diseases.

A constitutive model for the cortical bone that can capture the effects of relevant levels of oxidative damage observed in aging and diseases.

Faezeh Iranmanesh^a, John Montesano^b, Philippe Zysset^c, Thomas L. Willett^a

^aComposite Biomaterials System Lab, System Design Engineering Department, University of Waterloo, Ontario, Canada

^b Composites Research Group, Mechanical and Mechatronics Engineering, University of Waterloo, Ontario, Canada

^c ARTORG Center for Biomedical Engineering Research, University of Bern, Bern, Switzerland

Abstract

Oxidative stress (OS) is caused by an excess of reactive oxygen species that the body unable to control. OS has been demonstrated in a wide variety of chronic diseases and in aging. OS can damage components in the body including proteins, lipids, and DNA. Collagens, which are the most abundant proteins in the body, are good targets for these reactive oxygen species because they are relatively long lived. Very little research has been done on the development of oxidative damage and its deleterious impact on bone collagen and bone quality. In the current study, oxidative damage has been induced in bovine cortical bone by incubating bone specimens in neutral hypochlorous acid solutions. The mechanical properties of the oxidatively damaged cortical bone which were previously measured are calibrated in terms of oxidative stress biomarkers. The findings of this study provide new insights into the mechanics of oxidatively damaged bone. Building upon these findings, a previously developed constitutive model was extended for modeling oxidatively damaged bone. This extension involves substituting the constants in both the constitutive equation and the evolution equation with

relevant material functions of oxidative stress biomarkers. This model has been integrated into the commercial finite element solver Abaqus/Standard, allowing for accurate and reliable predictions of mechanical behavior. To validate the model, we have compared its predictions with experimental test data from single edge notched beam bending tests. Remarkably, the model closely aligned with the experimental load-deflection response, with an error rate of 7%. Additionally, the predicted microdamage process zone (MDPZ) area demonstrated 8% error when compared to experimental data. Also to demonstrate the capability of the model, the effect of variation in specimen size on the MDPZ has been explored using this model. These findings highlight the potential of our model to predict the mechanical behavior of cortical bone affected by aging and disease where oxidative damage plays a significant role in bone quality deterioration.

Keywords

Bone; Oxidative stress; Oxidative damage; Viscoplastic constitutive model; tensile deformation response

1 Introduction

Bone is a complex hierarchical tissue composed of inorganic mineral (mainly calcium deficient, highly substituted hydroxyapatite crystals), organic components (predominantly collagen type I), and water [54][53]. While the mineral phase of bone is known for contributing to pre-yield properties by providing stiffness [13][326], the collagen content, a major component of the organic phase, is crucial for bone's yield and post-yield mechanical behavior [14]–[16], [19], [326]. Studies by Wang et al. [14] revealed that heating and denaturing bone collagen significantly diminishes both bone toughness and strength, while leaving the elastic modulus largely unaffected. Similar research corroborates that collagen damage reduces toughness and strength without affecting stiffness [15][16][340]. For instance, Willett et al. [16] demonstrated a strong correlation between collagen network integrity and cortical bone fracture toughness, and Zioupos [15] highlighted that age-related collagen changes contribute to bone fragility. Moreover, research on genetically mutated collagen underscores its essential role in sustaining post-yield deformation and energy absorption prior to fracture [112].

Bone collagen is altered due to translational and post-translational processes over the lifespan. Aging and disease are among the most significant processes that can affect bone collagen, influencing both its content and structure [19]. In collagen, enzymatic crosslinking, typically enhances bone tissue mechanical properties. Conversely, non-enzymatic crosslinking, like advanced glycation end-products (AGEs), is often associated with increased bone brittleness [25]–[29]. While debates exist regarding a direct link between AGE accumulation and bone fragility [32] [33], conditions such as diabetes [20][21] and chronic kidney disease (CKD) [22][23], in which elevated levels of AGE have been reported, have been correlated with heightened fracture risks. This increased susceptibility does not consistently align with the measured areal bone mineral density (aBMD), prompting investigations into collagen changes as the fundamental reason behind the heightened fragility in diseases like diabetes and chronic kidney disease [29].

These post-translational modifications, especially alterations like AGE accumulation, are closely tied to oxidative reactions [30][15][31]. Oxidative reactions not only substantially amplify AGEs but also may compromise beneficial enzymatic crosslinks within collagen [32] and lead to chain fragmentation [33]. Oxidative stress, a pivotal factor in chronic diseases and age-related complications [10], [34]–[36], can detrimentally impact collagen's structure and function in bone tissue. This factor may be the underlying cause of numerous pathologies that evolve gradually over time [149].

Oxidative stress (OS) is characterized by an increase in reactive oxygen species (ROS), leading to an imbalance between ROS production and a cell's antioxidant defenses [38]. These ROS, natural by-products of cellular metabolism, can be beneficial or harmful depending on their concentration [125]. When ROS levels exceed the body's capacity to neutralize them, OS occurs, causing oxidative damage to various body constituents and contributing to disease development [41]. ROS play a significant role in aging and related complications, with chronic diseases often evolving due to oxidative stress as people age [42][43]. Evidence shows that bone biology is particularly affected by oxidative stress [24],[34]–[40]. Amazingly, despite the fact that oxidative stress has been demonstrated to damage bone collagens [44][45], very little

research has been done on the effects of oxidative stress towards the deterioration of bone tissue quality.

To evaluate oxidative stress and damage in collagen, several methods have been developed [341][150]. An effective approach involves measuring stable markers that indicate oxidative stress levels in specific tissues [329]. Among these, protein carbonyls are widely used indicators of oxidative protein damage [342]. Tissues under oxidative stress typically show increased carbonyl content, making protein carbonyls a reliable measure for assessing the impact of oxidative stress on proteins such as collagen [37][342]. In addition, oxidative stress can induce structural alterations in collagen molecules, such as cross-linking and/or fragmentation. As discussed above, oxidative stress can promote unfavorable non-enzymatic AGEs as well as deterioration of beneficial enzymatic cross-links. Various techniques [343][344] can be utilized to assess changes in collagen structure caused by oxidative stress and the resulting damage. As fully outlined in the experimental part of our previous experimental study [refer to experimental paper], we utilized thermo-analytical techniques such as differential scanning colorimetry (DIC) and hydrothermal isometric tension (HIT) to investigate collagen structural alterations in terms of collagen network nativity and connectivity.

To better understand the relationship between bone biology and bone tissue mechanical performance in circumstances such as aging and bone-affecting diseases, a constitutive model for damaged bone material under oxidative stress condition, i.e., oxidatively damaged bone tissue, would be helpful. Although in recent years, extensive research has been dedicated to understanding the complex biomechanical behavior of bone, aiming to unravel the underlying mechanisms that govern its damage behavior, a model of this nature has not been formulated to date.

In the pursuit of constitutive models tailored to simulate bone mechanical behavior, researchers have developed many computational models, providing a valuable supplementary tool to empirical investigations [345]. So far, several models capturing the damage characteristics of bone material at varying length scales [346][347], featuring differing material symmetries

(like [242] or orthotropic [205]), diverse loading conditions (such as monotonic [242] or cyclic loading [240], static or dynamic loading rates), and various constitutive responses (including elastic, plastic [240], rate-dependent [348], fracture [349][339], etc.), have been developed. Typically, these models are integrated into finite element methods to facilitate the study of intricate geometries, material distributions, and loading scenarios.

Notably, Schwiedrzik and Zysset [247] proposed an anisotropic elasto-viscoplastic damage constitutive model for bone. In this model, the viscoplasticity was implemented using Perzyna's formulation [248], while a scalar function of the accumulated plastic strain was used to model the damage, and a polynomial flow rule was introduced to capture the material behavior after viscoplastic yielding. Interestingly, this model can capture the evolution of damage and the anisotropic behavior of bone, from the elastic mode to the viscoplastic mode. This model is widely applicable on several length scales and describes bone porosity, mineral, and organic phase cohesive behavior, as well as viscoplastic and damage dissipation mechanisms. This model [247] is foundational for the present study.

Bone material exhibits intrinsic toughening mechanisms that play pivotal roles in delaying the initiation and propagation of cracks [84]. One such mechanism is the formation of microcracks [350][84]. When subjected to mechanical loading, pre-notched bone specimens exhibit the development of a distinct microcrack zone in close proximity to the crack tip, termed the microdamage process zone (MDPZ) [294], [297], [351]. This region, the size of which is significant compared to different bone length scales [351], serves to enhance the toughness of bone material by absorbing energy, blunting the stress field and therefore impeding crack growth[350][302].

Recent investigations have endeavored to model the onset of bone fracture by simulating the formation of the MDPZ through the application of continuum damage mechanics (CDM) [294], [297], [351]. This approach is particularly relevant as the accumulation of micro-damage within bone material leads to a progressive softening of the material and a subsequent reduction in stiffness [297].

One critical aspect that has emerged from the investigations of MDPZ is the size effect observed in bone tissue. The size effect, referring to the phenomenon where some aspects of material behavior exhibit variations with specimen size, has been particularly manifested in bone fracture toughness and accordingly the size of MDPZ [300][301]. This phenomenon has significant implications for the prediction of bone fracture behavior, as it underscores the need for a comprehensive understanding of bone mechanics beyond traditional fracture mechanics analyses.

Central to the size effect in bone is the concept of fracture toughness, which represents the material's resistance to crack propagation [302]. While fracture toughness is a fundamental property of bone, its dependence on specimen size has garnered increasing attention in recent years [301][300], [303], [304]. Studies have demonstrated that as the size of bone specimens changes, there is a notable change in fracture toughness, suggesting a size-dependent enhancement in resistance to crack propagation [301][300], [303], [304]. This observation challenges conventional assumptions about bone mechanics and underscores the importance of considering size effects in bone fracture analyses.

