Acta of Bioengineering and Biomechanics
Vol. 20, No. 3, 2018

Original paper
DOI: 10.5277/ABB-01134-2018-03

Effect of preservation methods on tensile properties of human femur-ACL-tibial complex (FATC) – a cadaveric study on male subjects

MUTHU MARIESWARAN[1], NASIM MANSOORI[2], VIJAY KUMAR DIGGE[3], SAROJ KALER JHAJHRIA[4], CHITTARANJAN BEHERA[5], SANJEEV LALWANI[2, 5], DINESH KALYANASUNDARAM[1, 6]*

[1] Centre for Biomedical Engineering, Indian Institute of Technology (IIT) Delhi, New Delhi, India.
[2] Cadaver Training and Research Facility (CTRF), Jai Prakash Narayan Apex Trauma Center (JPNTC) – All India Institute of Medical Sciences (AIIMS), New Delhi, India.
[3] Department of Orthopaedics, AIIMS, New Delhi, India.
[4] Department of Anatomy, AIIMS, New Delhi, India.
[5] Department of Forensic Medicine, JPNTC, AIIMS, New Delhi, India.
[6] Department of Biomedical Engineering, AIIMS, New Delhi, India.

Purpose: Deep freezing and storing in formalin are some of the common techniques of human tissue preservation. However, the preservation modes affect the biomechanical properties of the tissues. In this work, the effects of the above-stated preservation techniques are compared with that of fresh cadaveric samples. *Methods*: FATC samples from male cadavers of age between 60 and 70 years were tested under tensile loading at a strain rate of 0.8 s^{-1}. Fourteen FATC samples from soft embalmed cadavers were preserved for 3 weeks by two methods: (a) 10% formalin and (b) deep freezing at –20 °C followed by thawing. Seven FATC samples from fresh cadavers were experimented as control samples. The results were evaluated by a two-stage statistical process of Kruskal–Wallis H test and Mann–Whitney *U*-test. *Results*: It was observed that the failure force of fresh cadavers was the highest while that of preserved samples were approximately half the value. Failure elongation of frozen samples exceeded fresh samples while formalin samples failed at lesser elongations. Higher incidence of tibial insertion point or mid-section failures were observed in fresh samples while the higher incidence of ruptures at femoral insertion point was observed in the two preservation methods. *Conclusion*: Tensile properties of fresh tissues vary significantly from that of formalin preserved or frozen preserved samples.

Key words: *tissue preservation, storage in formalin, deep freezing, FATC, anterior cruciate ligament, biomechanical study, tensile testing*

1. Introduction

Cadaveric studies are important to evaluate biomechanical properties of human tissues [16]. Due to unavailability of fresh cadaveric samples within a short period, preserved tissue samples are often used for biomechanical testing [20], [23]. The common modes of preservation of tissue samples are storage in formalin or deep freezing [4], [7]. However, the variations in mechanical properties between preserved and fresh samples are not reported for human soft tissues. Kennedy et al. characterized fresh human cadaveric knee ligaments, however comparison with other preservation modes were not reported [11]. One of the tissues that is of significant interest to biomechanics researchers is the Anterior Cruciate Ligament (ACL) in the knee that helps in the stability during various kinematics, such as flexion, rotation and adduction. ACL is more prone to injury than other ligaments of the knee [10]. Hence, the biomechanical properties of ACL are of significant interest to researchers to enhance the injury prevention. In the past, biomechanical testing of ligament from preserved cadaveric sam-

* Corresponding author: Dinesh Kalyanasundaram, Indian Instittue of Technology Delhi, 298, Block 2, IIT Delhi, Hauz Khas, 110016 New Delhi, India. Phone: 01126597344, e-mail: dineshk.iitdelhi@gmail.com, dineshk@cbme.iitd.ac.in

Received: April 25th, 2018
Accepted for publication: July 9th, 2018

ples were carried out to understand the failure mechanics of ligament [3], [11], [18], [19], [21]. The cadaveric samples were stored either in saline [11] or in formalin or deep-frozen between –15 °C and –30 °C [3], [20].

Due to the lack of literature on the effect of storage methods on human cadaveric ACL, studies on other human tissues as well as animal ACL tissues are briefly described here. Herzog [9] studied the effects of storage chemicals (cialit) of human tendon grafts (finger flexor and extensor tendons) while Vidiik et al. [28] studied the effects of storage chemicals (formalin) on rabbit ACL. According to both Herzog and Vidiik et al., cell nucleus is the first structure to undergo degeneration after death in formalin-fixed samples, followed by the interstitial tissues [9], [28]. These chemical-induced changes affect the biomechanical properties of the tissues. Viidik et al. studied various preservation methods such as storage in 0.9% saline at 20 °C for 5 hours, storage in 0.9% saline at 4 °C for 24 hours, storage in a deep freezer at –20 °C for one week followed by thawing at 37 °C in water, and storage in formalin for 6 days [28]. The results were compared with fresh samples (no preservation). The key observations were: (a) the failure force of samples stored in 0.9% saline at 4 °C matched to that of fresh samples; (b) the elongation at failure of samples deep frozen at –20 °C closely matched with that of fresh samples. In another work, Vidiik et al. studied the effect of time duration elapsed after death on the tensile properties of rabbit ACL [29]. The authors reported the tensile properties at 0, 2, 6, 24, 48 and 96 hours. No significant variations in the failure force, failure elongation, stiffness, failure energy and rupture site were observed, thereby concluding that the mechanical properties of ACL tissue are not affected by death for the duration studied. Noyes and Grood [20] evaluated the effect of preserving femur-ACL-tibia-complex (FATC) samples of rhesus monkey in freezer (–15 °C) for 4 weeks. The contralateral knee was experimented immediately (control). No change in the tensile properties between frozen and fresh samples was observed. Barad et al. studied the preservation of rhesus monkey ACL at 4 °C overnight and –80 °C for 3 to 5 weeks. There was no significant difference in tensile properties of the ligament. Dorlat et al. [6] studied the behavior of canine ACL stored at –18 °C from 5 to 60 days. The authors found a marginal increase in stiffness of preserved samples. However, the average failure force remained unaffected. Stańczyk and Telega [15] reviewed the effect of cryopreservation of biological tissues on the mechanical properties. The authors focused on mechanical characterizations like compression, torsion, indentation, pull out etc. The authors did not report effect on tensile properties. The process of dehydration occurring in cells was explained. Effect of freezing on structural proteins, like collagen fibres, was not discussed. Effect of preservation (formalin storage and freezing) methods on biomechanical properties of bones have been studied recently by various research groups [17], [31], [32]. For no specific reasons, similar studies on ligaments and tendons were not reported recently.

All of the above-mentioned literature on preservation methods of tissues are refuting each other's conclusion. Currently, no studies exist on the effect of sample preservation on tensile properties of human ACL. In this work, the effects of various preservation methods, such as 10% formalin and refrigeration at –20 °C of human FATC, on the biomechanical properties are evaluated by comparing with that of fresh samples. The results of the tensile tests were analyzed by two-stage statistical methods.

