

Preservation Methods Influence Human Lateral Menisci Biomechanical Properties. An ex-vivo Comparative Study of Three preservation methods (Freezing, Cryo-preservation and Freezing+Irradiation).

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Preservation Methods Influence Human Lateral Menisci Biomechanical Properties. An ex-vivo Comparative Study of Three preservation methods (Freezing, Cryo-preservation and Freezing+Irradiation).

ABSTRACT.

Backgrounds: Three main menisci preservation methods have been used over the last decade: freezing, freezing with gamma-irradiation, and cryopreservation.

Hypothesis/Purpose: We hypothesized that all preservation methods will result in similar biomechanical properties as defined by traction and compression testing.

Methods: Twenty-four human lateral menisci were collected from patients operated on for total knee arthroplasty. The inclusion criteria were patients under 70 years of age, with primary unilateral (medial) femorotibial knee osteoarthritis. Cross sectionally each meniscus was divided into 2 specimens extending from the end of the central edge peripheral/capsular attachment to obtain 2 similar samples from the same meniscus. One sample was systematically cryopreserved constituting the control group (Cy;-140°c) and the other was used for either the simple frozen group (Fr;-80°c) or the frozen + irradiated group (FrI;-80°c + 25kGy irradiation).

Evaluation was performed using compression and tensile tests (Instron 5566 Universal Testing Machine) to analyze: 1) the Elasticity Modulus (Young's Modulus; YM) in compression, 2)the YM in traction, 3)the Tensile Force at failure, 3)the Rupture Profile of the tensile stress-strain curve.

Results: A significant difference of the mean compression elasticity's modulus was observed between Cy group and the Fr group (respectively 28.86±0.77MPa vs 37.26±1.08MPa; mean difference 8.40±1,33MPa; p <0,001) and between the Cy group and the FrI group (respectively 28.86±0.77MPa vs 45.92±1.09MPa; mean difference 17.06±1.33MPa; p<0,001).

A significant difference of the mean tensile elasticity's modulus was observed between Cy group and the Fr group (respectively 11.66±0.97MPa vs 19.97±1.37MPa; mean difference 8.31±1.68MPa; p=0.008) and between the Cy group and the FrI group (respectively

11.66±0.97MPa vs 45.25±1.39MPa; mean difference 33.59±1.59MPa; p<0,001).
We did not find any significant difference regarding the Tensile Force at failure between the different groups.
The analysis of stress-strain curve between groups revealed a slow-slope curve with a non-abrupt rupture (ductile material) for cryopreserved samples. A clear rupture of the stress-strain curve was observed for frozen and frozen + irradiated samples (more fragile material).
Conclusion: We rejected our hypothesis that all preservation methods will result in similar biomechanical properties. Cryopreservation allows to obtain a more elastic and less fragile tissue than the simple freezing or freezing plus irradiation**.**

Key Words: Meniscus; Allograft ; Conservation ; Storage ; Irradiation ; Cryopreservation ; Freezing ; Mechanical Properties.

Clinical relevance: The results of our study exhibit detrimental effect of simple freezing and freezing+irradiation on Human menisci's mechanical properties. If those effects occur in menisci prepared for allograft procedure, important differences could appear on graft's mechanical behavior and thus patients' outcomes.

What is known about the subject: Three main menisci preservation methods have been advocated: freezing, freezing with gamma-irradiation, and cryopreservation Gamma. Cryopreservation is the only method that preserves fresh meniscus architectural specificities. Freezing and freezing+irradiation methods modify histological properties of meniscal allograft. The results of those procedure have been not "directly" compared using adapted mechanical testing, in the actual literature.

What this study adds to existing knowledge:

Our study compared the three main preservations methods on identical samples using two different mechanical testings, aiming to approximate in-vivo loading.

Our results, first, confirmed that Freezing+Irradiation procedure should be used with caution, second, demonstrated that Freezing also have a detrimental effect on menisci mechanical properties, third, allowed us to conclude that Menisci Tissues preserved using Cryopreservation result in better mechanical outcomes.

INTRODUCTION.