It seems that the role that the MDPZ plays in enhancing the toughness of bone material caused the size effect to extend beyond fracture toughness to encompass the size-dependency of the MDPZ in bone material. Understanding the size-dependent behavior of the MDPZ, which has received very little attention so far, is essential for clarifying the mechanisms governing crack initiation and fracture processes in bone.

In this study, the elasto-viscoplastic damage model developed by Schwiedrzik and Zysset [30] for bone material was adapted and fine-tuned to incorporate the effects of oxidative stress. Also, the model was fully calibrated using the experimental findings in [the experimental paper] to integrate the effect of oxidative stress into the aforementioned model. This integration was made using multivariate linear regression using which different coefficients of the model have been proposed in terms of different oxidative stress biomarkers.

Using this model, the MDPZ has been simulated to study bone behavior under physiological and super physiological levels of oxidative damage and then was applied to investigate the size effect on MDPZ in both healthy and oxidized bone.

By investigating the effects of oxidative damage on bone tissue through experimental study [refer to the experimental paper] and subsequent constitutive modeling, this study aims to provide insights into how oxidative damage influences the mechanical behavior of bone tissue. The model developed in this study may provide a predictive framework for evaluating the mechanical responses of cortical bone under conditions of oxidative damage, which are relevant for scenarios where oxidative stress plays a significant role in bone strength degradation, such as aging and certain disease conditions.

2 Materials and Methods

2.1 Constitutive modeling and implementation

This study utilized an elasto-viscoplastic constitutive model to simulate the behavior of cortical bone. The model incorporates a viscoplastic deformation mechanism coupled with damage accumulation. The rheological model, illustrated in Figure 53, comprises a damageable elastic spring in series with a viscoplastic component, consisting of a plastic pad and a parallel dashpot, assuming an additive split of elastic and plastic strain [247].

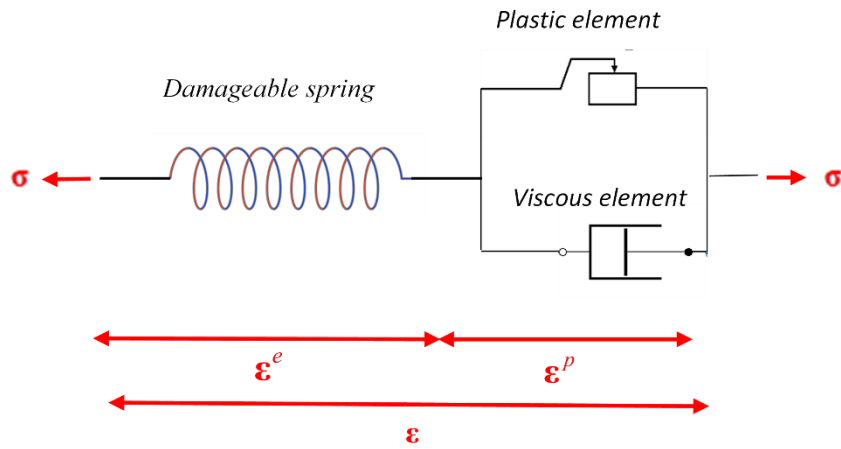


Figure 53. Rheological model of elasto-viscoplastic solid damage proposed by Schwiedrzik and Zysset [247].

While the material undergoes elastic deformation, the model's behavior remains independent of the strain rate. Transitioning into the viscoplastic regime, a Perzyna-type viscoplasticity formulation [248] is used for the accumulation of plastic strains. This model is based on two internal state variables $\boldsymbol{\varepsilon}^p$, a second-order tensor incorporating inelastic strains, and κ , representing the total accumulated plastic strain. It is assumed that damage accumulation is coupled with plasticity by a damage scalar function $D(\kappa)$ that reduces all elements of the elasticity tensor. This coupling is assumed on the notion that in bone, inelastic deformations typically coincide with the onset of damage, suggesting a synchronous initiation of these processes.

In the following equations, the symbols used for scalars, second order tensors, and fourth-order tensors are Z , $\mathbf{Z}$, and $\mathbb{Z}$ respectively. The double contraction operation, denoted by ":", is described as $\mathbf{Z}_{ij} = \mathbb{X}_{ijkl} \mathbf{Y}_{kl}$. The dyadic product is denoted by the operator $\otimes$ and is defined as $\mathbb{Z}_{ijkl} = \mathbf{X}_{ij} \mathbf{Y}_{kl}$. The symmetric product, denoted by $\bar{\otimes}$ is given by $\mathbb{Z}_{ijkl} = \frac{1}{2} (\mathbf{X}_{ik} \mathbf{Y}_{jl} + \mathbf{X}_{il} \mathbf{Y}_{jk})$.

The constitutive equation is described as follows [247]:

$$\boldsymbol{\sigma} = (1 - D(\kappa)) : \mathbb{E} : (\boldsymbol{\varepsilon} - \boldsymbol{\varepsilon}^p) \quad (22)$$

Where $\boldsymbol{\sigma}$ is the Cauchy stress tensor, $\mathbb{E}$ is the elastic tensor, $\boldsymbol{\varepsilon}$ is the total strain tensor, $\boldsymbol{\varepsilon}^p$ is the plastic strain tensor and D is the scalar damage variable defined by [244][264]:

$$D(\kappa) = 1 - e^{-k_p \kappa} \quad (23)$$

$$0 \leq D(\kappa) \leq 1$$

Where k_p is a constant assumed to be equal to 10.5 based on [244].

The rate-independent yield function is an anisotropic quadric yield criterion which is defined in 3D stress space [265] as

$$Y(\boldsymbol{\sigma}, \kappa) = \sqrt{\boldsymbol{\sigma} : \mathbb{A} : \boldsymbol{\sigma}} + \mathbf{A} : \boldsymbol{\sigma} - r(\kappa) = 0 \quad (24)$$

In this formulation, the hardening function, $r(\kappa)$, is an isotropic hardening function assumed to be an explicit function of the accumulated plastic strain:

$$r(\kappa) = 1 + (1 - y_r)g(\kappa) \quad (25)$$

$$g(\kappa) = 1 - e^{-k_s \kappa}$$

y_r is the ratio of the yield and ultimate properties, and k_s is a material constant representing the curvature of the exponential hardening function.

Also, in eq. (24), $\mathbb{A}$ is a fourth-order tensor, and $\mathbf{A}$ is a second-order tensor defining the origin, form and the orientation of the yield criterion [247][265].

$$\begin{aligned} \mathbb{A} = & \sum_{i=1}^3 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \mathbf{M}_i \otimes \mathbf{M}_i - \sum_{i,j=1; i \neq j; j \neq 3}^3 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j \\ & - \sum_{i=1; j=3}^2 \frac{1}{4} \left(\frac{1}{Y_j^+} + \frac{1}{Y_j^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j + \sum_{i,j=1; i \neq j}^3 \frac{1}{2T_{ij}^2} \mathbf{M}_i \bar{\otimes} \mathbf{M}_j \end{aligned} \quad (26)$$

$$\mathbf{A} = \sum_{i=1}^3 \frac{1}{2} \left(\frac{1}{Y_i^+} - \frac{1}{Y_i^-} \right) \mathbf{M}_i$$

In this formulation, Y_1^+, Y_2^+, Y_3^+ and Y_1^-, Y_2^-, Y_3^- are the uniaxial tensile and compressive yield stresses in the principal material directions, and T_{12} , T_{23} and T_{13} denote the shear yield stresses. Also ξ_{12} , ξ_{23} and ξ_{13} are the interaction coefficients [265]. Since cortical bone is usually assumed to be a transversely isotropic material, the material properties in the two transverse directions are assumed to be equal ($\begin{bmatrix} 1 \\ 1 \end{bmatrix} = \begin{bmatrix} 2 \\ 2 \end{bmatrix}$). In the following sections, for simplicity, the

indices for the transverse directions are replaced by t , and the indices for the longitudinal direction are replaced by l . so $[\sigma]_1 = [\sigma]_2 = [\sigma]_t$, and $[\sigma]_3 = [\sigma]_l$. Also, $\mathbf{M}_i$ is defined as follows:

$$\mathbf{M}_i = \mathbf{u}_i \otimes \mathbf{u}_i$$

Where $\mathbf{u}_i$ are the unit vectors of the principal material coordinate system of the cortical bone.

During the viscoplastic flow, the stress state of Perzyna viscoplastic materials can be outside of the rate-independent yield surface. In the inelastic regime ($\dot{\gamma} \geq 0$), the rate-dependent yield surface can be defined by expressing a new condition restricting the viscoplastic flow which will be a generalization of the rate-independent yield condition ($\dot{\gamma} = 0$) :

$$\bar{Y} = Y + \frac{m}{2} - \left(\frac{m^2}{4} + \frac{\eta}{\Delta t} \frac{\Delta \kappa}{h} \right)^{1/2} = 0 \quad (27)$$

Where η and m are two constants controlling the viscoplastic flow; the parameter η accounts for the viscosity and m is the degree of non-linearity of the viscoplastic strain evolution. Also h is the norm of the normal of the yield surface. As one would expect, when $\eta \rightarrow 0$, the rate-dependent yield surface, $\bar{Y}$, transforms into the rate-independent yield surface, Y .

