Cortical bone quality affectations and their strength impact analysis using holographic interferometry

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Abstract: It is now accepted that bone strength is a complex property determined mainly by three factors: quantity, quality and turnover of the bone itself. Most of the patients who experience fractures due to fragility could never develop affectations related to bone mass density (i.e. osteoporosis). In this work, the effect of secondary bone strength affectations are analyzed by simulating the degradation of one or more principal components (organic and inorganic) while they are inspected with a nondestructive optical technique. From the results obtained, a strong correlation among the hydroxyapatite, collagen and water is found that determines the bone strength.

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1. Introduction

According to the American Association of Clinical Endocrinologists (AACE), the osteoporosis is defined as a condition characterized by a low bone mass. Under this affection, the micro architectural deterioration of the bone tissue leads to a bone fragility and increases the probability to suffer a fracture [1]. The bone structure is biphasic, meaning that there is an organic matrix and an inorganic mineral component in it. The three primary constituent elements of the bone are: (1) fibrillary type 1 collagen (35 to 45% of the volume), (2) mineral linking of calcium-phosphate (35 to 45% of the volume) in the form of semi-crystalline hydroxyapatite, and (3) water (15 to 25% of the volume) [2, 3]. As a person ages, its bone's susceptibility to fracture increases, however, it has been shown that there is no change in the bone’s mineralization with aging, but it becomes less tough [4]. The aging osteoporosis is defined as a condition characterized by a low bone mass. Under this affection, the micro architectural deterioration of the bone tissue leads to a bone fragility and increases the probability to suffer a fracture [1]. The bone structure is biphasic, meaning that there is an organic matrix and an inorganic mineral component in it. The three primary constituent elements of the bone are: (1) fibrillary type 1 collagen (35 to 45% of the volume), (2) mineral linking of calcium-phosphate (35 to 45% of the volume) in the form of semi-crystalline hydroxyapatite, and (3) water (15 to 25% of the volume) [2, 3]. As a person ages, its bone's susceptibility to fracture increases, however, it has been shown that there is no change in the bone’s mineralization with aging, but it becomes less tough [4]. The aging
effect and the bone’s risk to fracture are therefore related to the organic phase and to the inorganic part. The property of the bone to resist fractures is known as strength [5], recently described as a complex factor determined by the integration of three factors: quantity, quality and turnover of the bone [6]. The quantity is generally related to bone density, mineral and collagen content, although common clinical procedures focus just on the mineral amount. The bone quality depends on the structural and material properties of the bone. The structural properties include its geometry (size and shape) and microarchitecture. The material properties include the organization and composition of the mineral and collagen components of the extracellular matrix [6]. On the other hand, the bone turnover is related to a metabolic response which allows a continuous renewal of the tissue by means of a resorption and formation processes. The balance between resorption and formation helps the bone to remove fatigue damage and replace it with new bone that reinforces its integrity. Since an imbalance between the resorption and formation results either in a loss or gain of bone, the turnover process affects the bone quantity and the bone quality, and as consequence, it affects the bone strength [5, 6]. The bone mineral density (BMD) is a clinical rating based on the dual energy X-ray absorptiometry technique (DXA) which is a technique that obtains relative values of the bone quantity. A common practice is to express the BMD in terms of the T-score. The latter reports the number of standard deviations for a patient’s BMD value compared to a reference BMD value for a healthy 30 year old adult of the same gender and similar ethnic group [7, 8]. Even though BMD value is used for medical practitioners to assess the risk of fracture, it has been found that less of the 50% of the whole bone strength is attributable to its variations [9–11].