The long-term damaging effects of total meniscectomy include: pain, potential instability and osteoarthritis [12,13,16]. Menisci allografts have been advocated to treat these issues and potentially slow the onset of osteoarthritis. Mid-term results of this procedure demonstrate significant improvement in patient's pain scores [26,27], as well as increasing survivorship without failure (85%) of meniscal allografts[10,28]. To play its biomechanical role, meniscus allograft tissue must resemble the qualities of native fibrocartilage[25]. As such, graft preservation methods will play a vital role in the biological, mechanical and thus clinical success of menisci allograft techniques[5]. Three main menisci preservation methods have been used over the last decade: freezing, freezing with gamma-irradiation, and cryopreservation[25].

In a recent comparative study Jacquet et al [14] observed that Cryopreservation does not cause significant histological alterations as compared to fresh tissue. On the other hand, significant differences were only found comparing between freezing and freezing with irradiation processes to fresh tissue or cryopreserved samples.

These ex-vivo microscopic findings need to be validated to estimate their clinical implication. This biomechanical study was designed to compare "preserved menisci allografts"

mechanical properties defined by the elasticity's modulus during traction and compression testing, as there is nothing in the literature to confirm that preserving meniscal architecture preserves the biomechanical properties of the graft. We hypothesized that all preservation methods will result in similar biomechanical properties.

METHODS.

Following local board approval, twenty-four human lateral menisci were collected from patients operated on for total knee arthroplasty from September to October 2017. All patients gave written consent prior to their inclusion into the study. Inclusion criteria were: patients aged <70 years undergoing Total Knee Arthroplasty with isolated medial femoral-tibial arthritis or femoral-patellar and medial femoral-tibial joint degeneration (but with an lateral femoral-tibial compartment graded Kellgrenn and Lawrence <2 [15]) and no history of prior surgery, trauma, or developmental disease of the operated knee. An MRI was systematically performed 1 month pre-operatively to verify the absence of radiological meniscal lesion. If a grade <1 lesion was detected, the patients were not included in the study Patient's characteristics are summarized in *Table 1*.

Patients	Age (yr)	Gender	Weight (kg)	Height (cm)	BMI ($kg.m^{-2}$)
01	63	M	77	182	23.2
02	65	M	82	186	23.7
03	61	F	68	175	22.2
04	64	F	56	158	22.4
05	66	M	84	186	24.3
06	67	M	79	181	24,1
07	60	M	77	184	22.7
08	59	F	63	161	24.3
09	64	M	79	178	24.9
10	62	F	57	159	22.5
11	63	F	61	164	24,3
12	61	F	63	165	22.7
13	67	F	62	164	23.1
14	63	F	56	159	22.2
15	62	M	74	182	22.3
16	69	M	77	179	24.0
17	68	M	79	180	24.4
18	62	M	77	177	24.6
19	62	F	63	162	24.0
20	64	F	59	167	21.2
21	67	M	73	177	23.3
22	68	F	64	164	23.8
23	67	M	80	180	24.7
24	61	F	68	169	23.8