2. Materials and methods

Ethical clearance for this study was obtained from Institute ethical committee (reference numbers: IEC/NP/-332/07.08.2015 & IEC-637/03.11.2017, RP-29/2017). A brief outline of the steps followed in this study is outlined in Fig. S1 in supplementary material. A note on the soft embalming procedure of cadavers is also provided in supplementary material.

2.1. Acquisition of cadaveric samples

Samples were cut four inches above and below the knee joint. Patella, quadriceps muscle and patellar tendon were removed at the time of dissection. FATC

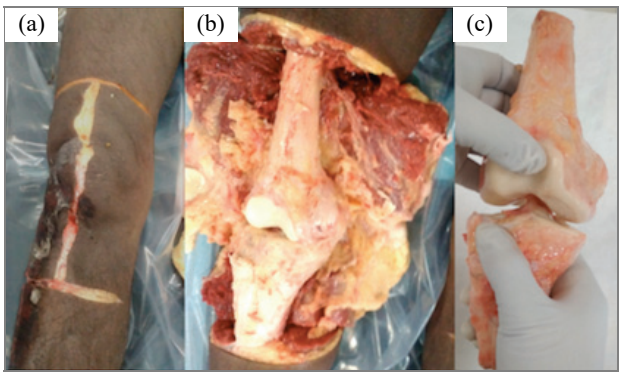

Fig. 1. (a) Marking in cadaver leg for dissection of knee, (b) knee joint, (c) dissected femur-ACL-tibia-complex (FATC)

samples of human male cadavers in the age 60 to 70 were dissected from four fresh cadavers (seven knee samples) as well as seven soft embalmed cadavers (fourteen knee samples). The control samples from fresh bodies were acquired during autopsy. Figure 1 briefs the stages of the dissection process.

2.2. Sample preservation methods

The fresh cadavers serve as control (Method A) while the two methods of preservations of storage in formalin and deep freezing are termed as Method B and C respectively. Of the fourteen samples harvested from soft embalmed cadavers, seven samples were preserved in 10% formalin at room temperature (~25 °C) for 3 weeks (Method B). Formalin is an aqueous solution of formaldehyde containing 37–40% formaldehyde and 60–63% water. The second set of seven FATC samples harvested from soft embalmed cadavers was pre-cooled at 4 °C (after dissection from the cadaver for 12 hours) before deep freezing at minus 20 °C for 3 weeks. Before the start of the experiment, the knee samples were defrosted at 4 °C for 12 hours, followed by thawing at room temperature for 12 hours. Medial collateral ligament (MCL), lateral collateral ligament (LCL) & posterior cruciate ligament (PCL) were cut leaving merely the ACL intact. The tissues around ACL (fat tissues, menisci etc.) were removed. The crosssectional area of the ligament was measured using digital vernier before testing.

2.3. Tensile testing of FATC

Conventional grippers were unable to grip FATC samples. A cylindrical gripper (as shown in Fig. 2c) was custom-designed and fabricated out of tool steel grade EN 31 to hold the samples at high strain rates in the UTM (Tinius Olsen model: H5K5, UK) fitted with a 5 kN load cell. Use of FATC over isolated ACL ensures (a) avoidance of slippage of samples at high strain rates; (b) avoidance of crushing of samples at the ends during gripping of isolated ACL preparations. A few research groups had removed small bone blocks and potted them into molds of resin [2], [14], [22], [27]. Maintaining the alignment of the tissue would be difficult in testing those potted samples. To obtain reliable and repeatable biomechanical properties of ACL, FATC was used for experimentation instead of isolated ACL. A cylindrical gripper that connects to the two bony segments was designed and fabricated. Further, the gripper was designed to reduce bending and axial stresses. Holes of 6 mm diameter were drilled in femur and tibia for clamping. The FATC sample was clamped using custom made circular grippers via hardened steel rods passing through the holes. The samples were prevented from any rotational movement or transverse movement. Figure 2c shows the fresh human FATC loaded in cylindrical grippers for tensile testing. The loading axis of the UTM is aligned with the longitudinal axis of the ACL tissue. The experiments were conducted at a strain rate of 0.8 s^{-1}, the highest permissible on the specific model of the UTM available. Human soft tissues are viscoelastic in nature, the differences if any between tissues, are amplified at high strain rates. Therefore, it shall help in evaluation of the effect of preservation methods. Hence, the strain rate of 0.8 s^{-1} closer to the highest reported strain rate of 1 s^{-1} [4], [18], [20] was used. Further, Bonner et al. have indicated that the frequency of occurrence of ACL injury is higher at high strain rates [2].

The current fundamental study is aimed at understanding the strength of the tissue along the longitudinal axis. The study does not relate directly to ACL injury or rupture conditions. Statistical analysis (Kruskal–Wallis *H*-test and Mann–Whitney *U*-test) of tensile properties was performed using IBM SPSS (IBM Analytics, Chicago, Illinois, USA).

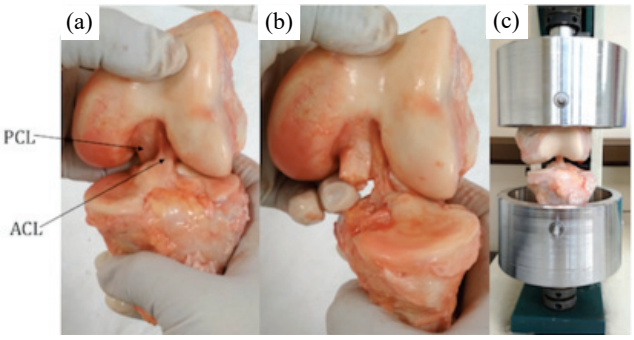

Fig. 2. (a) Human knee with ACL and PCL intact from fresh cadaver, (b) FATC sample, (c) FATC sample loaded in UTM using custom-made circular grippers for tensile testing

3. Results

A typical force-elongation graph for the tensile test of FATC samples with different preservation methods are shown in Fig. 3a. The difference in failure elongation between various samples are clearly evident in Fig. 3a. The numerical values measured from the experiments are provided in Tables 1 and 2. The mean, median, minimum, maximum, 25th and 75th percentile

values for failure force, failure elongation, stiffness, and failure energy are shown in Fig. 4 while the corresponding details for failure stress, failure strain, Young's modulus and volumetric strain energy are shown in Fig. 5. The frequency of occurrence of failure modes (or the point of failure) of the tensile tested samples for each of the preservation methods as well as the control method are shown in Fig. 7. The overall *p* values for the biomechanical were obtained using Kruskal–Wallis *H*-test are given in Table 3. The group-wise statistical analyses were performed for those cases in which the results of Kruskal–Wallis *H*-tests were significant (supplementary section; Tables S2 to S4). Failure force, failure elongation and failure strain of the tested samples were found to be statistically different for the combinations of methods mentioned in Tables S2 to S4. Other tensile properties were not observed to vary for the preservation modalities studied (Table 3). Failure modes observed during FACT tensile testing are presented in Fig. 6.