2.1.1 Integrating the effects of oxidative damage into the constitutive model of cortical bone

In this study, the oxidative damage does not evolve with time due to the in-vitro nature of the oxidation method (as opposed to in-vivo conditions). Although the mechanical damage in bone is affected by oxidative damage, and the rate of mechanical damage may accelerate or decelerate because of the oxidation damage, the essential mechanisms of mechanical damage in oxidized bone are assumed to be unchanged from those of a healthy bone. As a result of mechanical damage in either healthy or oxidized bone, the material loses its continuity via the formation of voids and microcracks. Consequently, the mechanical properties degrade [54]. To incorporate the impacts of oxidative damage into the bone material's constitutive model,

the method proposed in the study of Murakami and Mizuno [275] for integrating irradiation effects into a creep-hardening surface model was employed. Inspired from the study by Murakami and Mizuno [275], a simple way to incorporate the effects of oxidative damage into the constitutive model is provided by changing the coefficients of the constitutive and evolution equation in which these coefficients will be replaced by the corresponding material functions of oxidative damage.

Regarding the damage induced by oxidative stress on bone collagen molecules, an effective tool for quantifying the extent of oxidative damage is through the use of oxidative damage biomarkers [150]. These biomarkers can be molecules or compounds that are either directly involved in oxidative damage or are affected by it [150]. In the experimental phase of this study [refer to experimental paper], carbonyl content per collagen content - a stable and early indicator of oxidative damage - was measured in a significant number of oxidized and controlled specimens. Furthermore, to investigate the structural changes in oxidized bone collagen, including collagen network connectivity and nativity, two thermo-analytical techniques, namely differential scanning calorimetry (DSC) and Hydrothermal isometric tension (HIT) tests, were employed [refer to experimental paper]. To enhance the utility and interpretation of these tests' results, a previously proposed and verified mathematical model [291][352] was applied for evaluating collagen network characteristics using two model parameters, α and β .

Thus, the integration of the effects of oxidative damage into the constitutive model involves replacing the coefficients of the constitutive and evolution equations with material functions of carbonyl content measurements (CC), α , and β [refer to experimental paper].

The coefficients of the constitutive equations (reviewed in Section 2.1), can be divided into multiple classes. These parameters (listed in [Table 9](#)) include the elastic constants (the constants of the elasticity tensor $\mathbb{E}$ for transverse isotropy), parameters related to the cortical bone yield criterion (Y_i^+, Y_i^-), hardening parameters, (γ_r, k_s), the parameter of the damage law (k_p), and viscous parameters (η, m).

Table 18. Coefficients of the constitutive model

	Coefficients of the oxidized bone model
Elastic constants	$E_t, E_l, \nu_t, \nu_{tl}, \mu_{lt}$
Compressive yield strength	$Y_t^-, Y_l^-(CC, \alpha, \beta)$
Tensile yield strength	$Y_t^+, Y_l^+(CC, \alpha, \beta)$
Tensile ultimate strength	$U_t^+, U_l^+(CC, \alpha, \beta)$
Hardening law parameters	$y_r(CC, \alpha, \beta, \dots), k_s(CC, \alpha, \beta)$
Viscosity parameters	$\eta(CC, \alpha, \beta, \dots), m(CC, \alpha, \beta)$
Parameter of the damage evolution law	$k_p(CC, \alpha, \beta)$

According to our experimental findings [refer to experimental paper], the elastic properties of bone do not change meaningfully because of oxidation as changes in the organic phase of the bone mostly affect the yield and post-yield behavior of bone and hardly affect the elastic properties. Thus, the elasticity parameters were assumed to be constant in this study.

Also, according to earlier studies [292][293], the average collagen orientation found in lamellar bone is within 30° of the bone's long axis implying that the collagen is primarily aligned with the long axis of the bone. Thus, changes in collagen play a significant role in the longitudinal properties of cortical bone. With this assumption, the investigation focused solely on changes in the longitudinal properties following oxidation, as detailed in the experimental study [refer to experimental paper].

In order to determine parameters related to the cortical bone yield criterion ($Y_i^+(CC, \alpha, \beta, \dots), Y_i^-(CC, \alpha, \beta, \dots)$), a series of uniaxial quasi-static tension and compression tests were conducted under varying levels of oxidative damage, detailed in the experimental paper [refer to experimental paper]. Also, the uniaxial tests helped to define the hardening parameters ($y_r(CC, \alpha, \beta, \dots), k_s(CC, \alpha, \beta, \dots)$). To determine the parameter of the damage law, $k_p(CC, \alpha, \beta, \dots)$, load-unload tests in the tensile mode were implemented at different levels of oxidative damage. Furthermore, for assessing the viscous parameters

$(\eta(CC, \alpha, \beta, \dots), m(CC, \alpha, \beta, \dots))$, uniaxial tensile tests were performed at various strain rates and oxidative damage levels. For further insights into these tests, readers are encouraged to refer to the detailed experimental study (refer to experimental paper).

Here, since the constitutive model is furnished to account for the effect of oxidative damage, the fourth-order tensor $\mathbb{A}$, and the second-order tensor $\mathbf{A}$ in eq. (26) change as follows:

$$\begin{aligned}
 \mathbb{A} = & \sum_{i=1}^2 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \mathbf{M}_i \otimes \mathbf{M}_i \\
 & + \frac{1}{4} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} + \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right)^2 \mathbf{M}_3 \otimes \mathbf{M}_3 \\
 & - \sum_{i,j=1; i \neq j}^2 \frac{1}{4} \left(\frac{1}{Y_i^+} + \frac{1}{Y_i^-} \right)^2 \zeta_{ij} \mathbf{M}_i \otimes \mathbf{M}_j \\
 & - \frac{1}{4} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} + \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right)^2 \left(\sum_{i=1}^2 \zeta_{i3} \mathbf{M}_i \otimes \mathbf{M}_3 \right. \\
 & \left. + \sum_{i=1}^2 \zeta_{3i} \mathbf{M}_3 \otimes \mathbf{M}_i \right) + \sum_{i,j=1; i \neq j}^3 \frac{1}{2T_{ij}^2} \mathbf{M}_i \bar{\otimes} \mathbf{M}_j \\
 \mathbf{A} = & \sum_{i=1}^2 \frac{1}{2} \left(\frac{1}{Y_i^+} - \frac{1}{Y_i^-} \right) \mathbf{M}_i + \frac{1}{2} \left(\frac{1}{Y_3^+(CC, \alpha, \beta, \dots)} - \frac{1}{Y_3^-(CC, \alpha, \beta, \dots)} \right) \mathbf{M}_3
 \end{aligned} \tag{28}$$

Based on the above changes in the fourth-order tensor $\mathbb{A}$, and the second-order tensor $\mathbf{A}$ in eq. (5), the yield function defined in eq. (24) changes accordingly.

Also, the hardening function in eq. (25) transforms into the following form:

$$\begin{aligned}
 r(\kappa) &= 1 + (1 - y_r(CC, \alpha, \beta, \dots))g(\kappa) \\
 y_r(CC, \alpha, \beta, \dots) &= \frac{Y_3^+(CC, \alpha, \beta, \dots)}{U_3^+(CC, \alpha, \beta, \dots)}
 \end{aligned} \tag{29}$$

$$g(\kappa) = 1 - e^{-k_s(CC, \alpha, \beta, \dots)\kappa}$$

Where U_3^+ is the ultimate tensile strength in the longitudinal direction.

The rate-dependent yield function can be defined by modifying eq. (6), as follows:

$$\bar{Y} = Y(CC, \alpha, \beta, \dots) + \frac{m(CC, \alpha, \beta, \dots)}{2} \quad (30)$$

1

2.1.2 Numerical algorithm implementation

The numerical algorithm consists of two iterative processes. The initial process focuses on the local level, where it integrates the elasto-viscoplastic behavior over a specific time step by utilizing the return mapping algorithm (iterating over i). The subsequent process operates at a global level (iterating over increment number n), integrating the outcomes of the local step into the equilibrium equations. It also evaluates the equilibrium deviation by determining the residual. A visual representation of the numerical algorithm can be found in the flow chart of Figure 27.