Table 1 Patient's characteristics

BMI : body mass index

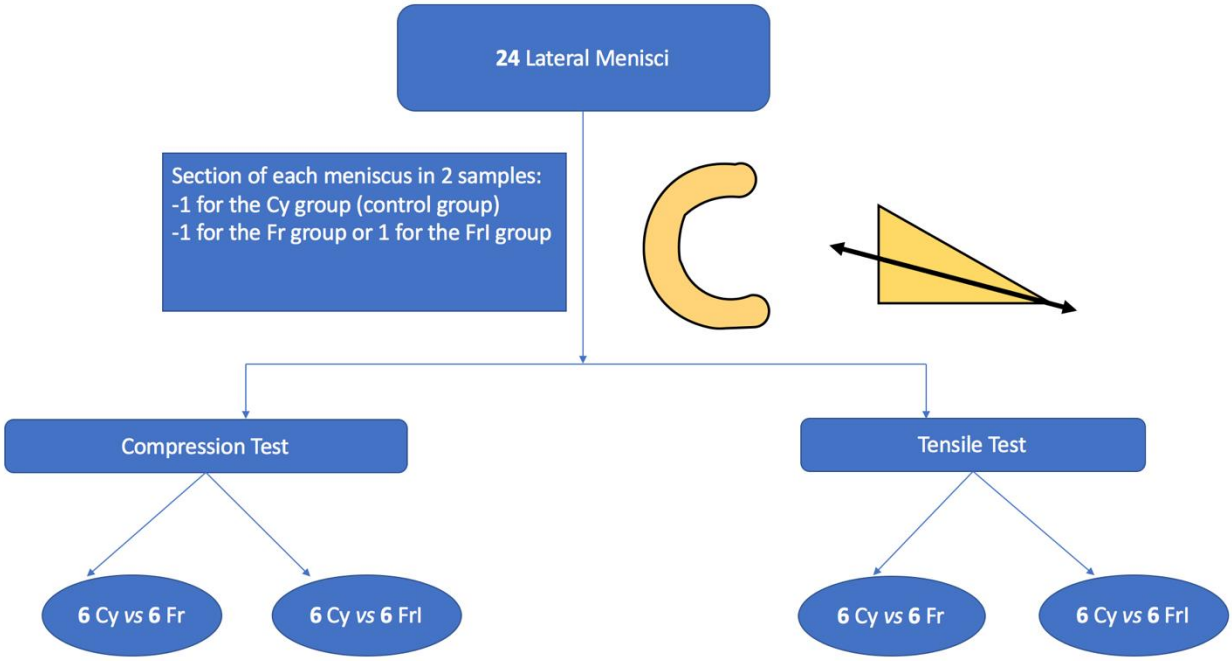


Figure 1 : Series' flow-chart

Cy : cryopreservation
Fr : Frozen

Samples Preparation

Cross sectionally each meniscus was divided into 2 specimens extending from the end of the central edge peripheral/capsular attachment to obtain 2 similar segments, one superior and one inferior (*Figure 1*). One sample was systematically cryopreserved constituting the control group (Cy) and the other was used for either the simple frozen group (Fr) or the Frozen + Irradiated group (FrI), Figure 1. The choice of the sample among the superior and inferior fragments was done randomly for each group.

For compression testing a parallelepiped specimen was harvested from each sample to obtain parallel flat surfaces at the central region of the meniscus *(Fgure 2*). Tensile testing did not require further preparation. Each sample was measured with a digital caliber (Absolute

Digimatic solar, Mitutoyo, resolution U = 0,01 mm) and only underwent tensile or compression testing.



Figure 2 Sample's preparation for the compression tests

Meniscus samples were plunged into a physiological saline solution and then placed in a cryo-kit (8°C) for transportation to the local tissue bank (<6 hours). Specimens were prepared with the following steps: (1) graft reception in clean room (controlled atmosphere zone); (2) decontamination of the graft with an antibiotic solution (Rifampicin + Thiophenicol); (3) rinsing with 0.1M cacodylate buffer for 5 min; and (4) bacteriological sampling. Following preparation, different conservation methods were applied. 1) For the cryopreservation group cryoprotective solution (10% of DMSO + SCOT 30 were added, the bag was vacuumed to extract the residual air, and progressively decreased the temperature (Starting at -4°C then decreasing at -2°C per minute to -40°C and then -5°C per minute to -140°C). Samples were stored in a nitrogen tank in a vapor phase at -145°C. 2) For the frozen group, a simple

freezing process was used, progressively decreasing the temperature (starting at -4°C then decreasing at -2°C per minute to -40°C and then -5°C per minute to -80°C). 3) For the frozen + irradiated group, a simple congelation with a progressive decrease in temperature (starting at -4°C then decreasing at -2°C per minute to -40°C and then -5°C per minute to -80°C) was performed. The grafts were then transported in a dry ice-controlled container (stored at -80°C) to be irradiated by gamma-rays by IONISOS factory ©. The doses received ranged between 22.7 and 27.8 kGy (2.2-2.7 Mrad). After this treatment, the samples were again stored at -80°C until analysis was undertaken. All samples were Stored at least 1 month prior to biomechanical testing

Biomechanical Testing

The compression and tensile tests were performed on an *Instron 5566 Universal Testing Machine* with a measurement error in displacement of 0.05% and the force transducer has a measurement error of 0.2% in tension and compression.

Compression test (Figure 3).

Each sample was subjected to 5 relaxation compression cycles with a maximum load of 50 N. The speed of progression was 3mm / min.

The Stress-strain curve was then obtained using pre-test relaxed measurement of section and thickness. Elasticity Modulus (Young's Modulus) was calculated in the relaxation elastic phase of the 5th cycle [23].