As a matter of fact, the majority of patients who experience fragility fractures have a BMD T-score above −2.5. If we consider that a normal bone density is above −1.0 and osteoporosis is below −2.5, those patients are medically diagnosed with osteopenia (low bone density); it means that although their bones reveal a BMD loss, they could never develop osteoporosis [12–14]. Another argument deals with the hip fracture probability which is five times greater at the age of 80 than at the age of 50 in women with a T-score of −2.5 [15]. This means that hip fractures in elderly population are produced by many factors which are not necessarily correlated to the bone mass loss caused by osteoporosis. Furthermore, BMD value is limited to diagnose the secondary causes (besides osteoporosis) of bone loss and to assess the response to a therapy. Then, the other two determinants of the bone strength: quality and turnover, should be included when assessing fracture risk in each individual rather than just the BMD score alone [16]. As a consequence, nowadays in specialized conferences, osteoporosis disease has been defined as ‘a skeletal disorder characterized by compromised bone strength leading to an increased risk of fracture’ [17]. This definition implies that understanding the bone strength may be the key to better understand the fracture risks [5].

In this work the effects of secondary affectations to the bone structure strength are analyzed in terms of its surface displacements. The objective of this study is to use an optical non-destructive technique to retrieve high resolution full-field information of the bone samples to estimate the impact of different affectations in their mechanical response (strength). This optical non-destructive testing (NDT) called digital holographic interferometry (DHI) is applied in ex-vivo samples [18, 19], in order to gain new knowledge to eventually complement diagnoses related to bone strength evaluation beyond those obtained by BMD score.

The study includes structural affectations to the bone by degrading one or more of their principal components: hydroxyapatite, collagen and water. The cortical bone probes are observed with DHI in an out-of-plane sensitivity set up, while a testing machine applies a controlled compression load. The out of plane sensitivity was selected considering that its magnitude is much greater than the in plane one in cortical bone [20] (here is where the mechanical response of the bone is mostly expressed). This configuration has the advantage to record the surface deformation information during the entire test (dynamic test) without the
need to remove the samples to analyze them (static test). In addition, the interferometer registers full-field information over the entire object’s surface, a feature required due to the bone’s anisotropy which results in non-repeatable or scalable tests which depend on the load value and its applied speed [21]. A precise characterization of the affectation procedures was carried out before each compression test is performed. Once characterized, a displacement comparison for each affected group is presented.

2. Method and materials

2.1 Bone affectation classification

Several conditions and diseases that directly and indirectly affect bone quality rather than just osteoporosis (secondary affectations) have been reported [5, 6, 22]. These disorders were categorized considering about 20 different conditions/diseases affecting bone quality [16], grouped as:

2.1.1 Disorders of bone mineral homeostasis

Bone mineralization is one of the major determinants of the bone quality and it is controlled by the mineral homeostasis process. Any possible condition that may interfere with the mineral homeostasis will affect the bone quality (leading to a fragility fracture) [6]. Patients who present an affectation to the mineral homeostasis are normally supplemented with an adequate calcium and vitamin D intake since these components are relevant to the bone mineralization [6, 16].

2.1.2 Imbalance of the bone remodeling

Some bone diseases such as osteodystrophy or disuse osteoporosis are caused by a high turnover state which provokes an increased activity of the osteoclasts [23–25]. As a consequence, the bone remodeling process is shifted towards the bone resorption, resulting in an imbalance of the bone turnover that causes fragility fracture. Moreover, if the defect is related to the osteoclast function such as osteoporosis, this could lead to a lack of bone heterogeneity, micro-damage accumulation and, consequently, fragility fracture [26, 27].

2.1.3 Collagen disorders

Type I collagen reaches approximately 95% of the organic matrix found in the bone. The collagen helices are crosslinked within and among others (increasing their strength). At the same time, this crosslinked collagen pattern forms a structural template for the bone mineralization [28]. This composition between collagen and mineral gives the bone its remarkable material properties. The bone strength and stiffness depend heavily on the mineral content in a wide range of anatomic locations [29–31], while the collagen plays a role in the bone structural integrity giving its tensile strength [32–34]. Usually the bone fails in tension, but collagen provides the main structural framework to prevent this failure. This is a paramount reason why a disruption of the collagen structure causes bone fragility (as it happens in osteogenesis imperfecta).