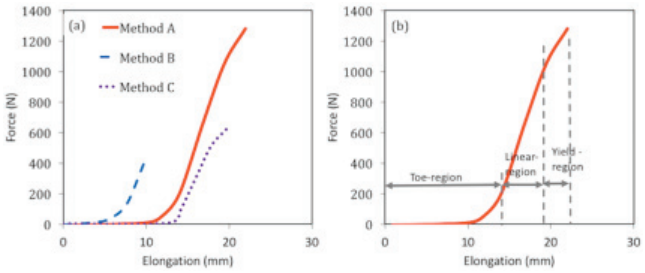

Fig. 3. (a) typical force-elongation graph for a ligament, (b) typical force – elongation graph for preservation methods followed in this study

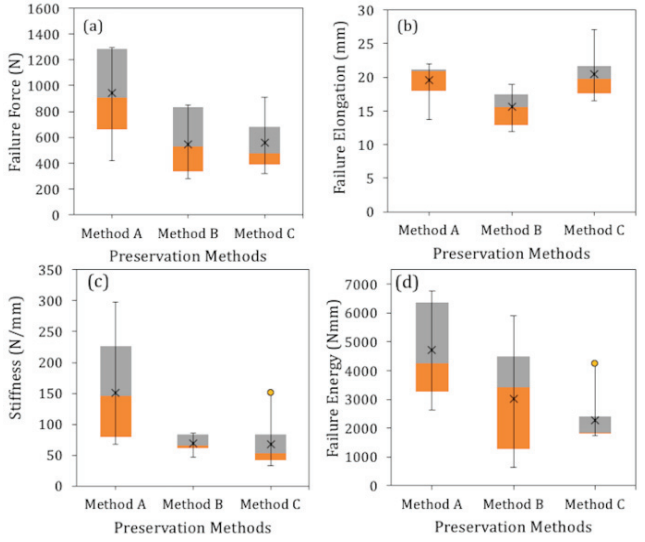

Fig. 4. Variation in structural properties of FATC for different methods of sample preservation followed: (a) failure force, (b) failure elongation, (c) stiffness, (d) failure energy. The boxes represent the values between 25th and 75th percentile. The horizontal line in the middle of each box is median of the corresponding data set. The vertical lines in each box extend from minimum value to maximum value for each data set. X represents mean

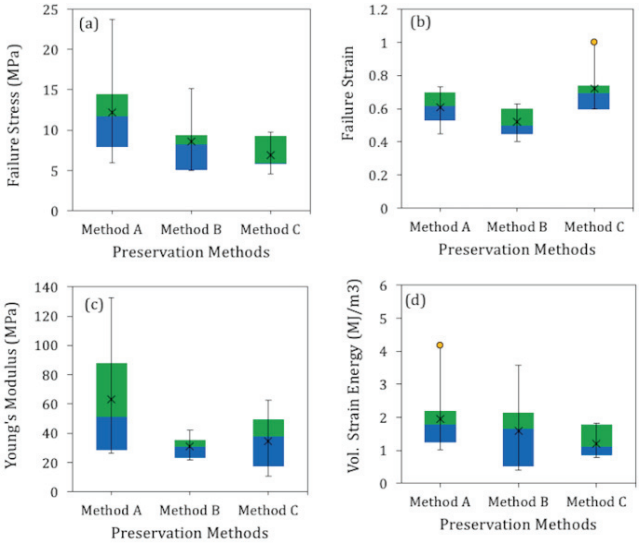

Fig. 5. Variation in structural properties of FATC for different methods of sample preservation followed: (a) failure stress, (b) failure strain, (c) Young's modulus, (d) Volumetric Strain Energy. The boxes represent the values between 25th and 75th percentile. The horizontal line in the middle of each box is median of the corresponding data set. The vertical lines in each box extend from minimum value to maximum value for each data set. X represents mean

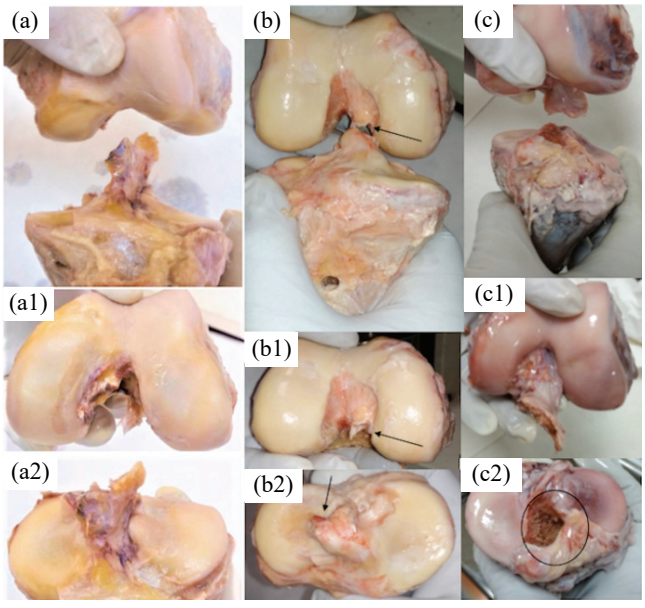

Fig. 6. Failure modes observed during FATC tensile testing: (a) femoral insertion failure, (a1) shows the footprint of ACL in femoral insertion region, (a2) view at the tibial region; (b) midsection failure, (b1) view in the femoral region, (b2) view in the tibial region; (c) tibial insertion failure, (c2) footprint of ACL in tibial insertion region

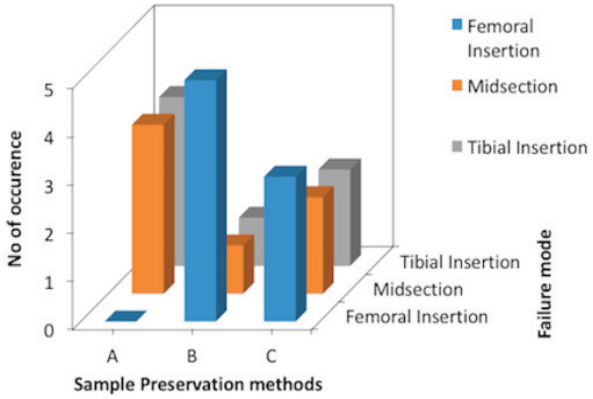

Fig. 7. Failure modes observed during FATC tensile testing: (a) femoral insertion failure, (a1) shows the footprint of ACL in femoral insertion region, (a2) view at the tibial region; (b) midsection failure, (b1) view in the femoral region, (b2) view in the tibial region; (c) tibial insertion failure, (c2) footprint of ACL in tibial insertion region

