Comparison Between the Hyperelastic Behavior of Fresh and Frozen Equine Articular Cartilage in Various Joints

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Fresh and frozen cartilage samples of the fetlock, carpus, and stifle were collected from 12 deceased horses. Half were measured immediately following extraction, and half were frozen for seven days and then measured. Seven indentations (various normalized displacements) were implemented with an indentation rate of 0.1 mm/s. Solid phase aggregate modulus (E_s), hyperelastic material constant (α), and fluid load fraction (F') of equine articular cartilage were assessed using the Ogden hyperelastic model. The properties were statistically compared in various joints (fetlock, carpus, and stifle), and between fresh and frozen samples using various statistical models. There was no statistical difference between the fetlock and carpus in the aggregate modulus ($p=0.5084$), while both were significantly different from the stifle (fetlock: $p=0.0017$ and carpus: $p=0.0406$). For the hyperelastic material constant, no statistical differences between joints were observed ($p=0.3310$). For the fluid load fraction, the fetlock and stifle comparison showed a difference ($p=0.0333$), while the carpus was not different from the fetlock ($p=0.1563$) or stifle ($p=0.3862$). Comparison between the fresh and frozen articular cartilage demonstrated no significant difference among the joints in the three material properties: $p=0.9418$, $p=0.7031$, and $p=0.9313$ for the aggregate modulus, the hyperelastic material constant, and the fluid load fraction, respectively.

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1 Introduction

A considerable amount of research on articular cartilage has been conducted over the past several decades to better understand and describe the characteristics of this biomaterial. Numerous works have been executed to determine the elastic characteristics of articular cartilage by quantifying the modulus or stiffness using various indentation techniques [1–5]. A few studies have investigated the stiffness measurements of different articular cartilage surfaces from different joints [6]. Many works focus only on the stifle joint because it is the foremost comparable joint to the human knee, and the joint most prone to osteoarthritis [7,8]. Mow et al. implemented a creep test on three cartilage surfaces of bovine stifle joints (equivalent to the human knee joint) to determine the aggregate modulus, Poisson's ratio, and permeability of bovine cartilage [9]. Nonetheless, a prevalent finding is that articular cartilage in different joints with various loadings or motions showed different properties [7,9]. This could suggest that the material properties of cartilage should be tailored to the specified conditions to design more realistically functioning biomimetic artificial cartilage joints. To verify the hypothesis that all comprehensive material properties reflecting either solid or fluid phase (or both, biphasic) elements vary depending on the joint, experiments were executed on equine articular cartilage surfaces in various joints in this work.

The other primary interest of this study was to investigate differences in mechanical behavior between fresh and frozen articular cartilage. Since it is often difficult to harvest fresh articular cartilage samples, frozen samples have been used in many works [2,10–14]. However, there have been various opinions on freeze-thaw effects causing significant differences between fresh and frozen samples [15–18]. Thus, the mechanical properties of fresh and frozen articular cartilage in various joints were compared under the hypothesis that articular cartilage material behavior changes even after a single frozen-thaw cycle. The equine model was selected since it is considered to most closely resemble human cartilage with various advantages: similar thickness of cartilage and subchondral bone, similar orthopedic diseases, convenience on handling calcified cartilage layer, and convenience on medical monitoring and treatments [8,19].

2 Materials and Methods

The equine articular cartilage, essential anatomy, and collection methods used for this research has been previously described

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[8,20,21]. Three cartilage surfaces (fetlock, carpus, and stifle) were studied here. The joints were harvested from 12 humanely euthanized horses for reasons unassociated with this study. The horses had no history or medical examination of joint surgery or lameness prior to collection. Their signalment information (breed, age, sex, and weight) can be found in our previous publication [8,20,21]. The cartilage samples from the horses were harvested for two simultaneous studies: signalment analysis (previous work [8,20,21], only fresh samples were used) and fresh and frozen sample comparison (this work). Fresh cartilage surfaces were harvested from 11 fetlocks, 10 carpi, and 10 stifles. Frozen cartilage surfaces were harvested from 12 fetlocks, 12 carpi, and 12 stifles. All cartilage surfaces were from the medial side. Each individual cartilage surface is independent from one another. Some fresh samples were excluded after they were accidentally damaged during sample preparation processes. Either the right or left set of joints were randomly selected and measured instantly after the dissection; the other set was frozen in saline at a temperature of approximately -20°C for an average of 7.3 days, and used for measurements after thawing for approximately 8 h at room temperature (20°C). Except for this, the fresh and frozen samples were handled using the same experimental protocol.