2.1.4 Drugs affecting bone quality

Some medications can bring a great therapeutic benefit; however, they normally involve collateral effects. Corticoids, anti-rheumatic drugs, cancer chemotherapeutic agents or even osteoporosis medications can adversely affect the bone [35–38].

2.2 Bone sample preparation

In order to study the bone strength, cortical bovine bone samples were treated by means of a demineralization [39–41] and air-drying [42–45] protocols, to affect the mineral and organic phases respectively (issues explained in sections 2.2.1 and 2.2.2). These procedures will result
in bone quality and quantity affectations as the bone turnover affectation is caused by affecting one of them or both. It is worth to mention that these protocols are independent from each other, i.e., the demineralization process does not affect the air-drying process and vice versa. The bovine femur bones were obtained locally with less than 24 hrs post-mortem and all of them come from male bovine strain Angus, which are regular sized with an average weight of 1000 kg and bred for human consumption. The slaughter age of the cattle was approximately 20 months. These cortical bovine femur samples were obtained from the middle diaphysis by hand saw avoiding the use of electric devices which induce heat. The average mean transverse section of the diaphysis is 1960 mm². All these femoral diaphysis were submerged in spring water for about 90 minutes to easily clean out other tissues by using a scalpel. After that, a low speed rotating diamond wheel saw is used, the cortical samples were carefully machined to a prismatic geometry of $5 \times 5 \times 17$ mm as seen in Fig. 1. The longest length of the samples ($b = 17$ mm) was selected to be aligned to the growth direction of the bone. The transverse section ($a\times a$, $5 \times 5$ mm) of the final samples was selected considering the temporal model for the demineralization process which acts in a radial form going from the periphery towards the center [41, 46–49].

![Fig. 1. Schematic of the cortical bovine bone samples machining steps. Opposite faces of the bone samples are parallel.](image)

It is important to point out that before, during and after the entire machining process, the samples were kept fresh and wet using a 0.1 M phosphate-buffered saline solution (isotonic substance to preserve cellular tissues due to its osmolality). Even when the rotating diamond wheel machine was used, the bone samples were always irrigated with this solution. Once machined, they were grouped into four groups, shown in Fig. 2.
The control group \((m + H2O)\) kept its normal mineral and hydration contents. The hydrated but demineralized group \((dm + H2O)\) was divided into two subgroups; one of them was demineralized for 4 hrs and the other for 6 hrs in order to check the demineralization process. In a similar way, the mineralized but dehydrated group \((m-H2O)\) has two subgroups too; one is air-dried during 24 hrs and the other one during 48 hrs. The fourth group is dedicated to demineralized and dehydrated samples for 48 hrs \((dm-H2O)\) with two demineralization subgroups of 4 and 6 hrs for control purposes. In order to verify the affectionation of the collagen, hydroxyapatite and water produced by the demineralization and air-drying protocols, a total of 24 cortical samples were tested using the Fourier transform infrared spectroscopy (FTIR) technique as will be shown in section 3.1.

2.2.1 Demineralization method

The bone is a composite material hierarchically structured and there are two types of it: cancellous and cortical. Both types are composed of type I collagen fibrils containing mineral (hydroxyapatite) dispersed [29, 49–53]. This organic and inorganic link requires treating the bone samples with a demineralization method that affects only the inorganic mineral component while the organic component is preserved. Bone demineralization studies based on Hydrochloric Acid (HCl) have shown a geometric dependence among the samples. The HCl solution penetration distance is a function of the square root of the time employed, giving a control of the cortical and cancellous bone demineralization while the collagen is preserved [39, 40, 54]. The in vitro demineralization method applied in this work has remarkable characteristics. The rate of demineralization increases according to the HCl solution concentration or temperature. The bone demineralization has three stages; in the first one, the constant demineralization rate increases as the HCl solution demineralizes the peripheral region of the sample. The second stage occurs on a steady state and the third one at the end of the reaction when the constant rate decreases. In order to check the demineralization method and its effects on the bone strength, two demineralizing times were employed, 4 and 6 hours. Each cortical sample was placed in a 50 ml clinic beaker containing 40 ml of 0.6N HCl at room temperature (21°C). According to the method’s protocol [41], the HCl solutions were refreshed for the samples after 0.5, 1.5 and 3 hours until 4 and 6 hours of demineralization were reached. The HCl solution parameters (concentration and temperature) were carefully selected to avoid a final bone’s dimensions change. The increase of these parameters is not recommended for mechanical tests because the bone final structure and dimensions are deeply compromised.
2.2.2 Dehydration method and its collagen affectation