Table 1. Structural properties of FATC specimens used in the study

Preservation method	Failure force [N]	Failure elongation [mm]	Stiffness [N·mm^{-1}]	Failure energy [N·mm]	Failure mode
Method A – fresh samples	1283.73	21.98	156.68	6770.23	tibial insertion
	1215.73	21.00	146.00	5519.56	tibial Insertion
	910.54	18.14	297.87	3270.27	tibial Insertion
	1293.82	19.77	225.82	6372.44	mid-section
	669.39	13.74	78.88	2617.96	tibial and mid-section
	418.35	21.16	68.14	4248.54	mid-section
	820.59	21.19	83.99	4245.71	mid-section
Method B (10% formalin)	850.09	17.33	85.33	4476.37	femoral insertion
	344.64	14.87	47.44	1309.99	femoral insertion
	281.29	11.96	79.05	639.54	tibial insertion
	536.97	17.46	65.22	3414.72	femoral insertion
	581.58	12.97	61.25	4124.56	femoral insertion
	400.27	15.70	83.55	1279.69	femoral insertion
	830.98	18.96	65.05	5901.53	mid-section
Method C (deep freezing)	477.02	27.14	33.127	1819.17	femoral insertion
	481.43	16.58	44.41	1820.77	femoral insertion
	678.00	17.69	150.15	1748.25	tibial insertion
	633.86	19.84	83.54	2398.24	femoral insertion
	909.46	18.69	101.90	5035.57	tibial and mid-section
	318.90	21.63	41.48	1925.94	tibial and mid-section
	395.39	19.46	53.73	1856.75	mid-section

Table 2. Material properties of FATC specimens used in the study

Preservation method	Failure stress [MPa]	Failure strain [–]	Young's modulus [MPa]	Volumetric strain energy [MJ·m^{-3}]
1	**2**	**3**	**4**	**5**
Method A – fresh samples	23.77	0.73	88.12	4.17
	14.47	0.70	51.11	2.19
	11.82	0.53	132.37	1.24
	13.19	0.56	79.45	1.85
	7.96	0.45	28.36	1.03
	5.97	0.62	37.62	1.78
	8.20	0.66	26.45	1.32

1	2	3	4	5
Method B (10 % formalin)	9.40	0.57	28.39	1.65
	8.30	0.50	35.70	1.03
	5.10	0.40	42.43	0.39
	8.60	0.60	21.80	1.88
	7.26	0.43	28.2	0.88
	5.00	0.50	30.54	0.51
	15.10	0.63	35.37	3.57
Method C (deep freezing)	6.00	1.00	10.44	1.82
	5.90	0.60	49.68	0.77
	9.70	0.60	62.39	0.86
	9.30	0.70	40.42	1.21
	12.99	0.6	44.21	2.32
	4.55	0.74	17.22	0.94
	6.58	0.69	24.37	1.1

Table 3. Overall *p*-value by Kruskal–Wallis *H*-test for tensile properties for five preservation methods

Tensile properties	Preservation methods						Kruskal–Wallis test	
	Method A		Method B		Method C		ψ^2	p
	Mean ± SEM	Median (Min–max)	Mean ± SEM	Median (Min–max)	Mean ± SEM	Median (Min–max)		
Failure force [N]	944.48 ± 127.17	910.54 (418.35–1293.82)	546.54 ± 85.47	536.97 (281.29–850.09	556.29 ± 75.54	481.42 (318.90–677.99)	6.078	0.048
Failure elongation [mm]	18.18 ± 1.40	19.77 (13.74–21.19)	15.61 ± 0.95	15.70 (11.96–18.96)	20.14 ± 1.23	19.46 (16.57–27.14)	6.063	0.048
Stiffness [N/mm]	166.45 ± 36.03	146.00 (68.14–297.87)	71.68 ± 5.05	76.15 (47.44–85.33)	72.61 ± 14.90	53.73 (33.12–150.15)	5.818	0.055
Failure Energy [N·mm]	4402.35 ± 479.65	4248.54 (2617.96–5519.56)	2736.04 ± 730.72	2130.42 (1279.69–5901.53)	2372.09 ± 422.25	1856.75 (1748.25–5035.57)	5.766	0.056
Failure stress [MPa]	12.19 ± 2.25	11.82 (5.97–23.77)	8.39 ± 1.28	8.30 (5.00–15.10)	7.86 ± 1.03	6.58 (4.55–12.99)	2.545	0.280
Failure strain	0.56 ± 0.04	0.56 (0.41–0.70)	0.51 ± 0.03	0.50 (0.40–0.63)	0.70 ± 0.05	0.69 (0.60–1.00)	7.160	0.03
Young's modulus [MPa]	71.50 ± 18.67	51.11 (26.45–145.19)	31.77 ± 2.52	30.54 (21.80–42.43)	35.53 ± 6.63	40.42 (10.44–62.39)	3.325	0.190
Volumetric strain energy [MJ·m^{-3}]	1.74 ± 0.23	1.78 (1.03–2.8)	1.41 ± 0.41	1.04 (0.39–3.57)	1.29 ± 0.20	1.10 (0.77–2.32)	2.456	0.290

4. Discussion

The force-elongation graph of ACL obtained under tensile loading shows a triphasic graph, consisting of (i) non-linear elastic toe region, (ii) linear elastic region and (iii) non-linear yield region as shown in Fig. 3 (b). The collagen fibrils at first are folded in a sinusoidal pattern (referred as crimp), that straightens out at low stresses, marking the toe region [8], [16]. The linear region is characterized with proportional force to elastic deformation. The start of non-elastic permanent deformation is marked by the yield region [25]. The three-standard failure sites in the tensile testing are femoral insertion point, tibial insertion point and mid-section of the ligament (Fig. 6). Insertion point failure includes avulsion type failures wherein the soft tissue pulls out from the point of attachment on the bone along with bone fragments [30]. Fresh samples fail either at tibial insertion point or at mid-section. Failure at femoral insertion site was not observed in fresh samples. In all other preservation modes, femoral insertion point failures were observed in higher numbers than other failure points. From this study, it is observed that preservation methods (followed in the study) alter the bone-ligament interface making femoral insertion region weaker than tibial insertion region and mid-section.

4.1. Effect of formalin preservation of samples on tensile properties

The chemistry behind the diffusion of formalin into tissue and its reaction with protein molecule is provided in the supplementary section. In the current study, formalin-preserved samples (method B) fail at lower elongation values than fresh (method A) and frozen ones (method C). Also, the toe-region of formalin preserved samples were smaller, compared to fresh and frozen samples. The failure force was observed to be higher in fresh samples than all the preserved samples by at least twice the value. The elongation of formalin-treated tissues decreased in comparison to fresh samples. As preservation was performed for an average duration of three weeks, it was expected that high number of covalent bonds be formed (i.e., stable irreversible cross-linking) in both inter and intra-fibrillar locations, due to the action of formalin on primary amines of collagen [26].

