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Rheological Experimentation to Investigate History Dependent Viscoelastic Properties of ex-vivo Ovine Brain Tissue

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Abstract: Much controversy exists around the issue of repetitive Traumatic Brain Injuries (TBIs) and long-term brain health. Little is known about the mechanical response of brain tissue to traumatic impacts and head accelerations. While history dependent characteristics of other biological tissues have been investigated experimentally, no methodology currently exists for investigating the mechanical response of brain tissue to cyclic loading and its fatigue properties. This investigation presents sample preparation, conditioning and rheological methodology for undertaking repetitive loading of *ex-vivo* ovine brain tissue. Rheological amplitude sweep tests undertaken at 3 Hz on ø10 mm by 5 mm ovine brain tissue samples, employing a periodic moisturisation and a normal force of 50 mN, yielded results agreeing with those reported in literature for low number of cycle loading and showed signs of history dependence at higher number of cycles. Understanding brain tissue response to repeated loading, fatigue properties and associated trauma mechanisms can be advances by undertaking high cycle rheological testing with the methodology presented in this article.

Keywords: neuro-systems, experiment design, quantification of physiological parameters for diagnosis and treatment assessment, bioinformatics, mammalian, insect and plant cell technology

1. INTRODUCTION

Traumatic Brain Injuries (TBIs) are a leading cause of mental deficiencies and death with recorded rates of TBIs increasing in The U.S. from 1995-2009 (Coronado et al., 2012). High instances of mental health issues and behavioural abnormalities have been reported amongst groups of individuals exposed to repeated head trauma, including high contact sports' athletes, military personnel, and sufferers of domestic abuse. A systematic review of the long-term implications of sports related mild Traumatic Brain Injuries (mTBIs), in particular, defined long-term effects as those evident at least 10 years post injury (Manley et al., 2017). Manley et al. concluded that depressive behaviours and cognitive deficiencies amongst some retired athletes investigated were correlated to the sustainment of multiple head injuries. A review of Australian rugby found that clinicians have legislative power to overrule in cases of suspected TBI, though they face multifaceted and ethical issues regarding return to play decisions (McNamee et al., 2016). Research indicates repeated sub-concussive impacts can cause chronic long-term symptoms (Bailes et al. 2013). Understanding the pathological implications of repeated brain trauma is imperative to bring forth legislation which focuses on prevention and recovery from concussive or sub-concussive injuries.

Fatigue is a means of mechanical failure of a material under cyclic loading (Callister and Rethwisch, 2014). The mechanical fatigue properties of load-bearing biological tissues has been investigated experimentally on specimens including tendons (Schechtman and Bader, 1994) and heart valves (Gilpin, 2005). The methodology for undertaking high cycle testing with brain tissue has not yet been reported in the literature. Computational models exist for predicting pathological response of collagenous soft tissue (Martin and Sun, 2011) and Finite Element Analysis (FEA) of repeated TBIs (Gerber et al., 2018). These rely on experimental data for biological tissue mechanical loading-specific properties.

While brain tissue is not a load-bearing tissue, in traumatic scenarios it is subject to dynamic stresses and strains. The repetitive brain tissue trauma, i.e. through concussive or sub-concussive head impacts, may be considered in the view of a cyclic loading scenario. Results from such testing could be used to obtain an understanding of the mechanics of brain tissue response to repetitive loading of brain tissue and trauma manifestation. The predictive models of repeated TBIs can thus be informed.

Viscoelastic brain tissue properties were investigated by a number of authors. An increase in strain was associated with a decrease in elastic properties (G') until the liquid response (G'') of brain tissue by (Nordin et al., 2012). No significant change in storage or loss moduli was observed using test frequencies below 1.6 Hz (Mezger, 2011). To observe frequency dependence of brain tissue, a relative increase in frequency of magnitude 5-10x was required. At higher frequencies, it is expected that a viscoelastic material will show a stiffer response. Rheological tests are typically

conducted in the recoverable region such that repeat trials can be performed with a given sample. For a given load, recovery percentage is decreased as loading time increases.

