Repeated Loading Behavior of Pediatric Porcine Common Carotid Arteries

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Rapid flexion and extension of the neck may occur during scenarios associated with traumatic brain injury (TBI), and understanding the mechanical response of the common carotid artery (CCA) to longitudinal stretch may enhance understanding of contributing factors that may influence CCA vasospasm and exacerbate ischemic injury associated with TBI. Immature (4-week-old) porcine CCAs were tested under subcatastrophic (1.5 peak stretch ratio) cyclic loading at 3 Hz for 30 s. Under subcatastrophic cyclic longitudinal extension, the immature porcine CCA displays softening behavior. This softening can be represented by decreasing peak stress and increasing corner stretch values with an increasing number of loading cycles. This investigation is an important first step in the exploration of fatiguelike behavior in arterial tissue that may be subjected to repeated longitudinal loads.
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1 Introduction

With over 250,000 emergency department visits, 15,000 hospitalizations, and nearly 1000 deaths annually in the U.S. [1], TBI in children aged 0–4 yrs is a major public health concern. Some TBIs are caused by rapid rotations of the head about the neck (whiplash, cyclic shaking-type injuries), and there is evidence that mechanical stretch of the cerebral arteries is a trigger for vasoconstrictive responses [2–4]. Most studies have focused on intracranial vasculature vasospasm in adult TBI patients, with a paucity examining vasospasm in pediatric TBI [5]. Recently, we reported that the CCA displays prolonged constriction after a single rapid sagittal head rotation in the immature pig [6]. It is possible that this CCA constriction and reduction in blood flow may mediate

decreased global cerebral blood flow [7], decreased brain tissue oxygenation [8], and increased lactate/pyruvate ratio [8] observed following similar injuries in this animal model.

Our goal was to elucidate the longitudinal mechanical behavior of the immature porcine CCA during nondestructive cyclic extension. Mechanical properties of carotid arteries are known to vary with age [9–14], and previous studies typically compare adult and advanced age. Most studies report the pressure–diameter and circumferential compliance relationship of the adult CCA [15–17]. Few have measured longitudinal mechanical properties [18–24], and only three have evaluated adult behavior during cyclic loading [25–27].

2 Methods

2.1 Specimen Preparation. All euthanasia and procurement procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Right CCAs were harvested from the 4-week-old female farm pigs, chosen to match subjects used in previous rapid nonimpact rotation studies [6,8], immediately after sacrifice ($n = 8$). Following removal of exterior fascia, each specimen was cut perpendicular to the longitudinal axis into three sections of near equal length and placed in serial dilutions of 2.5%, 5%, and 7.5% dimethyl sulfoxide (DMSO) in Dulbecco's modified Eagle media (DMEM) for 10 min each followed by a final concentration of 10% DMSO–90% DMEM for 20 min prior to freezing in Eppendorf tubes at -80°C . The location of each sample along the carotid axis (proximal, middle, and distal) was not recorded so that no systematic bias was introduced. Previous studies have shown that cryopreservation does not significantly alter the material properties of blood vessels [28–35].

On the day of testing, samples were removed from -80°C storage, thawed at room temperature, and rinsed of DMSO by placement in serial dilutions of 7.5%, 5%, 2.5%, and 0% DMSO in DMEM for 10 min each. After rinsing, the specimens were placed in fresh DMEM prior to testing. Cross-sectional area varied along specimen length. To determine average specimen cross-sectional area, the entire specimen was placed between two glass slides and the thickness and width of the specimen was measured in triplicate using calipers to find outer circumference (C , $C = 2\pi r_o$) and wall thickness (h , $h = r_o - r_i$). Artery cross-sectional area (A) was approximated as that of a hollow cylinder

$$A = \pi(r_o^2 - r_i^2) \quad (1)$$

where r_o is the outer radius and r_i is the inner radius.

2.2 Test Apparatus. In a setup similar to previous investigations [36,37], CCA test specimens were cannulated with 14G blunt-tipped needles and secured via silk suture (6-0) tied around the vessel circumference at the level of a groove machined in the needle. A small amount of cyanoacrylate was applied at both ends of the specimen distal to the suture fixation to secure the vessel on the needles. Each of the blunt-tipped needles was fastened to a Delrin block via a luer connection with a rotating lock ring to prevent specimen twisting. The blocks had channels machined through them to allow for fluid pressurization of the vessels and pressure sensor attachment (26PCBFM6G, Honeywell, Columbus, OH). Physiologic saline was inserted through a syringe into the channel in the first block, needle, vessel, opposite needle, and the second block, where the channel ended.