The increase in the number of covalent bonds was expected to result in higher failure force. However, the inverse was observed, suggesting the presence of competing mechanisms, such as weakening of other bonds that occurs during formalin preservation. There is no literature available on weakening of bonds in the presence of formalin as well as on the location of formalin-induced cross-links in the 3D structure of collagen molecule and fibrils. Detailed investigations into the above-mentioned phenomena are out of the current scope of the proposed study and shall be taken up in the future. Furthermore, the chemistry behind the formalin reaction with proteoglycan matrix is also not reported in the literature.

4.2. Effect of freezing samples on tensile properties

Water crystallization and thawing plays a critical role in deep frozen samples [28]. In comparison to other preservation methods, frozen based preservation is able to reproduce similar or equivalent elongation to that of fresh samples. Furthermore, the failure force is relatively higher than formalin-based preservation method, though lower than the fresh sample. Freezing of samples results in dehydration of tissue, leading to partial or total destruction [5]. Collagen fibres and ground substance are the largest contributors to the physical behavior of ligament [1]. Wet weight of ligament contains 60–80% of water, a major constituent of both collagen fibres and ground substance. Ground substance provides spacing and lubrication that helps in sliding of fibres. Ground substance is also attributed to the viscoelastic behavior of ligament. The properties of the collagen fibres and ground substance are severely affected by dehydration. Hence, samples preserved in deep freezer display low failure force in our experiments.

Summing up, both preservation techniques of storage in formalin and freezing has affected the physical structure of the tissue that, in turn, is well reflected in the biomechanical properties. This is the first study to compare the effect of preservation methods to fresh cadaveric ACL/ FATC samples during tensile testing. Fresh samples display higher tensile properties than preserved samples in our experiments. Formalin preservation is known to cross-link the protein further. But the interaction of formalin with proteoglycan matrix is not known. Freezing of samples results in dehydration of contents of tissue, leading to partial or total destruction.

4.3. Limitations of the study

In the current study, the strength along the longitudinal axis was examined, as a first level characterization. In real life situations, human ACL fails in the combined loading for majority of the times, such as flexed knee with inward rotation and load acting in the anterior-posterior direction [12], [13], [24]. In future, studies to exactly replicate ACL failures scenario shall be conducted.

5. Conclusions

This work reports and analyzes the effect of preservation methods of human cadaveric FATC on its biomechanical properties via tensile testing. Preservation in 10% formalin and deep freezing at –20 °C were the methods used for storing samples in this study. The samples were obtained from soft embalmed and fresh cadavers (control). Failure force, failure elongation and failure strain have been observed to significantly vary between the preservation methods studied. Higher elongation values were observed for fresh and deep frozen samples. Failure force of fresh samples was the highest. Failure stress, Young's modulus and volumetric strain energy were not observed to vary significantly across preservation methods used in this study. Failure at femoral insertion sites was found to be high in preserved samples while in the case of fresh

samples no failure at femoral insertion site was observed.

Conflicts of interest

The authors do not have any conflicts of interest.

Ethical clearance

Ethical clearance for this study was obtained from Institute ethical committee (reference number: IEC/NP/-332/07.08.2015 and IEC-637/03.11.2017, RP-29/2017).

Funding information

The authors would like to acknowledge the funding agencies for their continued support: Department of Science and Technology (YSS/2014/000880, and IDP/MED/05/2014), Indo-German Science and Technology Centre (IGSTC/Call 2014/Sound4All/24/2015-16), Naval research board (NRB/4003/PG/359), BIRAC, Department of Biotechnology (BIRAC/BT/AIR0275/PACE-12/17). The funding agencies have no role neither in the design of the study nor in the results of the work. No funding has been received for writing assistance. The equipments purchased from the above funds were used partly for the experiments.

Acknowledgements

The authors would like to thank the souls for donating their bodies for research. The authors also thank R.M. Pandey for assisting in the statistical evaluation of the data.

Authors contributions

MM, SKJ and DK designed the study. SL and SKJ acquired donated bodies and soft embalmed them. NM and VKD dissected the cadavers, acquired the FATC samples and disposed of the samples after the study. CB acquired fresh FATC samples during postmortem. MM preserved the samples, performed the tensile experiments and statistical analysis. MM drafted the first version of the manuscript. DK, SKJ, VKD and MM edited the various versions of the manuscript.

References

[1] AKESON W.H., WOO S.L.Y., AMIEL D., FRANK C.B., *The chemical basis of tissue repair*, [in:] Funk F.J., Hunter L.Y. (Eds.), *Rehabilitation of the injured knee*, St Louis, Mosby CV, 1984.

[2] BONNER T.J., NEWELL N., KARUNARATNE A., PULLEN A.D., AMIS A.A., BULL A. M.J., MASOUROS S.D., *Strain-rate sensitivity of the lateral collateral ligament of the knee*, J. Mech. Behav. Biomed. Mater., 2015, 41, 261–270.

[3] BUTLER D.L., NOYES F.R., GROOD E.S., *Ligamentous restraints to anterior drawer in the human knee: a biomechanical study*, J. Bone Jt. Surg., 1980, 62–A, 259–270.

[4] CHANDRASHEKAR N., MANSOURI H., SLAUTERBECK J., HASHEMI J., *Sex-based differences in the tensile properties of the human anterior cruciate ligament*, J. Biomech., 2006, 39, 2943–2950.

[5] CLAVERT P., KEMPF J.F., BONNOMET F., BOUTEMY P., MARCELIN L., KAHN J.L., *Effects of freezing/thawing on the biomechanical properties of human tendons*, Surg. Radiol. Anat., 2001, 23, 259–262.

[6] DORLOT J., SIDI A.B., TREMBLAY G., DROUIN G., *Load Elongation Behavior of the Canine Anterior Cruciate Ligament*, J. Biomech. Eng., 1980, 102, 190–193.

[7] FESSEL G., FREY K., SCHWEIZER A., CALCAGNI M., ULLRICH O., SNEDEKER J.G., *Suitability of Thiel embalmed tendons for biomechanical investigation*, Ann. Anat., 2011, 193, 237–241.

[8] FREEMAN J.W., WOODS M.D., LAURENCIN C.T., *Tissue engineering of the anterior cruciate ligament using a braid-twist scaffold design*, J. Biomech., 2007, 40, 2029–2036.

[9] HERZOG K.H., *Die Konservierung yon Sehnengewebe*, Langenbecks Arch. Klin. Chir., 1963, 513–523.

[10] JOHN R., DHILLON M.S., SYAM K., PRABHAKAR S., BEHERA P., SINGH H., *Epidemiological profile of sports-related knee injuries in northern India: An observational study at a tertiary care centre*, J. Clin. Orthop. Trauma., 2016, 7, 1–5.

[11] KENNEDY J.C., HAWKINS R.J., WILLIS R.B., DANYLCHUCK K.D., *Tension studies of human knee ligaments. Yield point, ultimate failure, and disruption of the cruciate and tibial collateral ligaments*, J. Bone Joint Surg. Am., 1976, 58, 350–355.