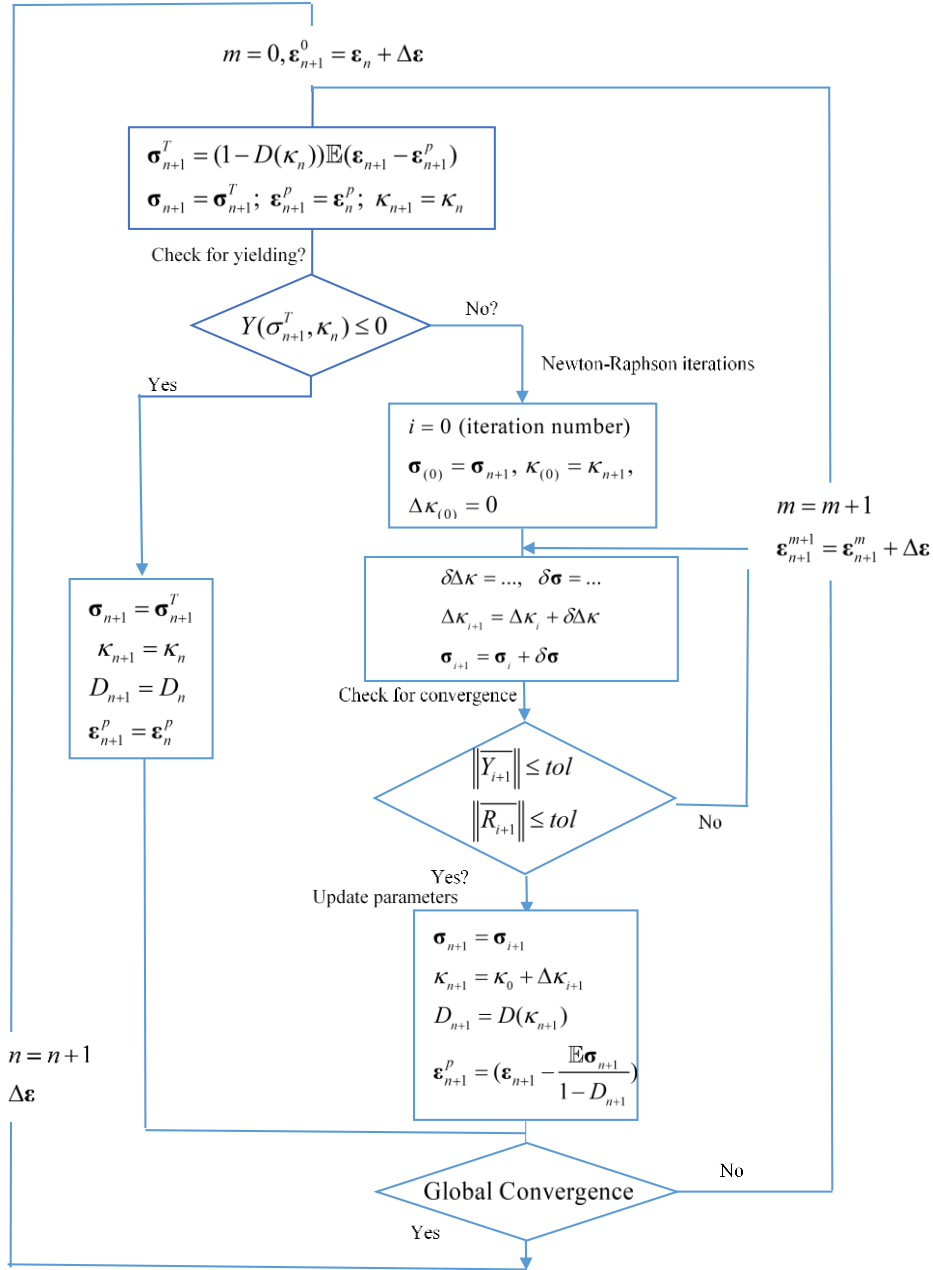


Figure 54. Flow chart of the updating stress-strain tensors and the state variable procedure

For the return mapping algorithm, the residual error of elastic strains and the yield function are linearized with respect to their variables $\boldsymbol{\sigma}$ and $\Delta\kappa$. This linearization results in a system of

equations that is solved using the Newton-Raphson algorithm. By solving this system, the variable increments ($\delta\sigma$, $\delta\Delta\kappa$) are determined, which are then used to update the values of σ and $\Delta\kappa$ during the return mapping process [247].

2.1.3 Identification and illustration

For the parameter identification purposes, a series of in vitro mechanical, chemical and thermo-analytical tests, the details of which are fully outlined in [exp. paper], were carried out on bovine cortical bone at different oxidation levels in order to identify various coefficients of the constitutive equations outlined in *Table 9*. The mechanical tests included uniaxial tensile test at three different strain rates, compression tests and load-unload tests.

The Young modulus in the longitudinal direction, E_l was determined using the quasi-static uniaxial tensile and compressive tests. The tensile strength in the longitudinal direction, Y_l^+ , as well as the hardening law parameters, y_r , and k_s were identified using the quasi-static uniaxial tensile test which was the uniaxial test performed at the lowest rate. The compressive strength in the longitudinal direction, Y_l^- , was identified using the compression tests. The parameter of the damage evolution law, k_p , was identified using the load-unload tests and for identification of the viscous parameters, η and m the tensile tests at three different strain rates were utilized.

For parameter identification, a series of mechanical test simulations were performed to model the uniaxial tensile tests. To simulate the uniaxial tensile tests, the same geometry and dimensions used to generate the G-code for the CNC mill were imported into Abaqus FEA software (refer to Figure 55). The model was meshed using 7,007 four-noded tetrahedral solid elements. Boundary conditions were applied to simulate the experimental setup, with one shoulder of the specimen subjected to a prescribed displacement while the other shoulder was fixed to prevent translational movement. The numerical algorithm described in Figure 3 was implemented as a user-defined material subroutine (UMAT) in the finite element software Abaqus Standard.

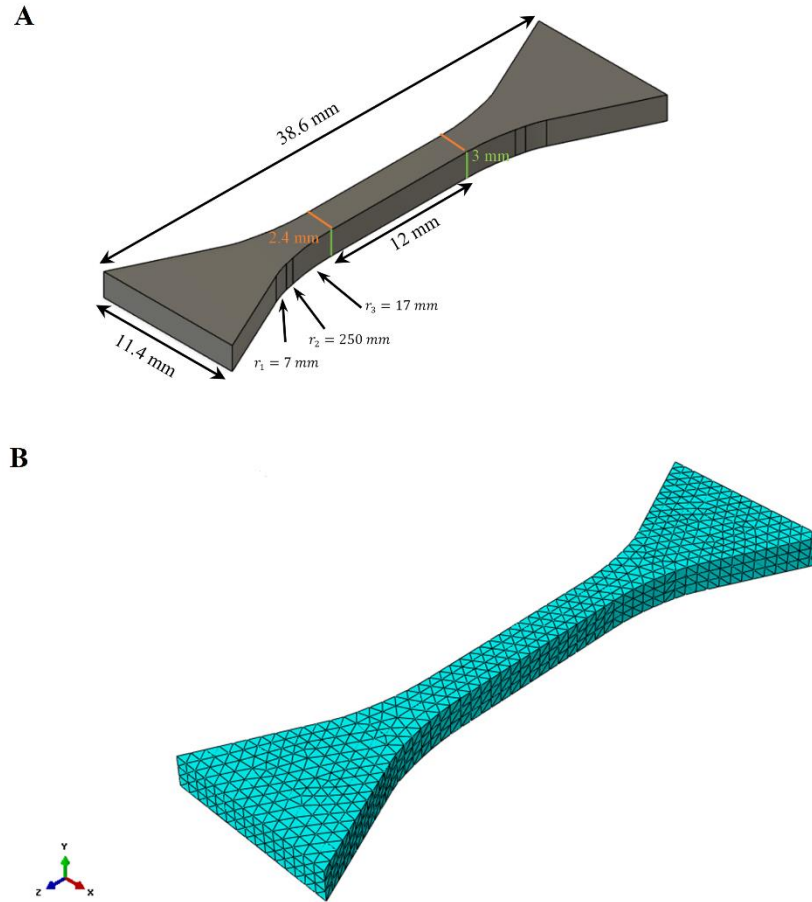


Figure 55. A) Geometry of the tensile test specimens. B) Finite element mesh of the tensile test specimens.

To model the microdamage process zone (MDPZ), 3-point single edge notched bending (SENB) tests were simulated. The specimen ($50 \text{ mm} \times 4 \text{ mm} \times 4 \text{ mm}$) had a pre-notch with a prescribed length (2mm), and only half of the specimen was modeled due to symmetry. The model length (Z direction) was aligned in the longitudinal direction of the bone microstructure. The mesh consisted of 57,607 unstructured tetrahedral elements, with a finer mesh around the notch tip. Boundary conditions were applied to simulate the supports and prevent translational movement. A prescribed displacement was applied on the symmetry line of the sample. Also,

one pinned support was fixed to prevent translational movement in the vertical direction, while the nodes on the symmetric face were fixed to prevent translational movement in the horizontal direction and rotation around the axis perpendicular to the page. Noted that to simulate the experimental tests at the location of the two supports, the translational movement in the direction perpendicular to the page was fixed, otherwise the model was unstable.

To check the validity of the model utilizing SENB results, the model outputs were compared with the corresponding experimentally derived measures. These evaluations encompassed the comparison of microdamage zone shapes and sizes at the peak load—typically indicative of the DIC strain field in the longitudinal direction—as well as the load-deflection behavior for $T_{inc}=0$.

In order to provide additional validation of the model, the MDPZ sizes of the simulated control and gamma-irradiated specimens were compared, and the size reduction was evaluated against the results reported by Burton et al. [192]. Burton's study employed a barium sulfate staining technique coupled with high-resolution micro-computed tomography (μ CT) to visualize and quantify the MDPZ. It should be noted that to find the equivalent incubation time for the gamma-irradiated specimen, the load-deflection curve from the irradiated specimen was used and aligned with the model data.

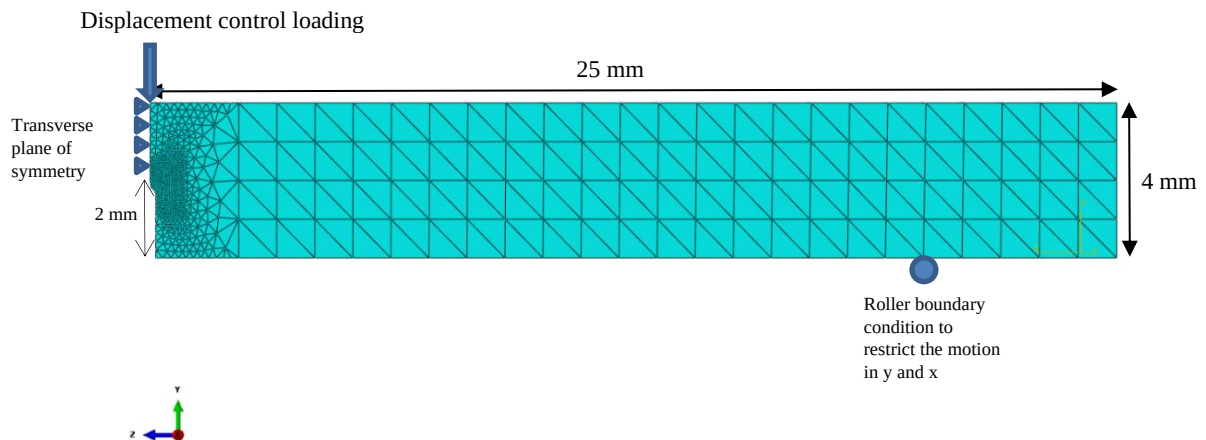


Figure 56. Mesh pattern and boundary conditions as well as the dimensions of the SENB model.