Figure 3 compression test

Tensile test (Figure 4)

Each sample was attached to the ends of the tensile testing machine by jaws dedicated to handle soft tissue to prevent inadvertent movement (*INSTRON 2716_015, force max 30kN with jaw face 0-0.25/25T/IN*) [21]. The positioning required 1/3 of the specimens' length in each jaw, the central 1/3 defining the initial length (L_0) before traction. An increasing load (10 mm / min) was applied until the specimens' failed. A stress-strain curve was obtained for each specimen using the dimensions of the samples. Then, we calculated Young's modulus in the elastic phase of the testing curve. Moreover, tensile force at failure was noted.

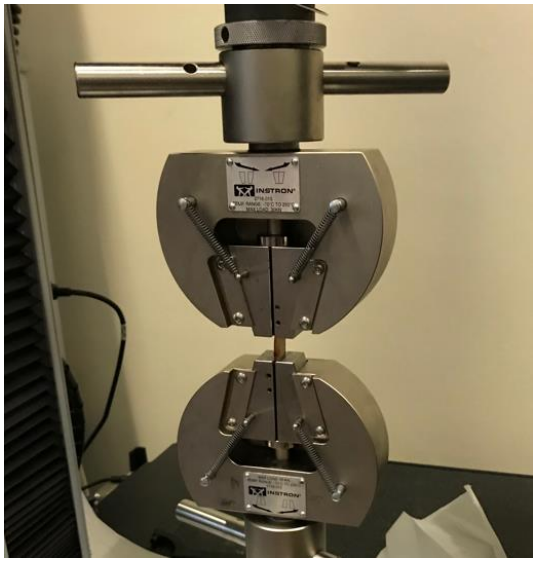


Figure 4 Tensile test

Statistics

Prior to the initiation of the study a sample analysis estimated that 6 samples for each group will be necessary to be powered (80%) to distinguish Δ: 5±3 nm Young's modulus values.

Patients characteristics were expressed using the appropriate descriptive statistics for the type of variables. Descriptive statistics included mean with SD, or median with interquartile range, as appropriate, for continuous variables. The Student t tests were used to compare the distribution of continuous parameters between groups (or the Mann-Whitney test when the data were not normally distributed or when the homoscedasticity assumption was rejected). All reported p values were 2- sided, with a significance threshold of \.05. Statistical analysis was performed using SPSS/JMP software (version 13; Microsoft software).

Results :

Compression test (Table 2)

		Absolute value of Mean difference (MPa)	IC-95% (MPa)	P value
Cryopreserved	Frozen	8.40	[5.40-11.41]	p<0.001
Cryopreserved	Frozen + irradiated	17.06	[14.05-20.07]	p<0.001

Table 2 Compression Elasticity's Modulus (Young's modulus)
MPa: MegaPascal

A significant difference of the mean compression elasticity's modulus was observed between Cy group and the Fr group (respectively 28.86 ± 0.77 MPa vs 37.26 ± 1.08 MPa; mean difference 8.40 ± 1,33 MPa and p <0,001).

A significant difference of the mean compression elasticity's modulus was also found between the Cy group and the FrI group (respectively 28.86 ±0.77 MPa vs 45.92 ± 1.09 MPa; mean difference 17.06 ± 1.33 MPa and p<0,001)

Tensile test (Table 3-4)

		Absolute value of Mean difference (MPa)	IC-95% (MPa)	P value
Cryopreserved	Frozen	8.31	[4.50-12.12]	p=0,008
Cryopreserved	Frozen+ irradiated	33.59	[29.78-37.39]	p<0.001

<u>Table 3</u> Tensile Elasticity's modulus

N: Newton

A significant difference of the mean tensile elasticity's modulus was observed between Cy group and the Fr group (respectively 11.66 ± 0.97 MPa vs 19.97 ± 1.37 MPa; mean difference 8.31 ± 1.68 MPa with p = 0.008)

A significant difference of the mean tensile elasticity's modulus was also noticed between the Cy group and the Fr group (respectively 11.66 ± 0.97 MPa f vs 45.25 ± 1.39 MPa; mean difference 33.59 ± 1.59 MPa with p<0,001) , Table 4.