A custom device was constructed to pull vessels cyclically (Fig. 1). Briefly, a computer-controlled closed loop stepper motor (Vexta #AS46AAP, Oriental motor, Braintree, MA) coupled to a gear-chain system was used to rotate threaded shafts to move a plate up and down at a specified frequency. The bottom grip was attached to the moving plate, while the top grip was attached to a 1000 g capacity load cell (Model 31, Honeywell-Sensotec, Columbus, OH). The load cell was fastened to a lab jack mounted on the ceiling of a cage surrounding the entire device, which

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Fig. 1 Vessel testing device including (A) lab jack, (B) load cell, (C) saline inlet, (D) pressure sensors, (E) 14G blunt-tipped needle grips, (F) gear-chain system, (G) rotary motor, (H) threaded shafts, (I) translating plate, and (J) laser displacement sensor

allowed for gage length adjustment of the specimen. A laser displacement sensor (LC 1607-50, Micro-Epsilon, Ortenburg, Germany) measured the global elongation of the vessel by tracking the position of the translating plate. The lab jack was adjusted until the artery specimen was taut and the load cell just began to register a load. The specimen gage length was measured from suture tie to suture tie with calipers, as the *in vivo* length was not recorded at excision.

2.3 Test Protocol. Specimens were pressurized with physiologic saline to a target physiological pressure, ~ 100 mm Hg [38–40], a stopcock on the saline inlet was closed, and stretch tests were conducted at a peak stretch ratio of 1.5 and a cycling frequency of 3 Hz (yielding average stretch rate of 3 s^{-1}) for 30 s (~ 90 cycles). For real-world scenarios with sustained cyclic loads, we used previous measurements of vigorous shaking (3 Hz, 30 s) seen in child abuse [41,42]. The cycling stretch limit was chosen as a comparative benchmark for future studies where, at extremes, vessels may reach a stretch ratio of 1.5. Load and displacement values (sampled at 1 kHz) were recorded on a computer.

Fig. 2 Representative plot of cyclic stress versus stretch ratio and a single cycle loading portion (inset) defining the toe and linear regions. Peak stress decreases and corner stretch increases with successive cycles. A large amount of hysteresis is observed in the first cycle compared with subsequent cycles.

2.4 Data Analysis. Recorded load and displacement signals were low-pass filtered (second order Butterworth) at 35 Hz and 20 Hz, respectively. Stretch ratio and first Piola–Kirchhoff stress were then calculated from the displacement and load traces for each cycle from displacement minimum to load maximum as follows:

$$\lambda(t) = \frac{l(t)}{l_0} \quad (2)$$

$$\sigma(t) = \frac{F(t)}{A} \quad (3)$$

where λ is stretch ratio, t is time, l is the instantaneous length of the specimen, l_0 is the gage length, σ is stress, F is instantaneous load, and A is cross-sectional area as defined in Eq. (1). Stretch rate was defined as the slope of the stretch–time relationship over the loading portion of each cycle.

The stress–stretch behavior was nonlinear and biphasic, with stiffer behavior at higher stretch magnitudes (Fig. 2). We calculated modulus in the toe (low stretch) and linear (high stretch) regions as well as the approximate corner stretch where the transition to stiffer behavior occurred (Fig. 2, inset). The toe region was defined in two steps. First, a linear regression was calculated between 20% and 40% of the length of the loading portion of each cycle, omitting the unloading segment (Fig. 2). Next, the regression was applied to the entire loading portion of the cycle, and the magnitude of the residuals (or absolute value of the difference) between the measured stress values and the linear fit were extracted. The toe region was defined as the segment where

Table 1 Mean $\pm$ standard deviation of second cycle (2), sixth cycle (6), and fatigued values (∞) of E_{toe} , E_{linear} , λ_{corner} , and σ_{peak} . ANOVA and post-hoc Tukey–Kramer analyses show that $E_{\text{toe},2} > E_{\text{toe},6} = E_{\text{toe},\infty}$, $\lambda_{\text{corner},2} = \lambda_{\text{corner},6} > \lambda_{\text{corner},\infty}$, and $\sigma_{\text{peak},2} > \sigma_{\text{peak},6} > \sigma_{\text{peak},\infty}$, while there is no significant difference among E_{linear} across cycles; $p < 0.006$. Bracketed letters [A], [B], and [C] indicate which levels are significantly different. Those not sharing the same letter are significantly different from one another.