Removing the water present in the bone by an air-drying method represents a process that has been previously employed in order to analyze bone structure at different scales [42, 43]. Room temperature and dehydration periods of 24 and 48 hours were applied. Water conforms about 20% of the bone’s volume and it is a key factor for the mechanical behavior of the bone. It is found basically in two parts: within the pores and bounded to the matrix where it is responsible for giving the collagen its ability to give the bone’s ductility or plasticity [42]. Thus, affecting the bound water level results in a collagen affectation and, as a consequence, the natural structural bone response is altered. This dehydration method has been employed in different bone samples such as bovine, porcine, equine, human tibia, femur and humerus [43–45].

2.3 Digital holographic interferometry (DHI)

DHI is a non-contact, remote and non-invasive optical technique [55–59], capable of recording fast and non-repeatable dynamic events such as the bone compression [21]. The deformation of the sample is recorded from the overlapping, at the camera’s sensor, of the backscattered light that illuminates its surface and a reference beam (an undisturbed beam coming from the illumination light source, e.g., a laser, that contains the undisturbed spatial coherence of the source), forming an image hologram [60]. This image hologram has the amplitude and the optical phase information when a two state correlation is performed (one image hologram is taken before and another one after the sample’s has suffered a deformation). Upon comparing these two image holograms, the relative optical phase difference $\Delta \phi$ contains the full field sample’s deformation information between these two states. $\Delta \phi$ is obtained using a Fourier transform algorithm [61] and it can be expressed as $\Delta \phi = \phi - \phi'$, where $\phi$ and $\phi'$ are the optical phases before and after the deformation, respectively. This phase difference $\Delta \phi$ is retrieved as a wrapped phase map with the deformation information codified within a range of $-\pi$ to $\pi$ (black and white, respectively) that typically is scaled from 0 up to 255 gray scale levels (for a 8 bit-depth camera sensor). This phase map is then unwrapped using an unwrapping algorithm that stitches the discontinuity jumps to obtain a smooth and continuous optical phase map ($\Delta \phi_u$), which is then converted into a displacement map ($w$) using Eq. (1):

$$\Delta \phi_u = \frac{2\pi}{\lambda} (1 + \cos \theta)w$$  \hspace{1cm} (1)

where, $\lambda$ is the light source’s wavelength, $\theta$ is the angle between the illumination and the observation directions and $w$ is the out of plane displacement (perpendicular to the object’s surface) [61, 62]. The use of the out of plane configuration makes the optical system sensitive in the direction where most of the bone’s surface displacement is expected during the compression tests.

3. Experimental procedure

3.1 Fourier transform infrared spectroscopy (FT-IR) validation

In this technique, electromagnetic radiation corresponding to the infrared band (400 to 4000 cm$^{-1}$) is directed onto the sample. The energy associated with these wavelengths is absorbed by the bone and converted into molecular vibration energy depending highly on the chemical groups present in the sample, thereby demonstrating their chemical structure. Many FT-IR studies have been made on bone identifying characteristic bands for mineral matrix groupings in hydroxyapatite at 500 - 700 cm$^{-1}$ and 900 - 1200 cm$^{-1}$; the organic matrix (collagen) in the 1200 - 1700 cm$^{-1}$, and also for bound and pore water molecules in 3427 cm$^{-1}$ [63, 64]. In order to prove qualitatively the effect of bone demineralization and collagen affectation
protocols, a Cary 670 FT-IR system was employed to test cortical samples with the specified geometry shown in Fig. 1. The samples were grouped as it was described in section 2.2 (Fig. 2) and each of them was transversally sectioned and observed in several points within the transverse section. The FT-IR spectroscopic system obtains each spectral value with 32 repetitions per sample and its analysis is necessary to guarantee that samples under compression are independently affected in the mineral, collagen and water components. Figure 3 depicts a comparison among the mean spectral transmittance for samples with 4 and 6 hours of demineralization ($\text{dm} + \text{H}_2\text{O}$) with respect to the control samples ($m + \text{H}_2\text{O}$).