		Absolute value of Mean difference (N)	IC-95% (N)	P value
Cryopreserved	Frozen	78.33	[16.02-131.33]	p=0.186
Cryopreserved	Frozen + irradiated	40.50	[28.95-107.25]	p=0.1993

<u>Table 4</u> Force at Failure

N: Newton

With the number available we did not find any significant difference regarding Force at failure between the different groups, the mean difference being 78.3N IC95% [16.02-131.33] between cryopreserved and frozen specimens (p=0.186) and 40.5 IC95% [28.95-107.25] and between cryopreserved and Frozen+Irradiated specimens (p=0.199) *(Table 4)*

The analysis of stress-strain curve between groups revealed a slow-slope curve with a non-abrupt rupture (ductile material) for cryopreserved samples (*Figure 5A).* A clear rupture of the stress-strain curve was observed for frozen and frozen + irradiated samples (more fragile material) *(Figure 5B*).

In addition, failure seemed to happen quicker for the frozen storage and frozen + irradiated specimens than in cryopreserved samples where the failure was more gradual, which is most probably due to the delamination of the fibers.

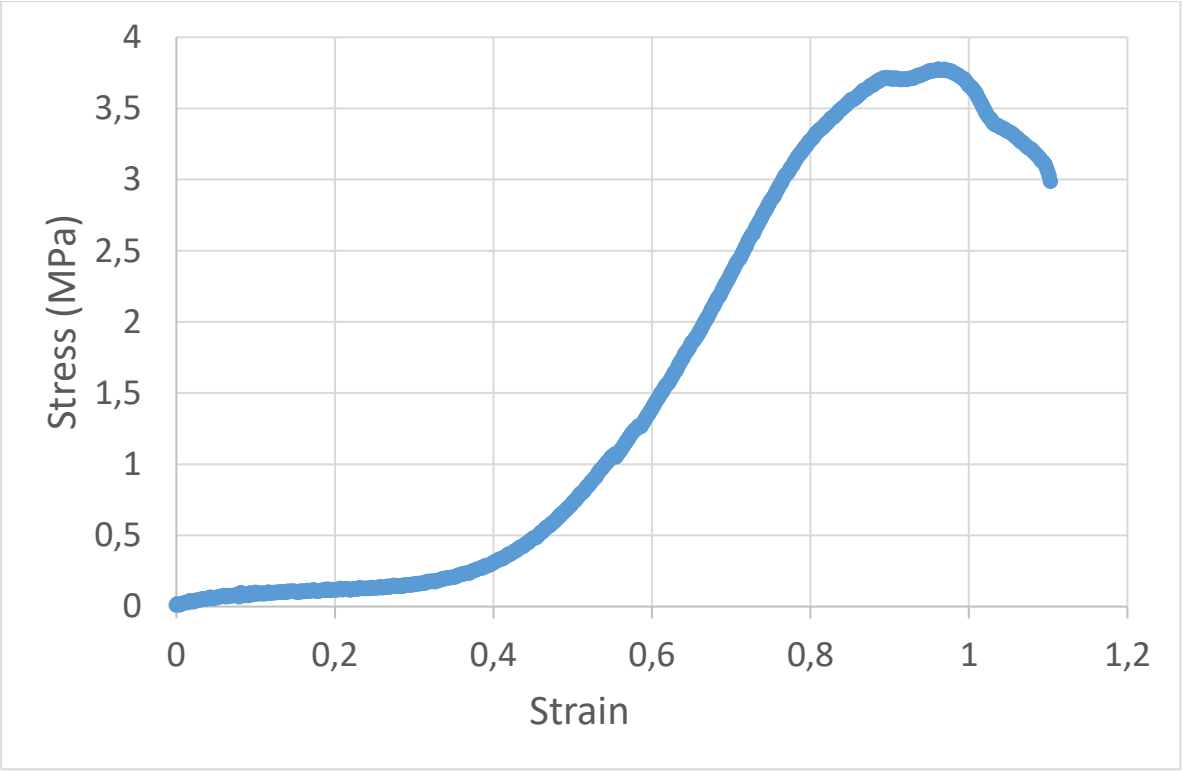
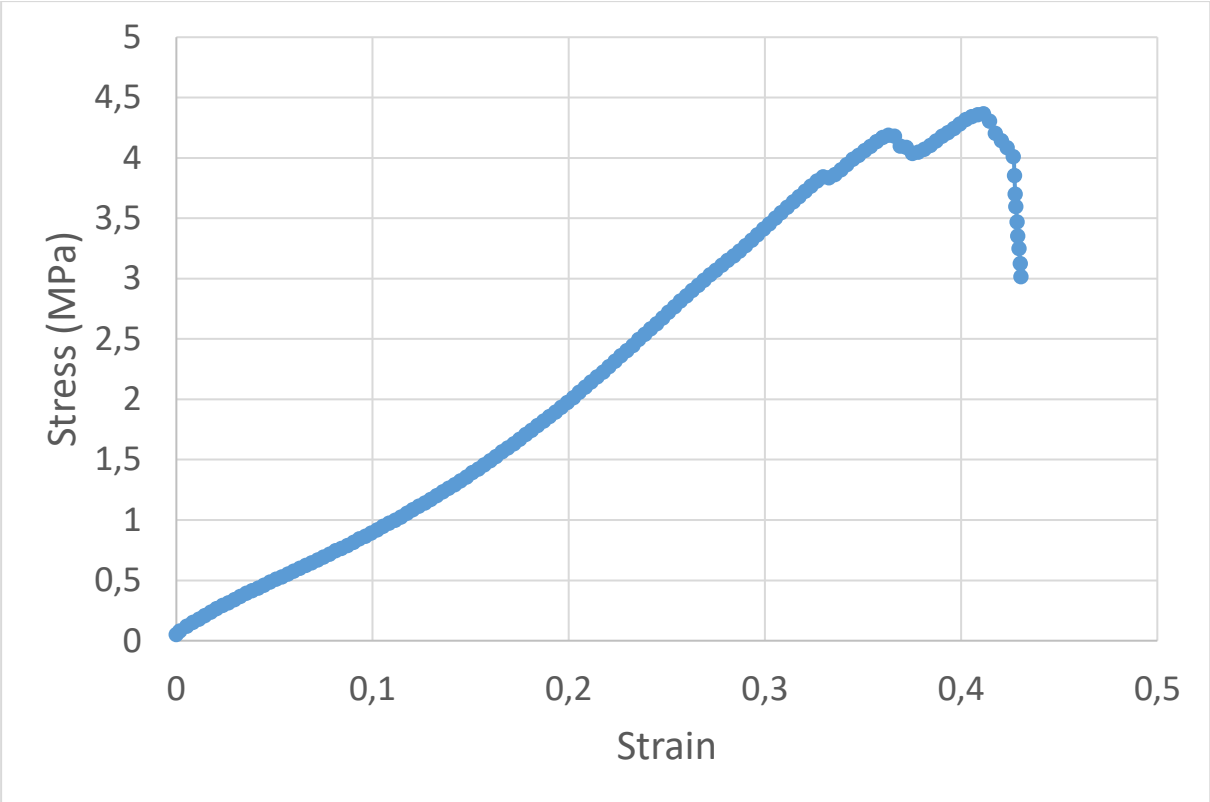