	E_{toe} (MPa)	E_{linear} (MPa)	λ_{corner}	σ_{peak} (MPa)
Second cycle (2)	0.069 ± 0.025 [A]	1.72 ± 0.96 [A]	1.37 ± 0.04 [A]	0.21 ± 0.07 [A]
Sixth cycle (6)	0.037 ± 0.022 [B]	1.68 ± 0.92 [A]	1.38 ± 0.04 [A]	0.17 ± 0.06 [B]
Fatigued (∞)	0.041 ± 0.011 [B]	1.67 ± 0.91 [A]	1.42 ± 0.03 [B]	0.14 ± 0.05 [C]

residual magnitudes were not continuously above 0.05 MPa. Finally, the modulus of the toe region, E_{toe} , was defined as the slope of the stress–stretch relationship in the toe region. A similar two-step process was used to find the linear region for a given cycle, except this time a linear regression was taken between 75% of the average peak stress of the last ten cycles and the peak stress of the cycle being analyzed. After evaluating residual magnitudes over the entire loading portion of the cycle, the slope of a second linear regression of linear region stress–stretch was defined as the linear region modulus, E_{linear} . The corner stretch, λ_{corner} , was defined as the stretch at which the toe region line of best fit and the linear region line of best fit were equal. Peak stresses (σ_{peak}) were also extracted for each cycle. Hysteresis was defined by the area encompassed by each cycle's loading and unloading curves. Because in some experiments (four out of eight) we observed yieldlike behavior during the first loading cycle occurring before reaching a stretch ratio of 1.5, we report E_{toe} , E_{linear} , λ_{corner} , and σ_{peak} for the second cycle through the last cycle (88 or 89).

A series of one-way analyses of variance (ANOVAs) were performed to examine differences between the second cycle ($E_{\text{toe},2}$, $E_{\text{linear},2}$, $\lambda_{\text{corner},2}$, and $\sigma_{\text{peak},2}$), sixth cycle ($E_{\text{toe},6}$, $E_{\text{linear},6}$, $\lambda_{\text{corner},6}$, and $\sigma_{\text{peak},6}$; after preconditioning), and corresponding fatigued parameters, defined as the average value of the last ten cycles ($E_{\text{toe},\infty}$, $E_{\text{linear},\infty}$, $\lambda_{\text{corner},\infty}$, and $\sigma_{\text{peak},\infty}$). For all tests, significance was defined as $p \leq 0.05$. If significance was achieved, post-hoc Tukey–Kramer analysis was performed to determine groupwise differences.

3 Results

Peak stretch magnitude and cycle frequency were maintained for the duration of the cyclic tests. With successive cycles, peak stress and hysteresis decreased and corner stretch increased, moving rightward (Fig. 2). Typically, when testing the mechanical properties of soft tissues, a few (~ 5) preconditioning cycles are performed at experimental or subexperimental load levels to obtain repeatable results. We observe continued changes in peak stress and corner stretch beyond the typical preconditioning realm.

We find that $E_{\text{toe},2}$ is significantly higher than $E_{\text{toe},6}$ and $E_{\text{toe},\infty}$, while $\sigma_{\text{peak},2}$ is significantly higher than $\sigma_{\text{peak},6}$, which in turn is significantly higher $\sigma_{\text{peak},\infty}$. In addition, $\lambda_{\text{corner},2}$ and $\lambda_{\text{corner},6}$ are significantly less than $\lambda_{\text{corner},\infty}$. On the other hand, no difference was observed between $E_{\text{linear},2}$, $E_{\text{linear},6}$, and $E_{\text{linear},\infty}$ (Table 1, means and standard deviations). Taken together, these data support evidence of classic fatigue behavior in the stress–stretch curve, with softening sustained past the traditional preconditioning regime.

4 Discussion

We investigated the longitudinal behavior of CCAs from “toddlerlike” 4-week-old pigs [43] under cyclic extension. Previous studies have reported axial displacement of the CCA wall in vivo during the normal cardiac cycle [44–53], with longitudinal strains between 1% and 8% (stretch ratios of 1.01–1.08) in the human CCA [46,47] and between 1% and 6% (stretch ratios of 1.01–1.06) in the adult pig [45]. Strain rate associated with this axial motion was only reported in human CCAs, on the order of $0.2\text{--}0.4\text{ s}^{-1}$ [46]. Furthermore, the CCA is under axial tension in vivo, and one study found axial prestretches of approximately 1.347 in adult porcine CCAs [54]. This prestretch (defined as the elongation from the traction-free length to in situ length) has also been shown to increase with age during postnatal development in dogs [55]. Finally, in a study of ferret CCAs, the longitudinal ultimate stretch ratio as calculated from the in vitro relaxed length varied between 2.53 for low rate ($0.2\text{--}2\text{ s}^{-1}$) and 2.60 for high rate ($\sim 200\text{ s}^{-1}$) tests [18]. Thus, our prescribed maximum stretch ratio (1.5, relative to the traction-free state) is slightly larger than in vivo pulsatile extension, and our stretch rate (3 s^{-1}) is about 1 order of magnitude higher than pulsatile rates, anchoring our

experiments as a nondestructive traumatic event rather than a typical physiological condition.