2.1.4 Exploring the size effect on the microdamage process zone (MDPZ)

In order to explore the specimen size effect on the size of the MDZP, three geometrically similar SENB models with size ratios of $(1:\sqrt{6}:6)$, similar to were simulated under 3-point bending test and three different oxidation levels. The widths (W) of the specimens were 3 mm, 7.35 mm, and 18 mm and the thickness (B) was a fixed value of 4 mm. For all specimens, the span-to-width ratio (S/W) was 0.2.

For this case, the whole model was simulated, and three boundary conditions were used. Two supporting rigid cylinders were fixed through their reference points which means all translational and rotational degrees of freedom were restrained. On the other hand, the loading cylinder was fixed in all directions except the loading direction where a displacement loading was applied at the direction of loading. Also 8-node linear brick elements were used to mesh the geometry. The “frictionless” tangential behavior and “hard” normal behavior were used to simulate the interaction between the beam and cylinders.

Table 19. The dimensions of SENB specimens use to investigate the specimen size effect [301]

Specimen size	$W(mm)$	$S(mm)$	$a(mm)$	thickness(mm)
Large	18	72	3.6	4
Medium	7.35	29.4	1.47	4
Small	3	12	0.6	4

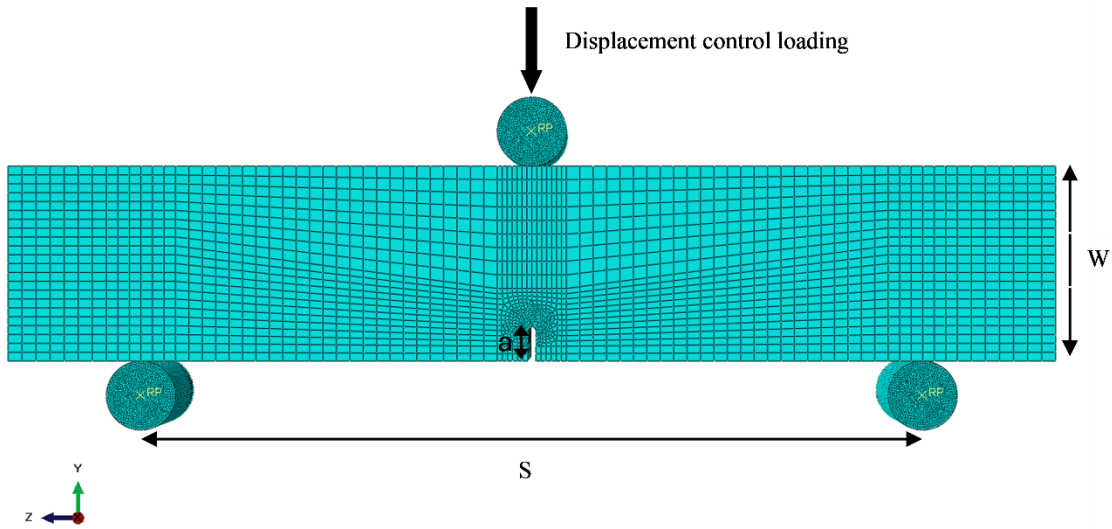


Figure 57. Mesh configuration, boundary conditions, and dimensions of the SENB model for studying size effects.

3 Results

3.1 Implementation of the constitutive model for the oxidized bone

To implement the constitutive model for oxidized bone, it was essential to define the coefficients in *Table 9* as functions of oxidative damage biomarkers, which were derived from experiments detailed in our previous experimental paper [1]. This involved using a multivariate linear regression approach to analyze the relationships between coefficients obtained from mechanical testing and biomarkers of oxidation gleaned from chemical assays and thermo-analytical assessments. The specific oxidative damage biomarkers considered in this analysis are carbonyl content (CC), α , and β , which are the outputs of a model mapping DSC to HIT [291][352]. These biomarkers are crucial because they quantify the extent of oxidative damage to bone tissue.

The regression analysis provides coefficients that describe how changes in these biomarkers affect the mechanical properties of the bone. The resulting linear regression models link the

mechanical degradation of bone tissue to measurable levels of oxidative damage, enabling the constitutive model to predict bone behavior under conditions of oxidative damage.

Table 10 presents the regression coefficients and the linear relationships derived from the regression analysis. These relationships form the basis for incorporating oxidative damage effects into the constitutive model, ensuring that the model can accurately reflect the influence of oxidative damage on bone mechanical properties.

Table 20. Multivariate linear regression relationships between coefficients of the constitutive model and oxidation biomarkers.

	Equation	R^2	$p - value$
Longitudinal tensile yield strength	$Y_l^+(CC, \alpha, \beta) = 129.25 - 0.003 CC - 2 \alpha - 13.44 \beta$	0.87	0.001
Longitudinal tensile ultimate strength	$U_l^+(CC, \alpha, \beta) = 148.41 - 0.0003 CC - 2.2 \alpha - 17.7 \beta$	0.88	0.001
Compressive yield strength	$Y_l^-(CC) = 159.24 - 0.0017 CC$	0.56	0.004
Hardening law parameters	$k_s(CC, \alpha, \beta) = 41.45 - 0.001CC + 1.8 \alpha + 2.9 \beta$	0.72	0.02
Hardening law parameters	$y_r = 0.88 - 4.099e^{-6}CC - 0.00044\alpha + 0.01\beta$	0.75	0.01
Viscosity parameters	$\eta(CC, \alpha, \beta, \dots) = 0.56349 - 1.69e^{-5}CC - 0.0049\alpha - 0.057\beta,$	0.87	<0.0001
Viscosity parameters	$m(CC, \alpha, \beta) = 20.72 - 0.0006CC - 0.47 \alpha - 3.76 \beta$	0.87	<0.0001
Parameter of the damage evolution law	$k_p(CC, \alpha, \beta) = 18.78 + 0.0018CC + 4.3\alpha + 9.2\beta$	0.86	0.03

It is important to note that, due to the small size of the compression test specimens [refer to the experimental paper], there was insufficient material to conduct thermo-analytical tests. Consequently, only the carbonyl content was measured for the compression specimens. As a result, the compressive yield strength was defined solely based on carbonylation.

According to the constitutive equations presented in section 2.1.1, in addition to the aforementioned coefficients, there are other material constants such as elastic and yield properties in transverse direction, interaction coefficients, etc. (Y_t^+ , Y_t^- , γ_{t3} , ζ_{tt} , ζ_{t3} , ...) that had to be defined. Since the material under study is bovine cortical bone, the mechanical properties of bovine cortical bone are utilized for these material constants (*Table 11*) [195], [197], [294], [295].

Table 21. Overview of the material constants

Elastic properties	E_t (MPa)	E_l (MPa)	ν_t	ν_{t3}	μ_{3t}
	10000	20000	0.46	0.35	4300

Material constants	Y_t^+ (MPa)	Y_t^- (MPa)	γ_{t3} (MPa)	ζ_{tt}	ζ_{t3}
	48	125	54	0.3	0.5

Just as an example of the calibration process, in Figure 58 , the results of the calibrated computational finite element model using the identified parameters along with the experimental uniaxial tensile tests at different strain rates are presented for three levels of oxidation in terms of hours of exposure to neutral hypochlorous acid. For each timepoint, there are three important regions in the experimental stress-strain curves used for the calibration, including the linear regime, the yield point, and the post-yield region. Also, in Figure 59, a typical stress field in the longitudinal direction is depicted. As one would expect, it shows a uniform distribution of uniaxial stress in the gauge length.