Indentation tests were performed to acquire hyperelastic material properties using a 10 mm spherical indenter. The measurements included various indentation (depth: 10–50% of the average thickness of cartilage in each joint (fetlock: 0.8606 mm, carpus: 0.8685 mm, stifle: 2.1466 mm) [21]. The details of the experimental methodologies can be found in previous research (Sec. 3.2 of Ref. [20]). This study was designed and implemented based on a popular theory to explain the fundamental material behavior of cartilage: the biphasic theory established by Mow and his coworkers [3,4,9].

The data analysis methodology used in previous research was also employed for the analysis of this study (see Sec. 4 of Ref. [20] for details). The Ogden hyperelastic model was chosen to fit the measurement data based on a biphasic model, as it showed the best fit [22]. As the Ogden hyperelastic model was fitted to the measurement data, three material properties of the articular cartilage were determined: aggregate modulus of the solid phase which is considered to be the stiffness of the cartilage matrix (E_s), hyperelastic material constant (α), and the fluid load fraction (F^f). The mixed model analysis of variance (ANOVA) was used to

Fig. 1 Comparison between the fetlock, carpus, and stifle in the solid phase aggregate modulus. The stifle showed significant differences from the fetlock ($p=0.0017$) and carpus ($p=0.0406$), while the fetlock and carpus were not different from each other ($p=0.5084$).

Fig. 2 Comparison between the fetlock, carpus, and stifle in the hyperelastic material constant. No statistical difference between joints were observed ($p=0.3310$).

statistically analyze the material properties. Each material property was compared between the three joints using Scheffe's test. Paired sample t -tests were also performed to compare mechanical properties between fresh and frozen samples in each joint. Since there were two samples without its pair in each case of the joint, a total of ten fresh and frozen pairs in each case of the joint were used for the paired tests. As an indicator to detect statistical difference in each comparison, p -values were reported. Additionally, power analysis results (β values) were reported for cases with p -values lower than 0.2 to check the probability of type II error.

3 Results

The Ogden hyperelastic model was fitted to data points of the solid phase forces from all measurements on each sample to acquire the solid phase aggregate modulus (the fit had an average $R^2=0.9684$).

Fig. 3 Comparison between the fetlock, carpus, and stifle in the fluid load fraction. The fetlock and stifle showed a difference between them ($p=0.0333$), while the carpus was not different from either the fetlock ($p=0.1563$) or stifle ($p=0.3862$).

Fig. 4 Material properties comparison between the fresh and frozen articular cartilage samples from the (a) fetlock, (b) carpus, and (c) stifle in the solid phase aggregate modulus. No statistical difference between fresh and frozen samples was observed in all joints ($p = 0.9418$).

3.1 Comparison Between the Joints. The mechanical properties of the equine articular cartilage of three joints were statistically compared as shown in Figs. 1–3. The fresh samples from the joints were used for the comparison. For the solid-phase aggregate modulus, there was no observed statistical difference on the moduli between the fetlock and carpus ($p = 0.5084$), as shown in Fig. 1. However, the stifle showed highly significant difference from the fetlock ($p = 0.0017$) as well as carpus ($p = 0.0406$). It demonstrated that the carpus and stifle possess the stiffest and least stiff cartilage, respectively. In the hyperelastic material

Fig. 5 Material properties comparison between the fresh and frozen articular cartilage samples from the (a) fetlock, (b) carpus, and (c) stifle in the hyperelastic material constant. No statistical difference between fresh and frozen samples was observed in all joints ($p = 0.7031$).

constant, the three joints were not significantly different from each other ($p = 0.3310$) as shown in Fig. 2. As a result of the comparison in the fluid load fraction (Fig. 3), the carpus was not different from the fetlock ($p = 0.1563$, $\beta = 0.312$) and stifle ($p = 0.3862$). However, the fetlock and stifle were statistically different from each other ($p = 0.0333$).