In this case, all samples did not go through an air-drying treatment previous to the FT-IR test. From Fig. 3 it is possible to observe signals at the bands of the hydroxyapatite ($1010 \text{ cm}^{-1}$), collagen ($1600 \text{ cm}^{-1}$) and water ($3427 \text{ cm}^{-1}$) [63, 64]. It can be seen that the transmittance percent of the band at $3427 \text{ cm}^{-1}$ for water is almost the same for 4 and 6 hours, hence it is safe to assume that water content tends to be the same independently of the demineralization time. For each spectrum, Table 1 shows the transmittance percent ratio values between the three components hydroxyapatite ($H$), collagen ($C$) and water ($W$) for each group. The transmittance ratios of the hydroxyapatite with respect of the collagen has increased from the control group $R_{HC} = 0.95$ to 1.22 and 1.33 for 4 and 6 hours of demineralization respectively (i.e., to obtain $R_{HC} = 0.95$ for the control group it was divided its hydroxyapatite transmittance value of 88.5, by its corresponding collagen transmittance value of 93 according to information of Fig. 3). This means that in the bone sample the relative content of collagen increases, while the content of hydroxyapatite decreases as result of the bone demineralization and the corresponding ratio is greater when the time is larger.

![Fig. 3. FT-IR mean transmittance spectra comparison for samples with 4 and 6 hours of demineralization ($\text{dm} + \text{H}_2\text{O}$) with respect to the control group ($m + \text{H}_2\text{O}$).](image)

**Table 1. Transmittance ratios considering the bands of hydroxyapatite ($1010 \text{ cm}^{-1}$), collagen ($1600 \text{ cm}^{-1}$) and water ($3427 \text{ cm}^{-1}$) for the control and demineralization process spectra shown in Fig. 3.**

| Bone Sample Group | $R_{HC}$ | $R_{HW}$ | $R_{CW}$ |
|-------------------|---------|---------|---------|
| $m + \text{H}_2\text{O}$ | 0.95    | 0.96    | 1.01    |
| $\text{dm} @ 4\text{h} + \text{H}_2\text{O}$ | 1.22    | 1.04    | 0.85    |
| $\text{dm} @ 6\text{h} + \text{H}_2\text{O}$ | 1.33    | 1.08    | 0.81    |

$R_{HC}$ (hydroxyapatite / collagen)  
$R_{HW}$ (hydroxyapatite / water)  
$R_{CW}$ (collagen / water)
In a similar way the dehydrated samples (m-H$_2$O) are analyzed using the FT-IR technique and Fig. 4 shows their corresponding transmittance spectra. In this case the two subgroups of 24 and 48 hours of dehydration are compared. As in Fig. 3, the transmittance bands of the hydroxyapatite, collagen and water are observed [63, 64]. The transmittance ratios among these bands are shown in Table 2 and it is possible to observe that the relative content of collagen is reduced as the time increases. $R_{hc}$ is reduced from 0.95 of the control group to 0.90 and 0.89 for 24 and 48 hours of air-drying respectively. Analyzing further these ratios, it can be observed that the content of water has been reduced drastically as $R_{hw}$ from the control group was 0.96 and it is reduced to 0.88 for both 24 and 48 hours (see Table 2). As there is no difference between the two air-dried samples, for convenience the samples at 48 hours will be used in the compression tests.