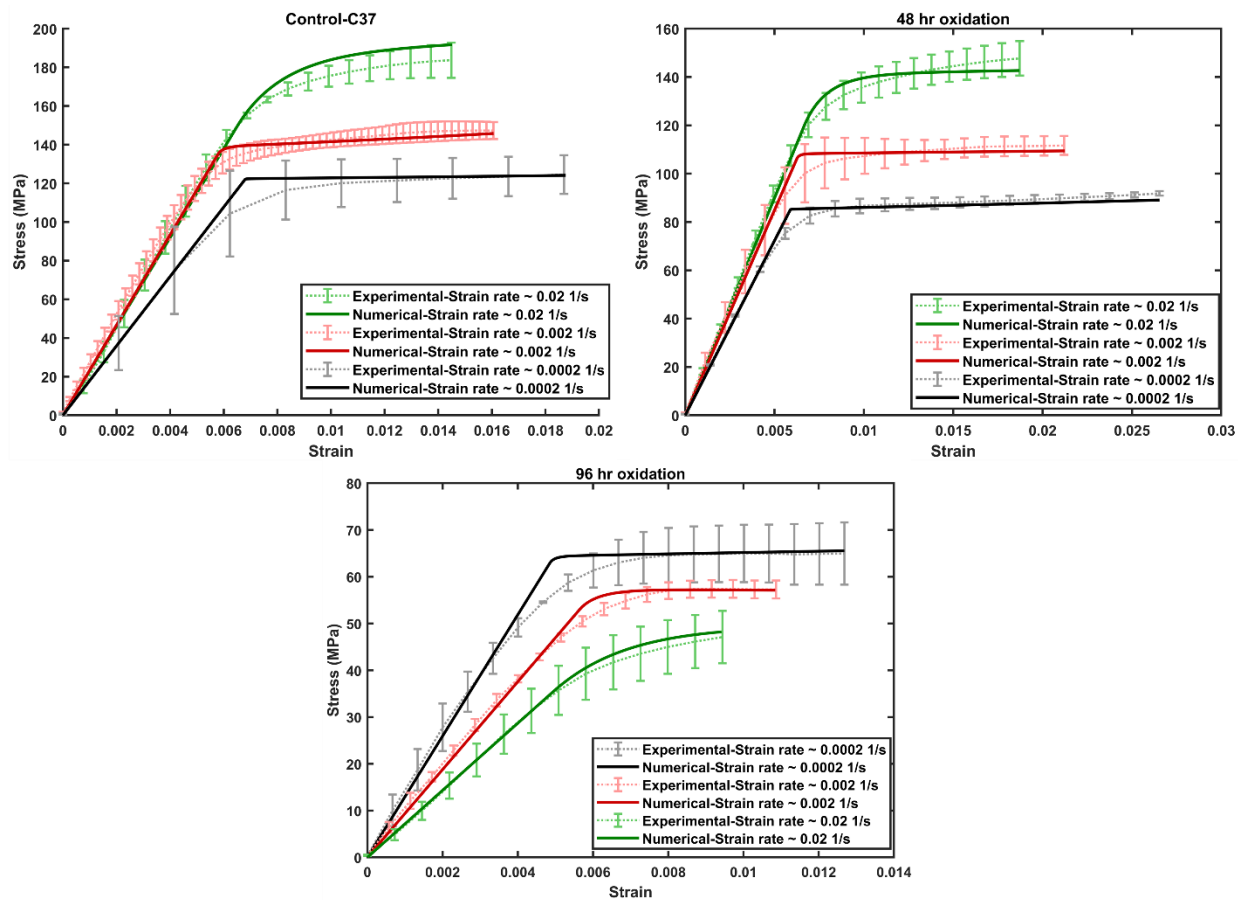


Figure 58. Experimental vs. computational uniaxial tensile curves for different incubation time points

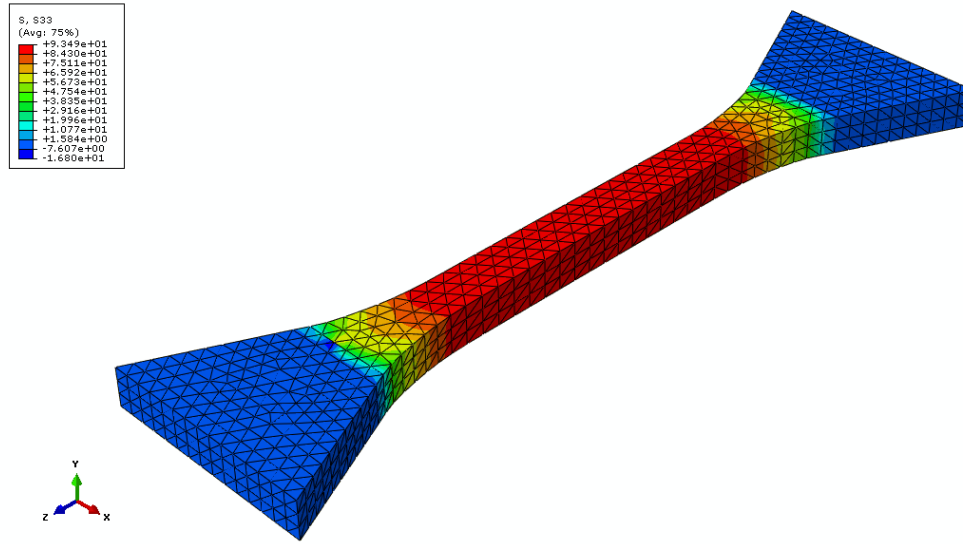


Figure 59. The stress field in the longitudinal direction (z) in the finite element model of a tensile test specimen.

3.2 Experimental validation of the model

Prior to conducting the validation simulations, single element verifications were performed in both the longitudinal and transverse directions, assessing tension and compression scenarios. The results are detailed in the supplementary material.

A 3-point single edge notched bending (SENB) test was modelled using the user-material subroutine. The material constants in Table 11 were used for this model. The bone was assumed in the healthy state so the parameters identified for the control specimen were used and the results were compared with an experimental SENB load-deflection curve from a previous study [296].

Figure 60 shows the load-deflection curves for the SENB model up to the peak load when significant crack growth would start during the test. Both the experimental and the computational results using the UMAT are presented. As depicted, there is a very good agreement between the experiment and the numerical model results (mean absolute error =7%).

In order to examine the effect of mesh size, a mesh sensitivity analysis was done and the results (not presented) showed that the mesh size was sufficient.

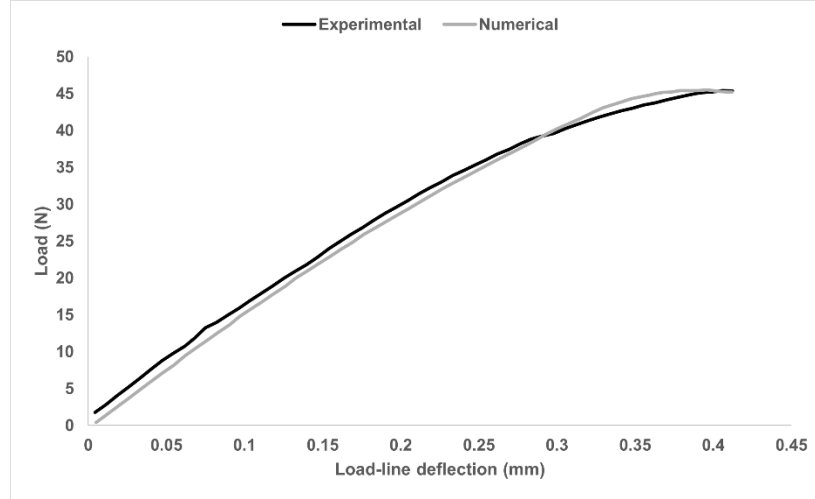


Figure 60. Experimental and computational Load vs. load-line deflection curves from SENB testing of bovine cortical bone.

DIC strain fields in the longitudinal direction of the SENB sample at peak load are shown in Figure 32 . The red region represents areas with strain exceeding a defined threshold (0.5%), referred to the onset of micro-damage in a previous study [297].

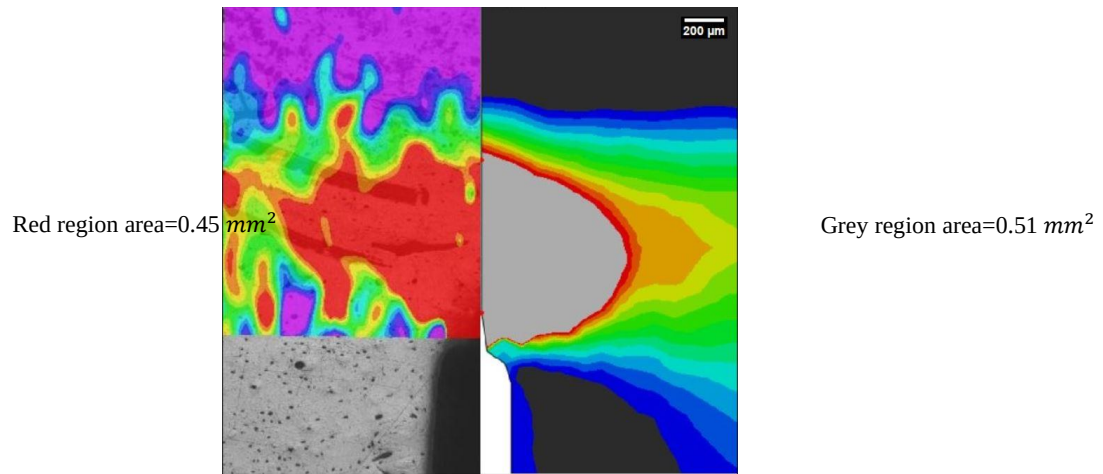


Figure 61. Comparison between the micro-damage process zones from the model (gray color area in the right) and from experimental DIC strain measurements (red color area in the left) for a SENB test of bovine cortical bone (Corresponding to peak load of load-line deflection depicted in Figure 10). Note the model does not account for tissue heterogeneity.

Figure 11 illustrates a comparison between the strain fields in the longitudinal direction obtained from the model (grey) and the experimental data (red). The comparison includes both the morphology and the area of the micro-damage process zone (regions where the strain has exceeded 0.5%).

As an additional validation step, the model was used to simulate the micro-damage process zone formation in irradiated bovine cortical bone samples. One set of data were taken from previous work [286] in which irradiated samples were prepared by irradiating healthy bovine cortical bone with a 33 kGy dose of gamma radiation. 3-point bending tests were then carried out on gamma-irradiated SENB specimens. Considering that the generated constitutive model in this study is based on incubation time, and oxidative damage in [286] was induced through gamma-irradiation, it is required to find the equivalent incubation time that creates the similar level of oxidative damage in gamma-irradiated specimens. Although the method of damaging the bone tissue differs between gamma-radiation and chemical oxidation, both processes share a similar damage mechanism being oxidative damage. To establish the equivalent amount of oxidation using our method for the gamma-irradiated samples, the load-deflection curves of these specimens were utilized, resulting in an approximate value of $T_{inc} \approx 50 \text{ hr}$ hours for the irradiated specimens.

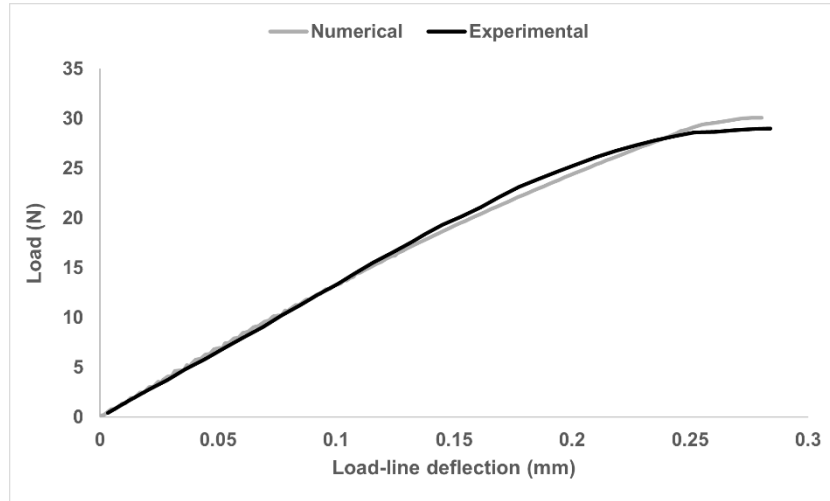


Figure 62. Experimental and computational (calibrated) Load vs. load-line deflection curves for SENB testing of irradiated bovine cortical bone.