3.2 Comparison Between Fresh and Frozen Samples. The characterized mechanical properties were compared between the fresh and frozen articular cartilage surfaces. The comparison of the solid phase aggregate modulus (E_s), hyperelastic material constant (α), and fluid load fraction (F') between fresh and frozen

Fig. 6 Material properties comparison between the fresh and frozen articular cartilage samples from the (a) fetlock, (b) carpus, and (c) stifle in the fluid load fraction. No statistical difference between fresh and frozen samples was observed in all joints ($p = 0.9313$).

samples in each joint are visualized in Figs. 4–6. Each fresh and frozen sample pair from each horse was plotted as a single dot pointing property values of both fresh and frozen samples. The black diagonal line in the graphs (merely $y = x$ plot) is a guideline; the closer a dot is to the guideline, the more identical the fresh and frozen samples the dot represents are. The number in the legend indicates the number of the horse (in Tables 1–3 in the Appendix) the sample was harvested from. The solid aggregate moduli of the fresh and frozen articular cartilage in every joint were not statistically different ($p = 0.9418$) as shown in Fig. 4. Paired sample t -tests displayed no statistical difference between

fresh and frozen samples in each joint (fetlock: $p = 0.3892$, carpus: $p = 0.6201$, stifle: $p = 0.5412$). The hyperelastic material constants of the fresh and frozen samples were also not significantly different ($p = 0.7031$) as shown in Fig. 5. Paired tests result indicated no difference from individual comparisons in different joints (fetlock: $p = 0.16/\beta = 0.311$, carpus: $p = 0.0621/\beta = 0.439$, stifle: $p = 0.3666$). A statistical difference in the fluid load fraction between the fresh and frozen samples in the three joints was not observed as well ($p = 0.9313$) as shown in Fig. 6. Individual paired test result supported the conclusion (fetlock: $p = 0.1752/\beta = 0.202$, carpus: $p = 0.6282$, stifle: $p = 0.9514$). From the Figs. 4–6, most data points positioned around the guideline for every material property and every joint, illustrating the fresh and frozen samples were more identical than different. In summary, any statistical difference between the fresh and frozen articular cartilage was not detected.

4 Discussion and Conclusions

It was assumed in the study that the curvature of the articular cartilage surface in indentation would minimally influence the characterization of the material properties according to an examination using Hertz contact [23,24]. However, the validity of this assumption that the cartilage is effectively flat was examined here. The following were the conditions used in the examination; the normal displacement is 0.4303 mm, the force is 10 N, and an elliptical contact occurs between a sphere (indenter) and a cylinder (cartilage). The effective modulus of the cartilage was acquired for each case with six different radii (10, 20, 30, 40, 50 mm, and ∞) of curvature of the cartilage that are considered the minor elliptical radius in the Hertz elliptical contact model. The effective moduli for the six cases were compared, and all differences between one another were less than 3.99%. Since the error is not significant, the curvature effect was not addressed in this work.

The closed form Ogden hyperelastic model clearly described the behavior of the solid phase of the articular cartilage (average $R^2 = 0.9684$), which illustrates that the articular cartilage matrix (solid phase) shows hyperelastic behavior. The hyperelasticity could be due to the substrate effect of the subchondral bone below articular cartilage. This is because the stress distribution and carried load in the cartilage layer vary with the variation of substrate (subchondral bone in this case) stiffness [22–26]. Other reports may not have observed this hyperelastic behavior because they considered much smaller strains than those in this work [10,27]. With large strain, the solid matrix of the cartilage may also become denser, hence effectively having a higher elastic stiffness. This would result in a hyperelastic behavior which was also previously reported. [28–31]. Note that the loads we used in the work aimed at being physiologically reasonable and therefore we believe this hyperelastic behavior would occur in the articular joints of a live animal.