![Fig. 4. FT-IR mean transmittance spectra comparison for samples with 24 and 48 hours of air-drying (m-H$_2$O) with respect to the control group (m + H$_2$O).](image)

**Table 2. Transmittance ratios considering the bands of hydroxyapatite (1010 cm$^{-1}$), collagen (1600 cm$^{-1}$) and water (3427 cm$^{-1}$) for control and air-drying process spectra shown in Fig. 4.**

| Bone Sample Group | Transmittance ratios |
|-------------------|----------------------|
|                   | $R_{hc}$ | $R_{hw}$ | $R_{cw}$ |
| m + H$_2$O        | 0.95     | 0.96     | 1.01     |
| m-H$_2$O@24h      | 0.90     | 0.88     | 0.98     |
| m-H$_2$O@48h      | 0.89     | 0.88     | 0.99     |

$R_{hc}$ (hydroxyapatite / collagen)  
$R_{hw}$ (hydroxyapatite / water)  
$R_{cw}$ (collagen / water)

### 3.2 Compression tests

Once the FT-IR spectroscopic signals were analyzed to validate the affectation processes on the bone samples, the selected groups were compressed in order to register their temporal behavior during this controlled compression tests. The load values to be compared are 100, 200 and 300 lbs because they represent load stages for the samples which include physiological (100 lbs) and overload conditions (200 and 300 lbs). The maximum compression load represents more than 40 times the normal load value considering mean weight and femoral transverse section of the selected cattle scaled to the area of the machined bone samples (please refer to section 2.2). The groups tested are: $m + H_2O$ (control),
$dm@4hrs + H2O$, $dm@4hrs-H2O@48hrs$ and $m-H2O@48hrs$. Figure 5 shows the average temporal response comparison of several affected samples for each cortical sample group during a full compression test.

Considering the control group ($m + H2O$) as a division parameter, two regions are observed in Fig. 5, one at the top and the other at the bottom. Samples which are air-dried ($m-H2O@48hrs$) show an opposition to the compression movement and they are located at the bottom region because more time is needed to reach the final load of 300 lbs under a linear compressive profile respect to $m + H2O$. Table 3 shows that this air-dried group has time delays corresponding to 7, 12.2 and 17.5 seconds for loads of 100, 200 and 300 lbs respectively, as compared to samples with normal conditions of hydration and mineralization ($m + H2O$). Observing this behavior it may be thought that the ($m-H2O@48hrs$) group presents more strength, but in fact as they have lessened their collagen volume their stiffness increases. In other words, they become more fragile in terms of the stress-distension material curve [65]. The latter results in a linear response that may fail or break suddenly before they experience any permanent deformation (e.g., brittle like a crystal) [66]. On the other hand, at the top region, groups appear which are treated for demineralization with and without air-drying procedure $dm@4hrs + H2O$ and $dm@4hrs-H2O@48hrs$. As the inorganic mineral structure provides the bones with a mechanical support, the lack of it is clearly observed by the time required for these samples to reach the final load value which is smaller with respect to $m + H2O$ (see Table 3). The opposition against the compression movement is diminished in the demineralized groups and they start to behave predominantly malleable due to the collagen nature even when it is also affected, especially in $dm@4hrs-H2O@48hrs$. In terms of the stress-distension curve, the bones are more flexible and they could be deformed considerably before a failure or break appears [66]. The latter is not a desirable condition because they lack of any mechanically stable morphism.

Table 3. Temporal comparison among sample groups.

| Load (lbs) | $m-H2O@48hrs$ | $m + H2O$ | $dm@4hrs + H2O$ | $dm@4hrs-H2O@48hrs$ |
|-----------|---------------|-----------|----------------|-------------------|
| 100       | 27.1          | 20.1      | 16             | 13                |
| 200       | 54.4          | 42.2      | 31.7           | 26.2              |
| 300       | 87.6          | 70.1      | 50.2           | 44.9              |
3.3 Opto-mechanical system