The influence of test conditions when investigating the mechanical properties of brain tissue was reviewed in detail by Hrapko et al. (2008). Hrapko et al. undertook rheological experiments with what was deemed from their research to be best experimental practice. The top and bottom rheometer plates were lined with sandpaper to prevent tissue slippage, testing the sample in a moisturisation chamber, controlling sample temperature with a base plate Peltier pump, and estimating sample height by lowering the top plate slowly until 5 mN of force was recorded. Brain samples tested at room temperature were observed to have a stiffer response than samples tested at 37°C. To best replicate in-vivo conditions, testing should be undertaken at body temperature. Sample plane affected measured response to loading. Measured response of coronal and transverse plane samples was 1.25-1.3x stiffer than that of sagittal plane samples. When comparing experimental results with those published in literature, effect of sample location should be considered. The degree of anisotropy is another cause of variation in measured material properties. Grey matter can be considered isotropic while white matter is anisotropic to varying degrees, hence the grey and white composition of each tested sample should be acknowledged. Anisotropy can be reduced by minimising sample size. The importance of post mortem time when undertaking rheological testing was also discussed. Autolytic processes, rigour mortis and osmotic swelling were given as potential causes for property degradation with increased post mortem time. Storing samples at lower temperature can decrease degradation while freezing samples can lead to cellular damage. The ideal window in which to test samples after death is suggested to be three hours; after this time, critical degradative changes in properties have already occurred and subsequent changed are hence less significant.

Hrapko et al. identified that human samples were 29% and 40% stiffer than porcine and bovine samples in stress relaxation, respectively. Conversely, frequency sweeps of porcine brain samples had a storage modulus 17% higher than human brain samples while loss modulus was similar for both species. This indicates limitation in using the animal brain to accurately represent those of human tissue.

Though rodents are more readily available for scientific testing than large animals, comparing the neuropathology and cognitive capacity of rodents with humans has inherent limitations. (Vink, 2018) identified that while extensive research has been done on rodent models of TBI, improving understanding and treatment of human TBI may be hindered by the lack of bio-fidelity between rodent and human brains. An area of particular concern was modelling the gyrencephalic brain of humans on lissencephalic rodent brains. This was largely because the presence of gyrus influences the degree of deformation and maximum stresses imposed on the brain upon mechanical loading.

Szotek, Kobielarz and Maksymowicz (Szotek et al., 2007) tested ovine samples within six hours of death. Samples were moisturised for indentation testing by misting with saline spray. Their results highlighted varying properties in different areas of the brain, reinforcing the importance of sample location when comparing results.

This article presents findings from an investigation into the sample preparation and rheological testing methodology for undertaking cyclic loading of brain tissue with the aim to simulate the mechanics of repetitive trauma. In particular, results from varying rheological frequencies were compared with the analysis of the effects of sample conditioning, such as the applied normal pre-compressive force and sample moisturisation.

2. METHODOLOGY

Ovine brain tissue was selected for this investigation as human brain tissue substitute due to its local availability. Frozen ovine crania were obtained from a local butchery and stored at approximately -4°C for less than a week. The crania were thawed at room temperature, and brain extraction was undertaken using a bone saw and small axe. Three cuts were made in the skull to access the brain; one coronally posterior of the orbits, and two bilateral to form a triangular shape towards the occipital condyle.

Two hemispheric 12 mm sagittal slices were made with a knife and cutting block. A 12 mm die punch was used to obtain cylindrical samples from the frontal cortex of ø10 mm. Samples were trimmed to a height of 5 mm by removing excess with a microtome blade after placing in a 5 mm deep ø10 mm opening in a cutting block. To reduce inhomogeneity, ø10 mm samples were chosen and taken from a hemispheric sagittal slice of the frontal cortex.

