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Mechanical, biological and structural characterization of in vitro ruptured human carotid plaque tissue

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ABSTRACT

Recent experimental studies performed on human carotid plaques have focused on mechanical characterization for the purpose of developing material models for finite-element analysis without quantifying the tissue composition or relating mechanical behaviour to preoperative classification. This study characterizes the mechanical and biological properties of 25 human carotid plaques and also investigates the common features that lead to plaque rupture during mechanical testing by performing circumferential uniaxial tests, Fourier transform infrared (FTIR) and scanning electron microscopy (SEM) on each specimen to relate plaque composition to mechanical behaviour. Mechanical results revealed large variations between plaque specimen behaviour with no correlation to preoperative ultrasound prediction. However, FTIR classification demonstrated a statistically significant relationship between stress and stretch values at rupture and the level of calcification ($P = 0.002$ and $P = 0.009$). Energy-dispersive X-ray spectroscopy was carried out to confirm that the calcium levels observed using FTIR analysis were accurate. This work demonstrates the potential of FTIR as an alternative method to ultrasound forpredicting plaque mechanical behaviour. SEM imaging at the rupture sites of each specimen highlighted voids created by the nodes of calcifications in the tissue structure which could lead to increased vulnerability of the plaque.

1. Introduction

Plaque rupture remains one of the most difficult clinical events to predict accurately and is one of the leading causes of stroke worldwide [1,2]. Despite significant advancements in thein vivo imaging of atherosclerotic plaque, the gold standard for predicting the likelihood of plaque rupture remains the luminal stenosis level and plaque type, both of which are determined through the use of duplex ultrasound [3–5]. However, it has been reported that duplex ultrasound measurement can underestimate the severity of the atherosclerotic plaque or under predict the risk of plaque rupture [6]. Tosi et al. [7] suggested that the clinical use of vibrational spectroscopy is ready to be developed as an in vivo analysis technique for characterizing atherosclerotic plaques. However, there are limited studies that relate the mechanical behaviour and rupture data of arterial plaques to vibrational spectroscopic data [8].

Fourier transform infrared (FTIR) is one of the most widely used vibrational spectroscopic techniques for the identification of biological components within tissue specimens [9]. FTIR functions by obtaining a broad range of infrared spectra from a sample based on the vibration of the molecules present, and therefore can be used to generate a positive identification of the composition of a biological tissue [8]. Previous work has shown the ability of FTIR to identify and quantify the presence of lipid, collagen and calcification within plaque tissue [9]. Ebenstein et al. [8] used FTIR analysis to correlate the mechanical data from nanoindentation to the biological content ratios calculated from FTIR. This study provides important data to aid in the use of vibrational spectroscopy in clinical applications. However, it is necessary to relate the FTIR analysis results to the mechanical properties of the plaques on a global level rather than localized areas as done by Ebenstein et al. [8] as carotid artery stenting (CAS) applies forces in the circumferential direction that will trigger a whole-plaque mechanical response. Also, such a study would demonstrate whether FTIR is truly predicting the biological composition of the plaque as a whole and the features that may increase plaque vulnerability.

The morphology and composition features that contribute to plaque vulnerability have been extensively studied [10,11]. However, the role of calcifications in plaque vulnerability is still under debate. Previous studies have suggested that calcifications stabilize the plaque [12–14]and that fibrous cap thickness[12,15] and peak circumferential stress [16,17]are the main contributors toward plaque vulnerability. Conversely, Wenk [18] demonstrated that the circumferential stress in fibrous tissue increases as the volume of calcifications increases and also that the presence of calcifications

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can significantly alter the distribution of stress and shift the peak stress from the main location of ruptures, such as the cap shoulder noted by Cheng et al. [12] and Versluis et al. [17]. Maldonado et al. [19] and Vengrenyuk et al. [20] have shown that microcalcifications have an effect on the Young's modulus of the surrounding tissue (inducing a 5-fold increase in the stress threshold at rupture) which can increase plaque vulnerability due to the increase in voids created by the growth of microcalcification clusters.

In order to examine the effect that calcifications have on the surrounding tissue structure, this study will use scanning electron microscopy (SEM) to observe the areas of rupture in the plaque in vitro induced by mechanical testing. SEM has, in recent years, been used in the biological sciences specifically to image the structure of tissue in great detail. Guasti et al. [21] and Dell'Orbo et al. [22] have demonstrated that SEM is capable of imaging the delamination and build-up of calcification in human carotid plaques. However, these studies are limited to one specific plaque specimen. Congiu et al. [23] examined a larger sample size ($n = 6$) and identified the delamination and dysfunction of the endothelium which led to the build-up of disease in the specimens. In comparison, this present study examines an even larger sample size of human carotid plaques in order to establish the key features present in all specimens in order to provide a better understanding of atherosclerosis development and in vitro plaque rupture. In parallel to SEM imaging, energy-dispersive X-ray spectroscopy (EDX), an elemental analysis tool, can be used to characterize the composition of biological tissue in the areas of interest observed during imaging. EDX spectroscopy can determine the main constituents at the rupture sites of each plaque [24] as well as validate the vibrational spectrums and trends produced by FTIR.