The force-indentation depth curve at each normalized displacement follows on nearly the same line as shown in Fig. 6(a) from Lee et al.'s work during the indentation step of the measurement [20]. This accounts for the articular cartilage not failing even under displacements with 50% of the articular cartilage thickness. Therefore, the articular cartilage completely recovered after each loading and demonstrated elastic behavior. This showed the total duration for seven indentations with different normalized displacements was not significantly influential on any changes of material mechanics. It also suggested that the 300 s time gap between measurements at each normalized displacement was enough for full recovery of the cartilage after indentation.

The stifle exhibited significant differences from both the fetlock and carpus in the overall characterized material properties except for α . The stifle showed the lowest E_s (0.177 MPa) and F' (63.8%) values. This demonstrated that the stifle is the least stiff, and the contribution of fluid in the stifle joint to support external force is

the lowest. Unique material properties of the stifle cartilage can be explained by its greater thickness compared to other joints. In fact, the stifle cartilage is thicker (more than twice) than cartilages from the fetlock and carpus, as shown in Lee et al.'s work [21]. Highly significant differences in the thickness of the cartilage bring about different mechanical behavior [22,25,26]. The stifle is also generally considered the most complex and is the largest equine joint [32]. This can enable the stifle to bear an applied load through the cartilage with smaller solid phase moduli and fluid load fractions than the other joints. The difference between the stifle, and both the fetlock and carpus, could also result from different magnitudes of load carried by the forelimb and hindlimb. Clayton et al. measured the ground reaction force under different normalized loads of trotting horses [33]. Nearly 55% and 45% of the load were carried by the forelimb and hindlimb, respectively. The fetlock and carpus are from the forelimb, while the stifle is from the hindlimb, and therefore, the stifle carries and bears a lower load than both the fetlock and carpus. These differences could also be analyzed by that the joints with higher loads rely more on boundary and elastohydrodynamic lubrication, while surfaces under less sliding motion could depend more on a full layer of fluid separating the surfaces via the squeeze film effect [23,24,34,35].

It was hypothesized that the fresh and frozen samples would show different mechanical behaviors because the cellular components ratio and structure of biological tissues generally change during freezing and thawing [15,18,36,37]. However, a significant difference between fresh and frozen articular cartilage in various joints was not observed: the p -values for E_s , α , and F' were 0.9418, 0.7031, and 0.9313, respectively. It appears that the results are not in favor of the hypothesis of this work based on the literature, as different thawing rates were used in this work than in previous reports; the frozen samples thawed gradually at room temperature (20 °C) in this work, while the samples were thawed rapidly at a higher temperature (37 °C) in the previous reports [15,18]. Damage to biological tissues during rapid warming could be attributed to osmotic pressure that could arise during rapid rehydration of cells after dehydration during slow cooling [38]. This is why slow warming may be beneficial for slowly cooled biological tissues. Since the cartilage samples in this work frozen and thawed slowly, the damage to the tissues may have been minimized [36–38], leading to no difference between fresh and frozen samples. It could be also resulted by that collagen network or glycosaminoglycans were not significantly damaged during the freeze-thaw cycle [36–42]. It is possible that under longer and more severe freezing conditions a difference might be found. Since the hyperelastic constant was also the closest to being statistically different, it is also possible that with a larger data set the difference would become significant. However, since the trend between fresh and frozen samples was not consistent (some were greater while others were less than) between the different surfaces, we do not believe this to be the case. The accuracy of the experimental apparatus or design could be also considered as possible reason for no difference between fresh and frozen cartilage. Based on the results with the employed sample treatment methodologies, frozen samples are recommended as appropriate alternatives for fresh samples to study mechanical behaviors of the articular cartilage, but should still be treated with some care [39–42]. Investigation of the multiple freeze-thaw cycle effect on mechanical behaviors of the articular cartilage could also be proposed as a further study to provide more of a profound understanding of this freeze-thaw mechanism. This study provides comprehensible and feasible ways of material preparation and preservation that may lead to improved protocols for articular cartilage preparation and preservation for clinical and scientific uses.