Rheological measurement of ovine brain tissue viscoelastic properties in rotational shear of ovine brain tissue in rotational shear was undertaken on an Anton Paar MCR 302 Rheometer. Storage and loss moduli, G' and G'', were calculated and plotted against strain amplitude γ. Tests were undertaken at 37°C to replicate in-vivo conditions. This was achieved by setting the temperature of the rheometer base Peltier pump plate and allowing a waiting period for the sample to reach 37°C. A bespoke 10 mm upper parallel plate attachment was loaded into the rheometer after initialisation. Amplitude sweep frequencies were undertaken at frequencies between 1 and 20 Hz from 0.01 % to 100 % strain. Preload of 5 and 50 mN was investigated with a 100 s preloading phase prior to the first amplitude sweep cycle. Further 100 s waiting periods were applied between each cycle from Hrapko to allow for sample recovery. Slippage was prevented by drying the outer faces of the sample with tissue paper. Moisturisation was achieved by applying approximately 1 mL of phosphate buffered saline to the outer edge of the sample between each cycle. Early methodology used 2 mm height samples, however the surface tension from the saline solution meniscus created from moisturising the samples skewed readings. Hence, 5 mm height samples were used. Mechanical properties and rheometer settings were recorded six times per shear strain decade.

Rheological amplitude sweep frequency was a key independent variable investigated experimentally. With motivation to better understand the pathological response of the brain to repeated cranial loading in sporting applications, amplitude sweep tests were undertaken at a frequency of 20 Hz reported for contact sports' athletes (Laksari et al., 2015). Amplitude sweeps were also undertaken at lower frequencies, particularly 3 Hz, to determine which frequency would be more appropriate for high cycle testing.

3. RESULTS

Sample preparation and rheological methodology was systematically varied in testing of more than 20 samples from 10 sheep brains. Rheological methods were first assessed by testing samples between 1 and 10 Hz and comparing results with those reported in literature. Methods were practised until consistent values of storage and loss modulus between samples were yielded in three amplitude cycle tests. At 3 Hz, storage modulus values in the range 800-2000 Pa at 1% strain and loss modulus of 400-600 Pa at the same strain were recorded consistently between frontal cortex samples. This fell within the ranges expected from a summary of data by multiple authors reported by Feng (2012) for animal tissue, particularly (Bilston et al., 1997) and (Bilston et al., 2001). Their findings reported storage and loss moduli of 800-2000 Pa and 300-500 Pa respectively for bovine unspecified white matter at 1, 5 and 20 Hz, and 1000-1500 Pa and 600-900 Pa respectively for bovine corpus callosum and corona radiata samples at 0.01-20 Hz. Consistency of results within an acceptable range identified from literature between samples indicated variation in grey and white matter composition and frontal lobe location did not cause significant variation in rheological results. Increased sample stiffness with increased testing frequency was observed with higher storage modulus values recorded at higher frequencies, as expected from Burstein and Frankel (1968), and Hrapko et al. (2008). The effect of sample moisturisation was investigated by subjecting a sample to an amplitude sweep at 5 Hz with and without application of saline. Elastic and loss modulus against strain for the two sweeps are presented against strain in Figure 1.

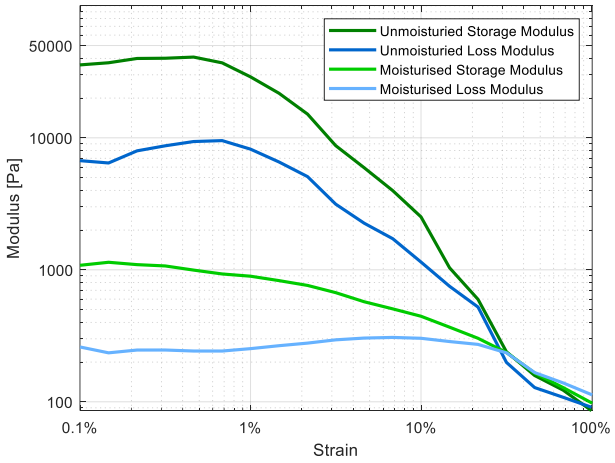

Fig. 1. Elastic and loss moduli against shear strain of grey and white matter sample tested at 5 Hz with and without moisturisation