[12] KOBAYASHI H., KANAMURA T., KOSHIDA S., MIYASHITA K., OKADO T., SHIMIZU T., YOKOE K., *Mechanisms of the anterior cruciate ligament injury in sports activities: A twenty-year clinical research of 1,700 athletes*, J. Sport. Sci. Med., 2010, 9, 669–675.

[13] LEVINE J.W., KIAPOUR A.M., QUATMAN C.E., WORDEMAN S.C., GOEL V.K., HEWETT T.E., DEMETROPOULOS C.K., *Clinically Relevant Injury Patterns After an Anterior Cruciate Ligament Injury Provide Insight Into Injury Mechanisms*, Am. J. Sports Med., 2013, 41, 385–395.

[14] LEVINE J.W., KIAPOUR A.M., QUATMAN C.E., WORDEMAN S.C., GOEL V.K., HEWETT T.E., DEMETROPOULOS C.K., *Clinically relevant injury patterns after an anterior cruciate ligament injury provide insight into injury mechanisms*, Am. J. Sports Med., 2014, 41, 385–395.

[15] STAŃCZYK J.J.T.M., *Thermal problems in biomechanics – a review*. Part III. *Cryosurgery, cryopreservation*, Acta Bioeng. Biomech., 2003, 5, 3–22.

[16] MARIESWARAN M., JAIN I., GARG B., SHARMA V., DINESH K., *A review on biomechanics of anterior cruciate ligament and materials for reconstruction*, Appl. Bionics Biomech., 2018, 1–14.

[17] MAZURKIEWICZ A., *The effect of trabecular bone storage method on its elastic properties*, Acta Bioeng. Biomech., 2018, 20, DOI: 10.5277/ABB-00967-2017-03.

[18] NOYES F.R., BUTLER D.L., GROOD E.S., ZERNICKE R.F., HEFZY M.S., *Biomechanical Analysis of Human Knee Ligament Grafts used in Knee Ligament Repairs and Reconstructions*, J. Bone Jt. Surg. Am., 1984, 66, 344–352.

[19] NOYES F.R., DELUCAS J.L., TORVIK P.J., *Biomechanics of anterior cruciate ligament failure: an analysis of strain-rate sensitivity and mechanisms of failure in primates*, J. Bone Joint Surg. Am., 1974, 56, 236–253.

[20] NOYES F.R., GROOD E.S., *The strength of the anterior cruciate ligament in humans and Rhesus monkeys*, J. Bone Joint Surg. Am., 1976, 58, 1074–1082.
[21] NOYES F.R., TORVIK P.J., HYDE W.B., DELUCAS J.L., FRANK N.R, PETER T.J, WALTER H.B, JAMES D.L., *Biomechanics of ligament failure.* II. *An analysis of immobilization, exercise, and reconditioning effects in primates*, J. Bone Joint Surg. Am., 1974, 56, 1406–1418.
[22] ÖHMAN C., BALEANI M., VICECONTI M., *Repeatability of experimental procedures to determine mechanical behaviour of ligaments*, Acta Bioeng. Biomech., 2009, 11, 19–23.
[23] PIOLETTI D.P., RAKOTOMANANA L.R., LEYVRAZ P.F., *Strain rate effect on the mechanical behavior of the anterior cruciate ligament-bone complex*, Med. Eng. Phys., 1999, 21, 95–100.
[24] SIEGEL L., VANDENAKKER-ALBANESE C., SIEGEL D., *Anterior Cruciate Ligament Injuries : Anatomy, Physiology, Biomechanics, and Management*, Clin. J. Sport. Med., 2012, 22, 349–355.
[25] SILVER F., *Biomaterials, Medical Devices and Tissue Engineering: An Integrated Approach*, Springer Netherlands, 2012.
[26] THAVARAJAH R., MUDIMBAIMANNAR V., RAO U., RANGANATHAN K., ELIZABETH J., *Chemical and physical basics of routine formaldehyde fixation*, J. Oral Maxillofac. Pathol., 2012, 16, 400.
[27] TRAJKOVSKI A., OMEROVIC S., KRASNA S., PREBIL I., *Loading rate effect on mechanical properties of cervical spine ligaments*, Acta Bioeng. Biomech., 2014, 16, 13–20.
[28] VIIDIK A., LEWIN T., *Changes in tensile strength characteristics and histology of rabbit ligaments induced by different modes of postmortal storage*, Acta Orthop. Scand., 1966, 37, 141–155.
[29] VIIDIK A., SANDQVIST L., MÄGI M., *Influence of Postmortal Storage on Tensile Strength Characteristics and Histology of Rabbit Ligaments*, Acta Orthop. Scand., 1965, 36, 3–38.
[30] WHITE E.A., PATEL D.B., MATCUK G.R., FORRESTER D.M., LUNDQUIST R.B., HATCH G.F.R., VANGSNESS C.T., GOTTSEGEN C.J., *Cruciate ligament avulsion fractures: Anatomy, biomechanics, injury patterns, and approach to management*, Emerg. Radiol., 2013, 20, 429–440.
[31] WIEDING J., MICK E., WREE A., BADER R., *Influence of three different preservative techniques on the mechanical properties of the ovine cortical bone*, Acta Bioeng. Biomech., 2015, 17, 137–146.
[32] ZHANG G., DENG X., GUAN F., BAI Z., CAO L., MAO H., *The effect of storage time in saline solution on the material properties of cortical bone tissue*, Clin. Biomech., 2018, DOI: 10.1016/j.clinbiomech.2018.06.003.

Supplementary information

S1. Protocol for sample extraction, preparation, and preservation

The protocol for sample extraction, preparation, preservation has been given in Fig. S1.

The anatomy act of 1959 (Indian government), allows usage of human dead body for teaching and research activities when (i) death happens in a state-run government hospital or in a public place within the prescribed zone of the hospital or (ii) police had clarified that the body is unclaimed (declared after 48 hours) (19). In addition, volunteers donate their bodies through voluntary body donation program. The medical institute has the rights to deny a body for use in education and research due to any of the following reasons (i) cause of death (suicide, homicide or due to