Overall, this work delivered novel results of comparison between articular cartilages from various joints as well as between fresh and frozen cartilage in material properties employing the Ogden hyperelastic model. The fetlock and carpus were different from the stifle in E_s , while the fetlock and stifle were different

from each other in F' . All joints were not different from one another in α . Fresh and frozen articular cartilages showed no difference for each material property.

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Appendix

Raw data of the fresh and frozen cartilage samples used in this work are attached in the following. Each table presents acquired raw data with respect to each material property. Age information of all horses the joints were harvested from was also added for possible interest of readers in the relationship between age and each material property. The relationships between age and hyperelastic material properties were reported in our previous work [8,20,21].

Table 1 Solid phase aggregate modulus (E_s)

No.	Age (year)	Fetlock (MPa)		Carpus (MPa)		Stifle (MPa)	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
1	15	0.8153	0.9658	0.5473	2.4162	0.0400	0.0056
2	12	N/A	N/A	2.4530	1.1671	0.6823	0.1793
3	1	1.3819	1.2053	1.1459	0.1552	0.0736	0.1855
4	9	0.4511	0.4199	0.2678	0.1249	0.1429	0.8789
5	10	0.5592	N/A	N/A	0.4905	N/A	0.2329
6	23	0.0923	0.2527	0.2440	0.0159	N/A	0.0073
7	15	1.4172	0.0001	0.3699	1.6115	0.4098	0.4156
8	24	1.3000	0.5315	N/A	0.1211	0.0218	0.0920
9	11	0.4323	0.7270	3.6787	0.7171	0.1084	0.0979
10	3 months	1.2208	1.724	1.1007	0.5331	0.0234	0.2700
11	3 months	0.2313	0.5543	0.0121	0.0195	0.2478	0.0366
12	16	0.8513	0.0359	0.8210	1.6476	0.0069	0.2544

Table 2 Hyperelastic material constant (α)

No.	Age (year)	Fetlock		Carpus		Stifle	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
1	15	-57.08	-56.81	-58.83	-41.48	-65.74	-104
2	12	N/A	N/A	14.43	-43.88	-31.06	-38.95
3	1	-45.53	-46.66	1.653	-85.9	-64.97	-56.71
4	9	-82.69	-66.76	-60.11	-101.5	-59.13	-29.19
5	10	-63.33	N/A	N/A	-47.97	N/A	-36.19
6	23	-27.8	-78	-14.72	-101.2	N/A	-61.83
7	15	-41.23	-207.1	-71.34	-47.08	-42.87	-34.66
8	24	-32.47	-55.07	N/A	-101.8	-71.94	-43.6
9	11	-47.95	-71.55	-25.62	-60.78	-51.63	-52.18
10	3 months	-34.02	-17.9	-28.44	-61.82	-78.15	-43.08
11	3 months	-84.91	-65.18	-120.9	-116.8	-44.65	-70.13
12	16	-56.7	-116.6	-50.13	-46.43	-85.59	-43

Table 3 Fluid load fraction (F')

No.	Age (year)	Fetlock		Carpus		Stifle	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
1	15	0.6889	0.7301	0.6847	0.6667	0.5931	0.6379
2	12	N/A	N/A	0.6616	0.6816	0.5669	0.5635
3	1	0.8348	0.7966	0.8226	0.8029	0.7394	0.6422
4	9	0.7622	0.8017	0.7589	0.6471	0.6984	0.6289
5	10	0.6760	N/A	N/A	0.8248	N/A	0.6474
6	23	0.6944	0.7905	0.5533	0.5131	N/A	0.4104
7	15	0.7840	0.7162	0.5426	0.5486	0.6643	0.6460
8	24	0.7452	0.8036	N/A	0.6146	0.5775	0.3832
9	11	0.7212	0.7248	0.5896	0.7041	0.6685	0.6740
10	3 months	0.8524	0.8305	0.8428	0.7438	0.7609	0.8029
11	3 months	0.7738	0.8181	0.7926	0.8233	0.7772	0.7474
12	16	0.6791	0.7953	0.6156	0.6297	0.3352	0.6835

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