From Figure 1, a significantly stiffer response was observed when the sample was tested without moisturisation. The peak elastic modulus recorded for the non-moisturised test of 40884 Pa was approximately 36 times greater than that of 1140 Pa from the moisturised test. The viscoelastic response of the sample at 5 Hz greatly exceeded that which has been recorded in literature, while the moisturised test fell within the expected range. This highlighted the importance of adequately moisturising samples to prevent stiffening.

Application of a normal force of 5 mN was trialled. It was found, however, that the lower limit of normal force application sensitivity of the rheometer was approximately 50 mN. Application of normal force below this value was inconsistent, introducing experimental variation in compressive force. A compressive normal force was required to ensure contact between the sample and the measuring system (upper rheometer plate) was maintained in testing. The observed reduction of sample height with subsequent loading cycles is shown in Figure 2.

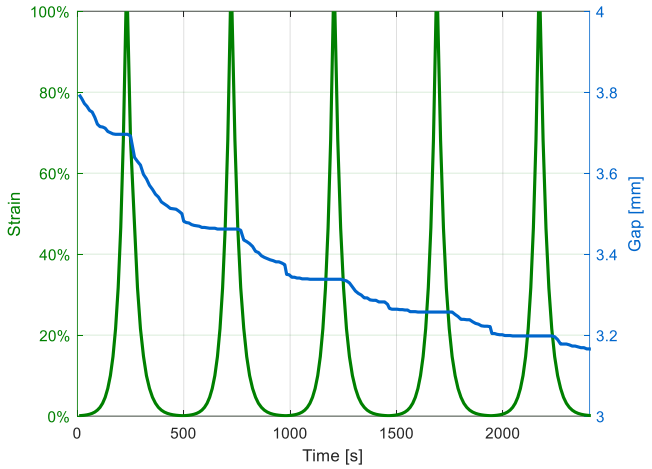

Fig. 2. Strain and sample height with time after 100 s initial load application phase of 65 % white matter brain sample across 5 amplitude sweep cycles at 3 Hz, initial height 5 mm

Figure 2 shows that maintaining a normal force of 50 mN slowly compressed the sample from an initial height of approximately 5 mm, to 3.8 mm after the initial 100 s loading period, and 3.2 mm after 5 loading cycles. Compression appeared to be greatest for the first cycle with the gradient of decreasing plate gap becoming less steep between cycles 2 and 5.

The following rheological results are from tests undertaken with samples from the frontal cortex of the right and left hemispheres of the same brain with an approximate white matter composition of 65% and 75%, respectively. The samples were subjected to 5 amplitude sweep cycles at 3 and 20 Hz, respectively. The tests resulted in a total of approximately 7,000 and 42,000 cycles at varying strains. The viscoelastic properties of the two samples across the 5 loading cycles are presented in Figures 3 and 4.

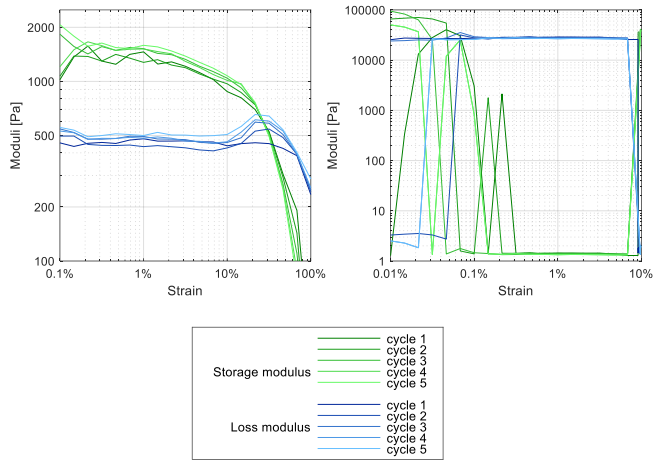

Fig. 3. Elastic and loss moduli against shear strain of 75 % white matter brain sample across 5 amplitude sweep cycles at 3 (left) and 20 Hz (right).