The schematic view of the DHI system is shown in Fig. 6 where the illumination source is a Verdi continuous wave (cw) laser at $\lambda = 532$ nm. This laser beam is divided using an 80:20 non polarizing beam splitter (BS), into the reference (OF1) and object (OF2) beams. These beams are conveyed and delivered by means of single mode optical fibers. The BS helps to bring most of the laser beam over the bone’s surface with an illumination angle of 5° with respect to the observation axis $z$. The backscattered light coming from the bone’s surface is collected and focused on the camera sensor by means of a lens (L) with 100 mm of focal length and an aperture (A) in front of it. A high-speed CMOS camera (PCO Dimax HD+) with $1920 \times 1440$ pixels at 12 bits dynamic range (bit-depth), working at 1000 frames per second (fps) is used to acquire and record the interference pattern coming from the overlapping of the reference and the object beams using a 50:50 beam combiner (BC) placed in front of the camera. The observed area of this optical arrangement is set to $20 \times 20$ mm which covers the entire bone sample dimensions. The cortical samples were prepared as it was described in section 2.2 and their flat and parallel faces adequately match the flat supports of a micro compression testing machine (MCTM) which was specifically designed and built for optical non-destructive testing. This testing machine gives the opportunity to analyze and compare the different bone responses in the same experimental load conditions.

The MCTM compresses each sample axially with a load range which covers from 0 up to 300 lbs and as it was mentioned previously, the maximum load will represent an overload of up to 40 times with respect to their normal value (overloads in bone are practically considered from about 10 times with respect to their physiological load) [65]. It is important to mention that the MCTM was previously calibrated under standard compression tests using known-material samples (aluminum 6061) with extension gauges sensors attached to them. The embedded control design of the MCTM allows a linear compression using a close loop method which is a relevant feature because it differentiates a non-linear structural response of the bone from the compression signal. The compression value is read by means of a load cell fixed at the bottom of the MCTM and helps to set the same preload value for each compression test. During the compression test the load cell readings are stored in a computer while simultaneously the high-speed CMOS camera is recording image holograms at 1000 fps. A homemade algorithm matches each load cell value with its corresponding image hologram in the continuous load application (the compression never stops during the image acquisition). The procedure to place each sample before the compression tests starts by placing the bone probe over the bottom support of the MCTM in front of the optical system. After that, the bone sample is compressed to a preload value of 30 lbs which simulates a physiological value. The machined bone’s parallel faces and the preload value avoid rigid body motion in the measurements. The latter was proven by recording images during long
periods with just the preload compression and observing the absence of any fringe pattern. As two image holograms are required to calculate a displacement map, the selected compression load uses the corresponding image hologram as the deformed one and a $\Delta l$ of $-3$ lbs is used to set the reference hologram. As an example, if the displacement map for 100 lbs of load is required, the holograms at 97 and 100 lbs will work as the reference and deformed holograms respectively. This helps to have the same $\Delta l$ for all the displacement maps allowing comparisons among the four bone groups.

4. Results and discussion

A comparison of the displacement maps at 100, 200 and 300 lbs is presented for each one of the analyzed groups (groups mentioned in section 3.2). Figure 7 shows the comparison between $m + H2O$ and $dm@4hrs + H2O$ for each one of the selected compression values.

![Figure 7](image)

Fig. 7. Average surface displacement map comparison between $m + H2O$ and $dm@4hrs + H2O$ left and right column respectively, for (a,b) 100 (c,d) 200 and (e,f) 300 lbs.
From Fig. 7 it is possible to notice that well hydrated and mineralized bone samples \((m + H_2O)\) have a decreasing magnitude as the compression load increases. This behavior is in good agreement with that reported in reference [21]. This surface response is replicated in the hydrated bone samples with a demineralization process of 4 hours. However, as these samples lost some of their hydroxyapatite, the water protects the natural anisotropy of the bone by keeping the internal collagen hydrated. The latter is observed with larger displacement magnitudes and a smaller time required to reach the selected loads (see Table 3) if it is compared with the control group (i.e., Fig. 7(a) compared with 7(b)). Figure 8 shows the surface displacement comparison of the two dehydrated groups: \(m-H_2O@48hrs\) and \(dm@4hrs-H_2O@48hrs\).