Subsequently, the micro-damage zone in the 33 kGy dose gamma-irradiated specimen was modelled and compared to the experimental micro-damage zone in [192]. The study in [192], reported a 49% reduction in the volume of the micro-damage process zone due to irradiation, as indicated by the decrease in damage volume (DV) divided by the volume of the region of interest (TV). In our case (refer to Figure 34), the area of the micro-damage decreased from 0.51 mm^2 to 0.21 mm^2 , indicating a reduction of 41%.

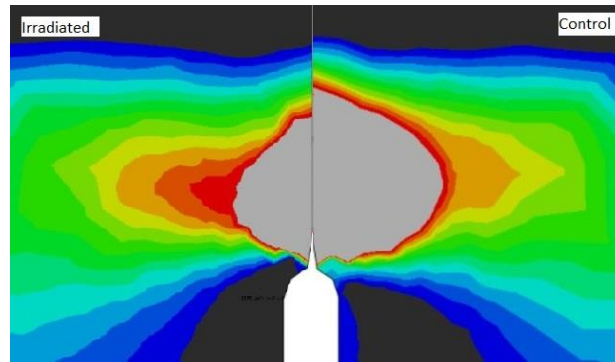


Figure 63. The micro-damage process zone models for control vs. irradiated SENB bovine specimens.

3.3 Exploring the specimen size effect on the microdamage process zone (MDPZ) using the constitutive model

To explore the specimen size effect on MDZP, 3-point single edge notch beam bending tests were simulated for three different sample sizes, illustrated in section 4.3.3, and three levels of oxidation in terms of oxidation hours (0, 48 hr, 96 hr). The area of the MDZP versus the specimen width (W) for the aforementioned levels of oxidation is depicted in [Figure 33](#). Based on this result, the increase in specimen size increases the MDPZ area. However, based on the fitted curves on these results, we predict that at some point, the mechanism saturates. This size dependency was also observed in the models of the oxidized specimens but with the saturation occurring at larger W values (48h) and perhaps not at all (96h).

To explore the effect of variation in specimen size on the microdamage process zone (MDPZ), we simulated three-point single edge notch beam bending tests for three different sample sizes, as illustrated in Section 3.3. These tests were conducted under three levels of oxidation, represented by oxidation durations of 0, 48, and 96 hours. Figure 33 presents the relationship between the MDPZ area and specimen width (W) for each oxidation level.

The results indicate that an increase in specimen size leads to a corresponding increase in MDPZ area. However, analysis of the fitted curves suggests that this size-dependent growth reaches a saturation point beyond which further increases in specimen size do not affect the MDPZ area. Notably, this size dependency is also observed in oxidized specimens, though saturation occurs at later points when compared to control specimens.

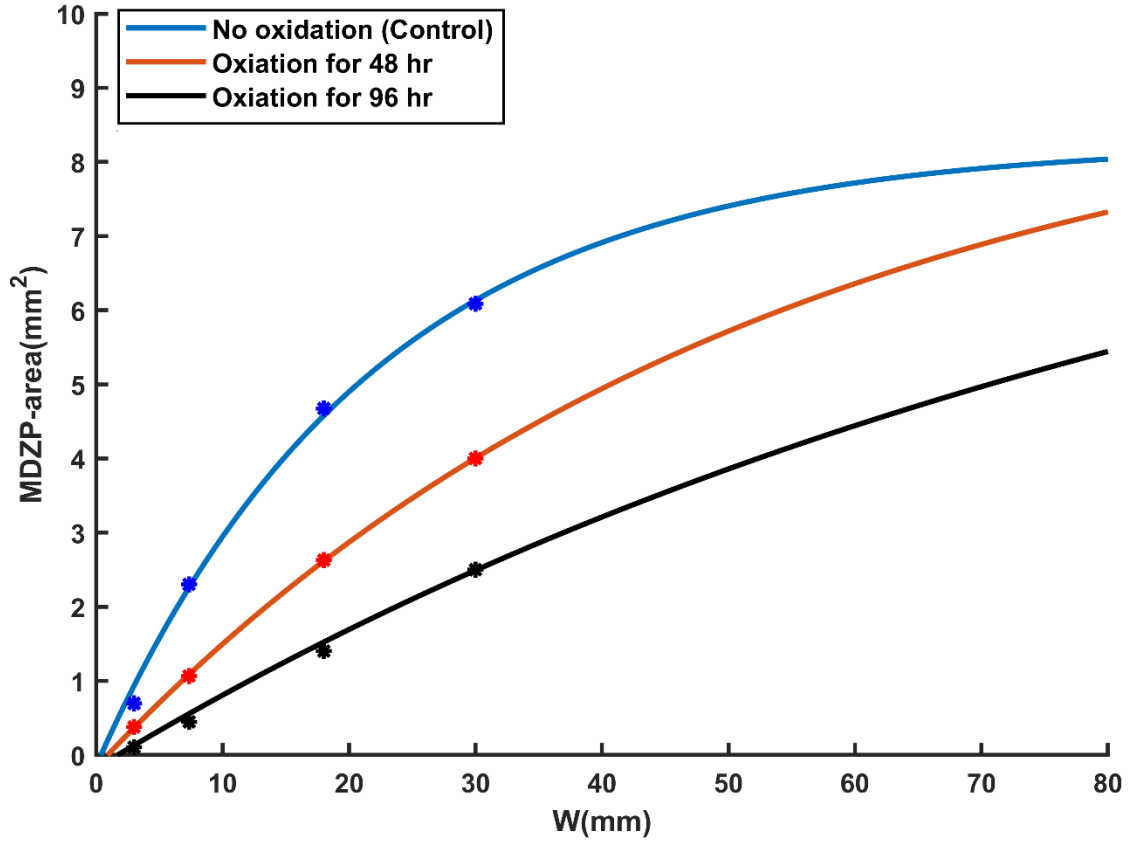


Figure 64. Effects of variation in size (W) of the specimen on the predicted size of the microdamage process zone.

4 Discussion

In this study, the mechanical behavior of oxidatively damaged bovine cortical bone which was previously characterized using different types of tests including uniaxial tensile at different strain rates, under compression and with tensile load-unload tests [refer to exp. Paper] have been suggested as mathematical functions of oxidative damage such as carbonyl content, α and β , which was measured in the tested specimen. Using these functions, a previously proposed elasto-visco-plastic constitutive model [247] was adapted to account for the oxidative damage and the model was fully developed into a user material subroutine UMAT code implemented in the commercial finite element solver Abaqus/standard. The model was verified and validated by implementing the algorithm in MATLAB for a single integration point under different

loading modes and directions and then by applying the model and identified properties to model SENB tests.

The model parameters, including hardening and viscosity parameters, were identified using the results of the uniaxial tensile tests. Interestingly, the amount of hardening (k_s) consistently decreased as oxidative damage increased, which is consistent with the literature, implying that the amount of stable ductility decreases with collagen degradation (see section 1). Also, the viscosity parameters (η, m) exhibit a consistent behavior as the oxidation increases;

The model was validated by simulating a SENB test using calibrated parameters and comparing the results to an experimental SENB test (Figure 31). To quantify the level of agreement between the experimental and numerical data, the mean absolute error was calculated. Given the range of the data, the mean absolute error of 7% indicates a very good agreement for validation purposes. Furthermore, a comparison was made between the micro-damage process zone sizes measured in the experimental results and the numerical model. As shown in Figure 12, the area of the experimental and numerical micro-damage zone exhibits only an 11% difference, indicating a relatively small error likely due to a lack of heterogeneity captured in the model.

As an additional validation step, the model was used to simulate the micro-damage process zone in an irradiated sample which is a severe case of oxidative damage. The micro-damage process zone area reduced by 41% in the numerical model of the irradiated sample compared to the model of the non-irradiated control. This area reduction is only 8% different from the reduction in MDPZ size measured experimentally by Burton et al. [192].

Finally, to demonstrate the capability of the model, we investigated the effect of specimen size variation on the area of the microdamage process zone (MDPZ), which acts as a toughening mechanism in bone. Previous studies have examined the influence of specimen size on bone fracture toughness, primarily employing fracture mechanics criteria [300][301]. These studies have shown that fracture toughness varies with specimen size and some semi-empirical relations were suggested to deal with this effect [300][301]. Our model's predictions align with these findings, confirming that the specimen size effect extends to the MDPZ as well. Based

on the prediction of our model, the size of the MDPZ reaches a saturation point beyond which further increases in specimen size do not affect the MDPZ area. This suggests a limit to this size-dependent toughening mechanism within bone. Additionally, our investigations revealed that greater oxidative damage reduces the size of the MDPZ. Despite this reduction, the size effect persists in oxidized bone until it too reaches a saturation point, although at a larger specimen size in terms of W . These findings underscore the impact of specimen size on MDPZ behavior and highlight the compounded effects of oxidation. The delayed saturation in oxidized specimens further confirms that oxidative damage influences the material's toughening mechanisms.