This paper examines the biological composition of arterial plaque material and how the composition of this relates to the mechanical behaviour and rupture potential of these plaques through the use of FTIR globally as well as SEM and EDX. In order to achieve this, 25 carotid plaques are examined, using each of these techniques as well as mechanical testing. This paper also analyses the ability of FTIR to predict plaque mechanical behaviour in order to assess whether FTIR is a viable technique for use as an in vivo preoperative imaging tool in the treatment of carotid atherosclerotic tissue as well as for assessing the contribution of calcifications toward plaque vulnerability. Finally, SEM imaging is carried out to investigate the morphology and structure of the in vitro rupture sites of the plaque specimens.

2. Materials and methods

2.1. Sample acquisition

Twenty-three carotid plaques were obtained from Limerick University Hospital, Limerick, Ireland in a manner that conformed to the Declaration of Helsinki and was approved by the hospital's Ethical Research Committee. The carotid plaques were collected from consenting patients who had undergone standard carotid endarterectomy surgery to treat high-grade carotid artery stenosis. Within this population 58% (12/23) of the patients were male, with a median age of 65.6 years (range 52–79) and the median age of the female population was 72.2 years (range 52–85). Plaques were surgically removed from the carotid artery with preservation of plaque structural integrity emphasized to minimize possible disruption of the plaque luminal surface (Fig. 1). The ultrasound preoperative identification was based on the type classification by AbuRahma and Bergan [25], according to which plaques are classified from Type I (soft echolucent plaque) to Type IV (hard echogenic plaque).

The plaques were frozen in phosphate buffer solution (PBS) immediately after removal at −20 °C. On the day of tissue testing, the plaques were equilibrated to room temperature in PBS and, after FTIR analysis, were further heated to 37 °C prior to mechanical testing. Each plaque underwent the process illustrated in Fig. 2.

2.2. Mechanical testing

Uniaxial mechanical testing was carried out on 25 whole specimens obtained from the 23 patients. Plaques 3 and 16 were divided into two separate pieces during the surgical removal and were sufficiently large to be tested individually. This increased the sample size to $n = 25$ (i.e. plaques 3a, 3b, 16a and 16b). Prior to mechanical testing, the specimens were placed into clamps designed for soft biological tissue, and a uniform force was applied to the clamps using a torque screwdriver [26]. Measurements of the gauge length, thickness and width were taken using a vernier calipers and also with a non-contact photography system to validate the values. The plaques typically had width-to-length ratios greater that 4:1 which was suitable for planar shear testing. Fig. 3 illustrates the mechanical testing process for each whole plaque specimen.

When the specimens were placed in the clamps, the geometrical ratio was taken into account to reduce the errors associated with unsuitable width-to-length ratios based on previous work by Mulvihill and Walsh [27]. As the samples were tested as a whole, the width and gauge lengths between each sample varied, 22.89 ± 5.94 and 4.157 ± 1.75 mm, respectively. However, there was a larger variation in the sample thickness which is inherent in these samples due to difference in fibrous cap thickness, level of stenosis and overall artery size between each specimen (1.521 ± 1.278 mm). This current study evaluates the circumferential stretch and stress value that the plaque can withstand prior to rupture, Fig. 3, at a physiological strain rate that replicates the instantaneous systolic pulse experienced by the plaque in vivo, i.e. 30% of the gauge length per second ($\%s^{-1}$) which is based on

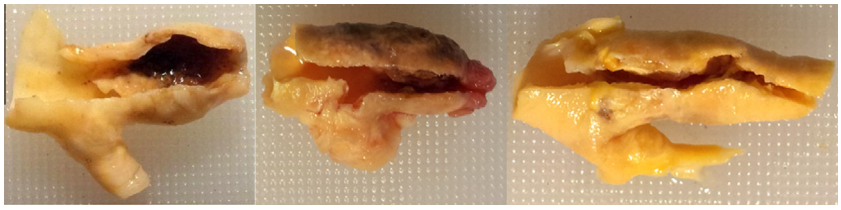

Fig. 1. Three carotid plaque specimens imaged post-endarterectomy and prior to mechanical testing which have been equilibrated to 37 °C, highlighting the random nature of the geometries and composition between each specimen acquired.

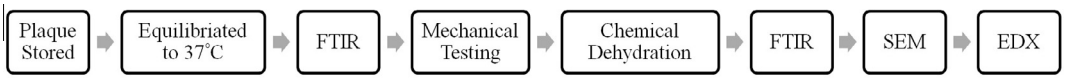

Fig. 2. Mechanical and biological characterization process for each plaque tested.