Qualitatively similar to previous investigations [18,19,24,27], we observed a biphasic response, with a low modulus in the toe region at small stretches where crinkled collagen fibers straighten and elastin fibers elongate from the traction-free state. At higher levels of stretch, the stiff collagen fibers become taut and dominate the mechanical response of the artery yielding much stiffer behavior. With continuous cycling, the biphasic behavior persisted, with a significant decrease in toe region modulus and peak stress, and an increase in corner stretch. The limited published data regarding the longitudinal properties of the porcine CCA under cyclic loading [25–27] show that the majority of stress softening occurs in the initial loading cycle [25,27], similar to our observations regarding first cycle hysteresis (Fig. 2). The same phenomenon was reported in human aorta [56]. We speculate that our observations of large amounts of softening occurring in the initial cycle and softer response in the low-stretch toe region with successive cycling may be attributed to irreversible (plastic) changes to matrix elements as collagen fibers are pulled apart from one another transverse to their axes, or due to reorientation or damage to collagen fibers. Indeed, several studies of ligament and tendon preconditioning have documented changes in collagen fiber alignment toward the direction of loading with successive cycling [57,58]. However, the levels of stretch examined in this study were not high enough to show any appreciable change in the high-stretch linear region response with increasing numbers of loading cycles. Future studies should investigate the role of fiber reorientation in cyclic deformation of CCAs and higher levels of peak stretch. To determine whether the observed softening may be attributed to transient changes associated with viscoelasticity rather than permanent alterations due to tissue fatigue, periods of relaxation may be prescribed between multiple loading protocols for comparison across the same sample. Finally, future studies may use histological sections to investigate if the prolonged softening, similar to the large amount of softening in the initial cycle, is due to structural alterations in the vessel wall: either to the matrix as collagen fibers are pulled apart or to collagen fibers themselves by comparing unloaded samples, samples undergoing a single cycle, and those undergoing many cycles.

Preconditioning is a procedure often used in mechanical testing of soft tissues in which a specified loading and unloading protocol is recommended to stabilize the mechanical response of the tissue. However, preconditioning is not a standardized procedure and the number of cycles, peak loading parameter, and the inclusion and length of viscoelastic recovery rest periods between cycles vary widely between studies [59]. Furthermore, it is generally accepted that a preconditioning loading protocol should be conducted under conditions as similar as possible to the test itself [59–65]. However, in the case of large deformation or failure testing, preconditioning tissue to test levels may damage the tissue prior to testing [66]. No preconditioning was performed on the samples tested in this study, as the aim of the experiments presented was to investigate the effect of prolonged cycling on tissue response. Nonetheless, future studies should determine the influence of a preconditioning protocol at subdamage loading levels, perhaps to in situ longitudinal stretches, on CCA behavior under cyclic loading.

5 Limitations

There are several limitations to our findings. First, vessels were moistened and tested at room temperature, as in other studies [18,21,25,26]. Future studies may evaluate properties at body temperature. Second, we measured needle-to-needle displacement to capture average vessel response. Investigators interested in edge effects near the grips [67] may use speckle patterning to capture local variations in vessel stretch. Third, we stretched our CCA specimens from their traction-free state. To capture in vivo conditions, future studies should characterize hysteresis and fatigue in

vessels stretched from their in situ length. Finally, we used a displacement-controlled study design and observed softening beyond the sixth cycle, as evidenced by the increasing corner stretch point (where stress begins to increase dramatically with each cycle) and decreasing peak stress. Future stress or force-controlled studies should be conducted to confirm fatigue by an increase in creep response beyond the first few cycles.

6 Conclusions

To the best of our knowledge, this study is the first to investigate cyclic loading of the CCA beyond five cycles [27]. We find that under subcatastrophic cyclic longitudinal extension, the immature porcine CCA displays softening behavior. This softening can be represented by decreasing toe region modulus and peak stress as well as increasing corner stretch with an increasing number of loading cycles. This investigation is an important first step in modeling fatiguelike behavior in arterial tissue that may be subjected to repeated longitudinal loads.

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