Storage and loss moduli were highly erratic below approximately 0.1 % strain at all frequencies tested. Though the amplitude sweep test specifications were set with the minimum and maximum strains of 0.01 % and 100 % for the 20 Hz test, strains above 10 % were not undertaken as the maximum electrical torque of the rheometer was reached. Between 0.1 and 1 % strain, the storage modulus at each cycle dropped below 10 Pa, indicating a substantial loss of elasticity at 20 Hz. There was a small window for collecting viable data above 0.1 % strain and below 0.5 % where storage modulus plummeted to less than 10 Pa. The maximum magnitude of the storage modulus of 25,000 to 55,000 Pa did not agree with the results collated by Feng (2012) which suggested that the storage modulus at 20 Hz would be of the order of 2000-2200 Pa. This indicated that for the small 10 mm diameter samples used, higher frequency amplitude sweeps yielded viscoelastic property data that did not match that found in literature due to equipment sensitivity and limitations.

Opposingly, amplitude sweeps undertaken at 3 Hz progressed to 100 % strain in each cycle and yield results expected from Feng (2012), stated at the start of this section. Noise was low for the amplitude sweep from 0.1 to 100 % strain with storage modulus dropping below the loss modulus between 20 and 40 % strain. This could indicate a flow point where the viscous properties begin to dominate over the elastic properties, or could be due to the onset of slippage observed at approximately 10 % strain at 3 Hz. Stress versus strain from the first 5 amplitude sweep cycles of the same samples at 3 and 20 Hz are presented in Figures 4.

Recorded shear stresses were an order of magnitude higher for a given strain at 20 Hz than at 3 Hz. The maximum stress recorded in the first 5 cycles of the 20 Hz test at approximately 10 % strain was 6061 Pa, compared with 119.1 Pa for the 3 Hz sweeps at the same strain. This agrees with Burstein and Frankel (1968) who predicted a stiffer response for a given tissue tested at higher oscillation frequencies.

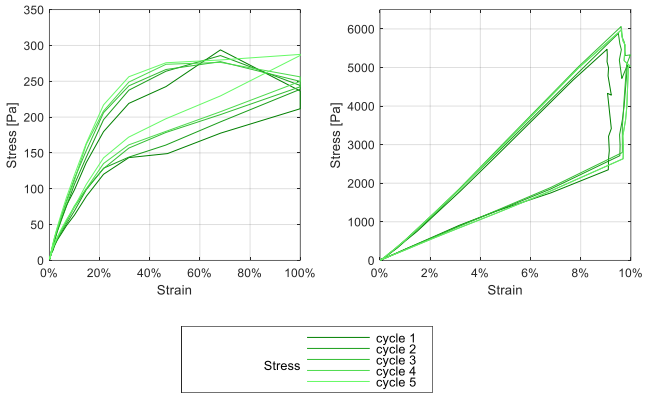

Fig. 4. (left) Shear stress against shear strain of 65 % white matter neurological sample across 5 amplitude sweep cycles at 3 Hz and (right) Shear stress against shear strain of 75 % white matter brain sample across 5 amplitude sweep cycles at 20 Hz

Data from Figure 3(left) shows considerably less erraticism than Figure 3(right), though the validity of conclusions made from the data after the significant recorded loss of elasticity is potentially low. At 20 Hz, stress strain hysteresis was not observed as the shear strain achieved was at a maximum of 10 % as well. From the 5 cycles presented in Figure 4(left), variation between the hysteresis response of the sample at 3 Hz with successive cycles can be observed.