Herein, the model parameters, some of which reflecting the bone mechanical properties, have been calibrated only in the longitudinal direction and the properties in transverse direction were assumed to be constant. This assumption is reasonable given that, as described in section 1, oxidative damage mostly affects the bone collagen which largely determines the yield and post-yield behaviour in the longitudinal direction whereas the strength and ductility in the transverse plane are dominated by the microstructure and its weak interfaces, making the transverse direction relatively brittle [48]. The main structural unit in cortical bone is the osteon, part of the Haversian system, which is aligned parallel to the bone's long axis (longitudinal direction). The wall of the osteon is made up of 20 to 30 concentric lamellae, each of which is made of mineralized collagen fibrils [48]. Also, according to earlier studies [292][293], the average collagen orientation found in lamellar bone is within 30° of the bone's long axis implying that the collagen is primarily aligned with the long axis of the bone. Thus, changes in collagen play a significant role in the longitudinal properties of cortical bone. However, for a more comprehensive understanding of the bone behavior under oxidative damage conditions, the changes in the transverse direction should also be measured and modeled in future work.

In this investigation, an established constitutive model was expanded, inspired by the concept presented in reference [275], involving the adjustment of coefficients within the constitutive and evolution equations. These coefficients were substituted with material functions relative

to the biomarkers of oxidative stress. By integrating these biomarkers into the constitutive model, we enhance our ability to simulate and predict how oxidative damage, such as that caused by aging or chronic diseases, impacts bone quality and mechanical performance. This improved model can be a valuable tool for understanding bone fragility in affected populations and for developing strategies to mitigate the detrimental effects of oxidative stress on bone health.

The model was validated against SENB test results for both control and irradiated scenarios in this research and the effect of specimen size variations on the MDZP area were investigated using the established model.

The study makes contributions to the comprehension of the mechanics of oxidatively damaged bone by examining the impact of oxidative damage on bone behavior. The proposed model holds promise for predicting the mechanical behavior of cortical bone in situations where oxidative damage is a significant factor in the decline of bone strength, such as aging and disease conditions.

5 Acknowledgements

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Appendix E

To verify that the model was able to predict the stress-strain curves, the algorithm was implemented in MATLAB and tested for two loading conditions—tension and compression—in both the longitudinal and transverse directions for one Gauss point. For this, the strain history of a single-element simulation was imported from Abaqus into MATLAB. The single element Abaqus results was compared with the MATLAB outputs (Figure 65). The material constants employed correspond to the ones listed in the Table 2 of the paper. Additionally, for the parameters that are functions of incubation time, $T_{inc} = 48$ hrs is utilized, with a value of $\eta = 0$. Just for the case of compression loading in the longitudinal direction, a value of $\eta = 0.02$ was used.

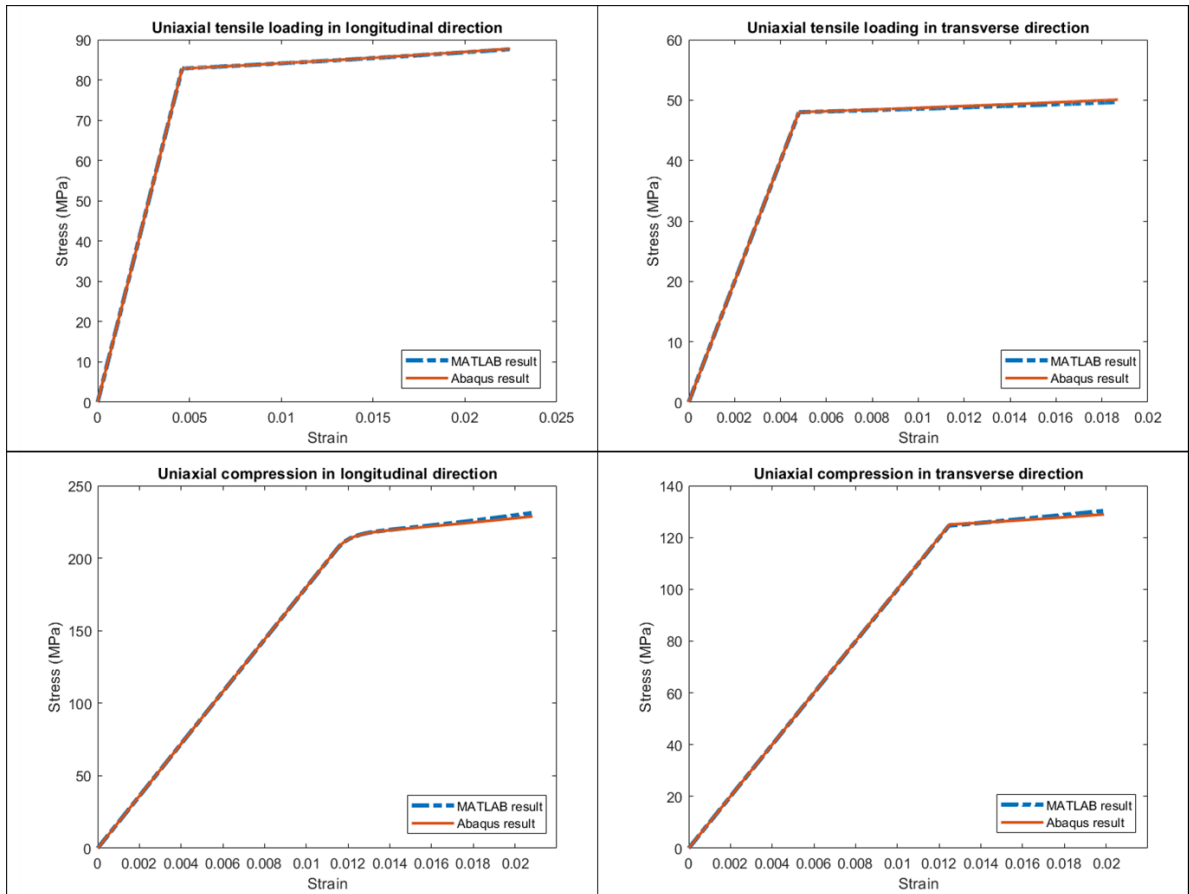


Figure 65 . Verification of the implemented algorithm for the uniaxial loading cases for a single integration point

Each loading case's numerical results are verified qualitatively and quantitatively. Thus, in each loading case, the stress increases linearly up to the yield point (Y_3^+ in tensile loading in longitudinal direction, Y_t^+ in tensile loading for transverse direction) and then the material hardens to the ultimate point. This code-to code comparison verifies the algorithm and checks that the subroutine has been implemented correctly.

Appendix F

In order to examine the effect of mesh size on SENB testing results, another simulation that used a very fine mesh with a total of 428713 tetrahedral elements was conducted. The results, which are depicted in Figure 66 for both fine and coarse mesh, clearly illustrate that the mesh size was sufficient.

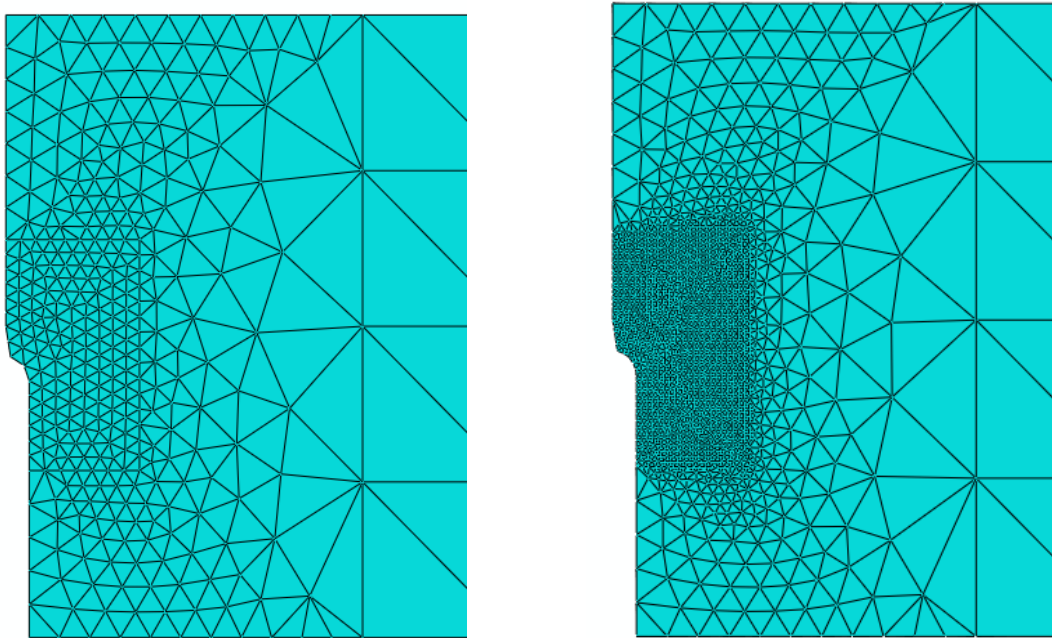


Figure 66. A) Two different meshes (fine vs. coarse) tested during model verification,

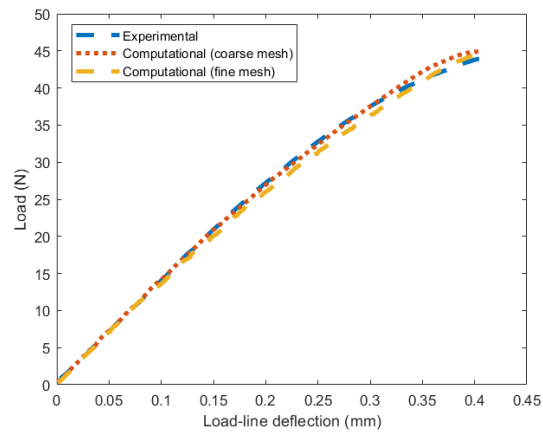


Figure 67. Experimental and computational Load vs. load-line deflection curves for SENB testing of bovine cortical bone for different mesh size.