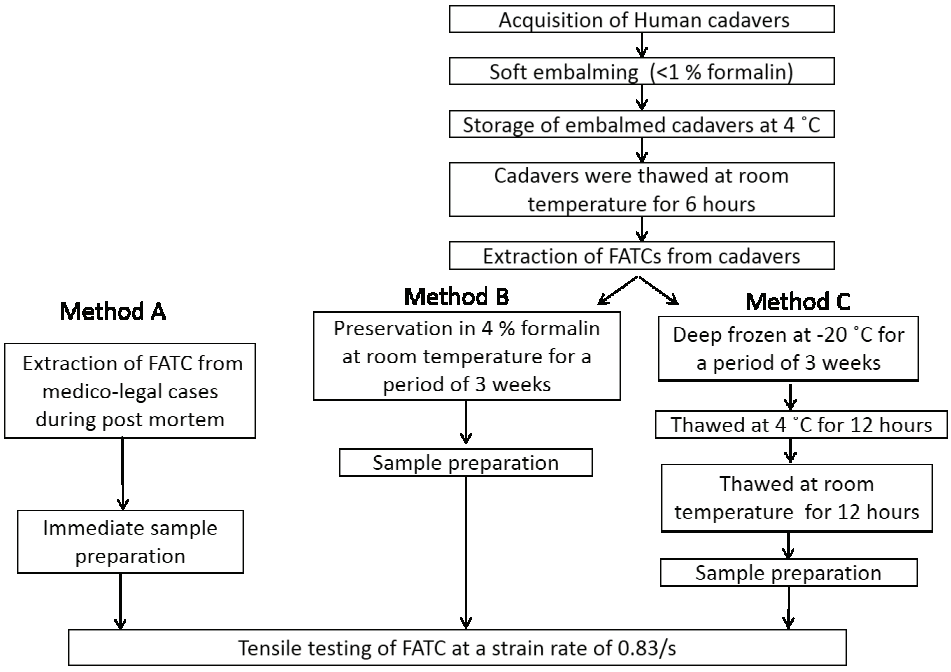

Fig. S1. Protocol followed for sample extraction, preparation, preservation and testing

contagious disease) (ii) autopsied bodies (iii) extremely thin or obese donors (iv) decomposed cadavers (v) removal of organs and tissues except for the eyes. The cadavers are tested for the risk of any contagious disease like Human Immunodeficiency Virus/AIDS, syphilis, Hepatitis B, C, active tuberculosis, spore-bearing organisms like Clostridium tetani, etc. The donors infected with any of the above diseases are rejected due to the risk involved in handling them [S1].

S2. Soft embalming of cadavers

Once donation is made, the medical institute either embalm the body immediately or preserve it in freezer. Embalmed bodies are preserved in formalin tank for long-term use of the bodies for teaching purposes. Soft embalmed bodies are preserved in freezer or formalin tank with modified solution.

Owing to the legal, ethical and cultural constraints, acquiring human samples immediately after death is impractical in the country of the study. The cadavers used in the experiments in the forthcoming chapters are soft embalmed. The embalming fluid (about 9 litres – composition of fluid is given in Table S1) is injected into the vascular system of the cadaver through femoral/carotid artery for 3 hours. Centrifugal pump is used for injecting embalming fluid. Blood is not drained out through vein. Blood is absorbed by the surrounding tissue when the artery is pumped with embalming fluid. Arterial embalming is the major embalming procedure followed. In autopsied bodies (medico-legal cases), cavity embalming is performed in addition to arterial embalming.

In this study, Thiel's solution was used for the soft embalming of 6 cadavers. Information on the composition of Thiel embalming solution is provided in Table S1 [S8, S14]. This embalming solution (solution A and solution B) was circulated in the deceased body through the femoral or coronal artery with the use of a pump. Embalmed cadavers were stored in the freezer at minus 40 °C [S16]. Perfusion of embalming fluid into the knee cavity and the cruciate ligaments was minimal as the synovial cavity is avascular. Formaldehyde has been in practice recently as it does not stain the tissues. However, it is a carcinogenic compound (under group 1, according to the international agency for research on cancer) and hence, merely 2 to 4% formaldehyde was used [S5, S6]. Thiel-based embalming methods make the tissue softer, whereas formalin based embalming method makes the tissue stiffer [S3].

Table S1. Composition of Thiel Injection solution

Solution A	Solution B
Boric acid 250 g	4-chloro-3-cresol 500 g
Sodium Sulphite 700 g	Ethandiol 500 ml
Ammonium Nitrate 1680 g	
Potassium Nitrate 420 g	
Ethandiol 2500 ml	
Spirit 1000 ml	
Morpholine 140 ml	
Hot water 8580 ml Formalin 280 ml	

S3. Chemistry of formalin with ligament tissue

Formalin penetrates the tissues in order to preserve them. With a molecular weight of 30, formaldehyde is expected to diffuse into tissue at a faster rate. Diffusion of formaldehyde into tissue follows Fick's law (20). The diffusion is directly proportional to temperature, concentration and the square of time. Formaldehyde penetrates the tissue quickly but takes longer time for fixation. In aqueous solution, formaldehyde gets hydrated to form methylene glycol [S12].

$$CH_2O\ (\text{Formaldehyde}) + H_2O \underset{RR}{\overset{FR}{\rightleftharpoons}} CH_2(OH)_2\ (\text{Methylene Glycol})$$

In aqueous solution, the forward rate (rate of formation of methylene glycol or formaldehyde monohydrate) is higher. When a tissue is immersed in formalin solution, large quantity of methylene glycol and small quantity of formaldehyde penetrates the tissues. When the penetrated formaldehyde is used up by the tissue for cross-linking, more formaldehyde is produced from dissociation of methylene glycol (rate of formation of formaldehyde increases). Hence fixation takes longer time.

The first step in the reaction of formaldehyde with tissue occurs at a faster rate. Permanent fixation is extremely slow at moderate conditions (25 °C and pH 7). Reactions rates are constant in the pH range 3 to 8. Above pH 8 the reaction rate decreases. The process of permanent fixation may take even weeks to complete.

Formaldehyde (or methanal) is the simplest of the aldehydes that react with amine groups present in proteins [S13]. Proteins are made of one or more linear chains of amino acids [S7]. These linear chains of amino acids are also termed as polypeptide chains. Formaldehyde reacts with proteins in the following steps.

Step 1:

$$R\text{-}NH_2 \text{ (protein)} + CH_2O \text{ (formaldehyde)} \longrightarrow R\text{-}NH\text{-}CH_2\text{-}OH$$

The methylol compound formed at the end of step 1 is highly reactive. It condenses with other similarly formed methylol compounds to form methylene bridges.

Step 2:

$$R\text{-}NH\text{-}CH_2\text{-}OH + NH_2\text{-}CO\text{-}R \longrightarrow R\text{-}NH\text{-}CH_2\text{-}NH\text{-}CO\text{-}R \text{ (methylene bridges)} + H_2O$$

These methylene bridges cross-link between 2 polymer chains [S10, S15]. Step 1 is the initial reversible stage while step 2 is an irreversible stage wherein stable, permanent endpoint fixation occurs due to the formation of a high number of covalent bonds. Contrary to the fact that formation of large number of covalent bonds due to formalin, the formalin-preserved tissues should require larger failure force. This fact needs an in-depth analysis into the type of bonds created due to formalin as well as dehydration-hydration effects of formalin on the tissues and the bones.

Initial cross-links are formed within 24 hours to 48 hours while the stable covalent linkages take around 30 days [S17]. Inter and intra-molecular cross-links are formed in the case of irreversible permanent fixation. Permanent fixation results in dehydration, resistance to microbes, enzymes and other chemicals, insolubilization, hydrophobicity and trapping of macro-molecules in the matrix of cross-linked proteins. Elasticity is partially lost but less extreme than in the case of other fixing methods (radiation, heat, acids, etc.) [S2].