![Fig. 8. Average surface displacement map comparison between \(m-H_2O@48hrs\) and \(dm@4hrs-H_2O@48hrs\) left and right column respectively, for (a,b) 100 (c,d) 200 and (e,f) 300 lbs.](image-url)
The dehydrated groups show higher displacement magnitudes than those observed in Fig. 7. In the case of the average response of the group \( m-H_2O@48hrs \) the displacement at 100 lbs is larger than the one at 300 lbs. This displacement reduction looks similar to that of the control group, but in this case the magnitudes are approximately 5 times larger in every case. The average response of the group \( dm@4hrs-H_2O@48hrs \) which is affected in mineral, water and collagen, shows displacement magnitudes opposite to the control group. Here, as the compression load increases the surface displacement also increases. This indicates that bone’s ability to deal with the compression load is no longer available. This can also be observed in terms of the time that each of these two groups consumes to reach the compressive load values. The demineralized groups are affected by the compression faster than the mineralized samples as Table 3 times indicate. The collagen is the main element in the \( dm@4hrs-H_2O@48hrs \) group and gives not enough support to the bone’s structure.

5. Summarize and conclusions

A study of the strength secondary affectations in bovine cortical bone was presented in terms of a compression load test. Several samples were machined for each one of four groups that emulate the secondary affectations of bone strength. Considering the conditions and diseases that may affect the bone’s strength in quantity, quality and turnover, the affectation protocols are designed to degrade independently one or more of the bone’s principal components: organic or inorganic. Demineralization and air-dried protocols were applied to modify the hydroxyapatite and the collagen-water contents. The effectiveness of these protocols was tested by means of the FT-IR transmittance spectral signal. An out of plane DHI system was used to record image holograms in order to retrieve the optical amplitude and phase that is transformed into the surface displacement map. This displacement information is used for comparison purposes among the four bone groups, and indeed to corroborate the mechanical behavior of the bones.

The compression test was performed by means of a MCTM specifically designed for optical non-destructive testing which simplified the matching with the high speed camera recordings. The compression load range between 30 and 300 lbs was selected to start in the physiological range up to an overload of 40 times for the selected cattle. Two types of analysis are presented for the bone samples: temporal response (Fig. 5) and surface displacement (Figs. 7 and 8). In the temporal loading tests two zones were clearly identified with respect to the control group if the mineral component is considered. These regions imply the required time for a bone sample to reach certain compression value, being the dehydrated-demineralized bones those that reach it faster. The mineralized and air-dried bones showed more opposition to be compressed increasing their stiffness but being more susceptible to a sudden break without permanent deformations before that (especially for intermediate loads as Fig. 8(c) shows). In this case they behave similar to a crystallized material and the strength is highly compromised with respect to the control group. The demineralized bones with compromised organic phases reduced their support capabilities; this is evident due the surface displacement which shows the dominance of the elastic properties in medium and high loads (Figs. 8(d) and 8(f) respectively).

The results presented in Figs. 7 and 8 are obtained with the average surface displacement response of several samples per group. The full field displacement information gives a better understanding about the mechanical response of the samples as an entire and complex structure. The affectation procedures help to separate the isolated contribution of a missing component and its influence during a compression test. The results obtained proved the theory for a strong integration existence among the hydroxyapatite, collagen and water, which determines the bone strength (more than just bone quantity as in the case of osteoporosis). How the strength is modified by removing any of these components is shown by the surface variation that the sample suffers during a compression. This work also showed that DHI is a functional technique to analyze strength in mechanical \textit{ex-vivo} biomaterials (bio and...
composite materials) where the presence/lack of each one of the essential elements determines the material mechanical response. Finally, in this proof of principle it was observed that hydration is the most relevant factor that modifies the bone’s mechanical structure to support compression loads.

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