4. DISCUSSION

From Figure 1, moisturisation had a significant impact on the measured viscoelastic response of the brain sample. Periodic moisturisation is required to preserve *in-vivo* sample properties and prevent a stiffening response.

Though the rate of sample compression appeared to decrease with subsequent cycles (Figure 2), a decrease in sample height from 3.8 to 3.2 mm was observed from cycles 1 to 5. Though 50 mN was deemed to be the minimum applicable normal force for this testing, validity of results could be improved by reducing normal force to minimise sample compression.

This investigation determined a suitable frequency for experimental determination of brain tissue properties in cyclic loading. In particular, 3Hz cyclic loading was more successful in measuring rheological parameters than 20Hz. Figure 3 shows unstable values that imply the rheometer was not capable of capturing coherent behaviour at 20Hz. In contrast, Figure 3 showed consistent behaviour at 3 Hz that was within the range of parameter described in the literature (Feng, 2012, Bilston et al., 1997, Bilston et al., 2001). This implies that the brain sample preparation and testing methods were able to reproduce expected values.

The measurements taken at 3 and 20 Hz were highly erratic at low strains as torque values fell below recordable limits of the rheometer with a ø10 mm upper plate. The low torque measured at low strains below 0.1% was associated with the limiting size of the sample and rheometer measurement range. While the rheometer was programmed to reach 100%

strain, it reached a maximum electrical torque limit at 10% strain at 20 Hz. The latter can be explained by the shear rate stiffening properties of brain tissue (Burstein and Frankel, 1968).

Figure 3(left) shows consistent rheological measurements for brain tissue during cyclic loading. A consistent stiffening trend observed over these few cycles imply high cycle testing at 3 Hz may be suitable for analysis of mechanical fatigue of brain tissue. Extending to more loading cycles will allow for better observation of history dependent elastic properties. If the number of cycles was increased, changing viscoelastic response characteristics of brain tissue could provide an implication of fatigue mechanics in multiple TBIs.

The rheological methods utilised allowed for continuous cyclic loading of brain tissue. Tensile methods have been used to measure mechanical properties of other biological samples such as tendons or arterial tissue (Schechtman and Bader, 1994, Gilpin, 2005). However, tensile methods typically require clamping and are thus not suitable for soft biological tissues such as brain tissue. In particular, tensile methods that require high forces to clamp the sample and potentially damage the material at the clamp regions. In contrast, the parallel plates of the rheometer impose a very low clamp force of 50 mN, inflicting significantly less damage on the material. For biological samples of bone and tendon, samples can larger, hence local damage can be ignored provided a smaller local region is kept intact. For brain tissue, samples tend to be smaller and hence any local damage would significantly affect recorded results. In addition, the grip on a fatty brain tissue is not sufficient and prone to failing.

Testing *ex-vivo* ovine samples as a means of gaining an understanding of living human brain tissue has inherent biological limitations. *Ex-vivo* tissue is unable to undergo repair or react in the same way as living tissue. Hence, this study is likely to overestimate the damage to *in-vivo* tissue subjected to multiple TBIs over a prolonged period.

5. CONCLUSIONS

From this investigation, brain tissue sample preparation, pre-conditioning and rheological cycling testing methodology have been explored. Methods utilising an amplitude sweep frequency of 3 Hz and sample geometry of ø10 mm by 5 mm was successfully used to obtain viscoelastic properties that agreed with previously reported in literature for low cycle testing when the sample was compressed with a 50 mN normal force and was periodically moisturised. The rheological methodology to measure brain tissue mechanical response to cycling loading was developed. The results from such testing can strengthen current understanding of the mechanics of brain tissue trauma. The latter can inform stronger TBI prevention and recovery measures, particularly in sporting and military applications.

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