The effect of embalming on tissues

Human body structures are made of protein molecules. Embalming forms numerous cross-links between protein molecules. This cross-linked protein cannot serve as food/substrate for bacteria and enzymes. Proteins have many reactive centres in them. These reactive centres are destroyed by the embalming process. The proteins lose their ability to hold water after embalming and become dry. Preservatives and germicides used in the process of embalming also react with the enzymes (bacterial enzymes and autolytic enzyme produced by body cells). Enzymes are protein molecules. Thus embalming preserves cadaver by acting on (i) protein molecules of cadaver by cross-linking them and making them rigid and inert to the action of enzymes (ii) bacterial enzymes and autolytic enzymes [S13].

S4. Ligament hierarchy

Fratzl and Weinkamer has provided a detailed description of tendon/ligament hierarchy [S9]. Only a brief note is provided here. Ligaments are made up of cells (fibroblasts) and extracellular matrix. Collagen fibres and ground substance constitute the extracellular matrix. Ground substance consists of adhesion proteins, polysaccharides and proteoglycans. Adhesion proteins such as fibronectin bind collagen fibres and cells to ground substance. Polysaccharides present in ligaments are known as glycosaminoglycans (GAG). The polysaccharides associated with protein molecules are known as proteoglycans [S18]. Proteoglycans are hydrophilic and have the ability to form a hydrated gel-like network. Above a critical value of shear stress, they tend to flow like a fluid [S9]. Collagen molecule is a helical structure with length of 300 nm and diameter of 1.3 nm. The molecules are arranged into fibrils (with parallel staggering of 67 nm). The fibrils show the presence of gap and overlap regions. Few collagen molecules make up one fibril. The diameter of fibrils is 50–500 nm. The molecules are held together by intra-fibrillar cross-links (covalent bonds). The proteoglycan-rich matrix between the fibrils forms the framework which provides support to the system [S9]. Numerous parallel fibrils embedded in a proteoglycan matrix forms a fascicle. The diameter of fascicle is 50 to 300 μm. A bundle of fascicle forms ligament.

When formalin reacts with ligament tissues, cross-links (additional to the natural cross-links present between the collagen molecules) are formed between the collagen molecules. The length of the cross-links formed between the collagen molecules is 0.23 to 0. 27 nm (spacer arm length of formaldehyde molecule) [S11] and occurs between neighboring molecules [S4]. No literature on the location of formalin-induced cross-links in the 3D structure of collagen molecule and fibrils is available. In our work, the tensile strength was observed to decrease in samples preserved in formalin. The chemistry behind the formalin reaction with proteoglycan matrix is also not reported in the literature.

S5. Statistical results

The statistical results of group-wise comparisons are provided in the Tables S2–S4 for failure force, failure elongation and failure strain.

Table S2. *p* value for failure force between different methods of preservation by Mann–Whitney *U*-test

	A	B	C
A	N.A		
B	0.038	N.A	
C	0.038	0.902	N.A

Table S3. *p* value for failure elongation between different methods of preservation by Mann–Whitney *U*-test

	A	B	C
A	N.A		
B	0 0.128. **128**	N.A	
C	0.710	0.011	N.A

Table S4. *p* value for failure strain between different methods of preservation by Mann–Whitney *U*-test

	A	B	C
A	N.A		
B	0.45656	N.A	
C	0.073	0.007	N.A

References for supplementary information

[1] AJITA R., SINGH Y.I., *Body Donation and Its Relevance in Anatomy Learning – A Review*, J. Anat. Soc. India, 2007, 56 (1), 44–47.

[2] BEDINO J.H., *Embalming Chemistry : Glutaraldehyde versus Formaldehyde*, J.H. Bedino, Res. Educ. Dep. Champion Co., 2003, 2614–2632.

[3] BRENNER E., *Human body preservation – old and new techniques*, J. Anat., 2014, 224(3), 316–344.

[4] CAWLEY A.T., FLENKER U., *The application of carbon isotope ratio mass spectrometry to doping control*, J. Mass Spectrom., 2008, 43(7), 854–864.

[5] COGLIANO V.J., BAAN R., STRAIF K., GROSSE Y., LAUBY-SECRETAN B. et al., *Preventable exposures associated with human cancers*, J. Natl. Cancer Inst. 2010, 103(24), 1827–1839.

[6] DIMENSTEIN I.B., *A Pragmatic Approach to Formalin Safety in Anatomical Pathology*, Lab. Med., 2009, 40 (12), 740–746.

[7] FALLIS A., *Fundamentals of Protein Structure and Function*, 2013, Vol. 53, 1689–1699.

[8] FESSEL G., FREY K., SCHWEIZER A., CALCAGNI M., ULLRICH O., SNEDEKER J.G., *Suitability of Thiel embalmed tendons for biomechanical investigation*, Ann. Anat., 2011, 193 (3), 237–241.

[9] FRATZL P., WEINKAMER R., *Nature's hierarchical materials*, Prog. Mater. Sci., 2007, 52(8), 1263–1334.

[10] GUSTAVSON K.H., *Aldehyde Tanning in the chemistry of tanning process*, Academic Press, New York 1956, 244–282.

[11] KAST J., KLOCKENBUSCH C., *Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin*, J. Biomed. Biotechnol., 2010.

[12] KIERNAN J., *Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do*, Micros. Today, 2000, 12 (c), 8–12.

[13] MAYER R.G., *Embalming History Theory and Practice*, 5th ed., The Mc-Graw Hill Companies, 2012.

[14] OTTONE N.E., VARGAS C.A., FUENTES R., SOL M. DEL., *Walter Thiel's Embalming Method. Review of Solutions and Applications in Different Fields of Biomedical Research*, Int. J. Morphol., 2016, 34(4), 1442–1454.

[15] PUCHTLER H., MEIOAN S.N., *On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions*, 1985, 201–204.

[16] SURI A., ROY T.S., LALWANI S., DEO R.C., TRIPATHI M. et al., *Practical guidelines for setting up neurosurgery skills training cadaver laboratory in*, 2014, 5–12.

[17] THAVARAJAH R., MUDIMBAIMANNAR V., RAO U., RANGANATHAN K., ELIZABETH J., *Chemical and physical basics of routine formaldehyde fixation*, J. Oral Maxillofac. Pathol., 2012, 16 (3), 400.

[18] TORTORA G.J., DERRICKSON B., *Principles of Anatomy and Physiology*, John Wiley & sons Inc., 12th ed., 2009.

[19] V SB. *Law in relation to medical men*, [in:] *Medical Jurisprudence and Toxicology*, Rai Bahadur Jaising, P. Modi (Eds.), 22nd ed., Butterworths, New Delhi 1999, 724–727.

[20] YONG-HING C.J., OBENAUS A., STRYKER R., TONG K., SARTY G.E., *Magnetic resonance imaging and mathematical modeling of progressive formalin fixation of the human brain*, Magn. Reson. Med., 2005, 54 (2), 324–332.