Figure 5A Stress-strain curve of a cryopreserved sample



<u>Figure 5B</u> Stress-strain curve of a frozen sample

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DISCUSSION

The key finding of this study is that Cryopreservation allows **for** more elastic and less fragile tissue than the simple freezing or freezing plus irradiation. We rejected our hypothesis that all preservation methods will result in similar biomechanical properties. We observed a significant change in the Young's modulus in both compression and traction testing when comparing Cryopreserved and Frozen specimens. These findings were more obvious when comparing differences between Cryopreserved and Frozen + irradiated specimens. All of our findings might be explained by an increased rigidity of the meniscal tissue related to the freezing and/or irradiation procedures.

The relatively large variability in tensile and compression stiffness amongst different preservation processes is multifactorial. In general, the tensile mechanical properties of biological materials depend on the relative contents of major extracellular matrix constituents, the organization of the matrix constituents and the interactions of these constituents meniscal. Prior studies have reported that different preservation methods can alter meniscal ultrastructure [8,9], which corroborates the differences we saw between cryopreservation, freezing and freezing + irradiation.

Whilst conducting this study we were also able to examine the meniscal Tensile Force at Failure and rupture profile of the tensile strain- curve. This is also defined as the ability of collagen tissue to absorb energy until it fractures. The Tensile Force at Failure of the Frozen and Frozen + irradiated samples were lower than for cryopreserved samples even if this difference was not statistically significant. This decrease in Tensile Force at failure could lead to more frequent lesions of Frozen and Frozen + irradiated grafts during transverse stresses occurring during flexion-extension movements.[17].

Our analysis of the stress-strain curves demonstrates that the cryopreserved meniscal tissue has a very gradual rupture profile reflecting a "ductile material", where Frozen and Frozen +

irradiated samples, present an acute rupture often found in “fragile material”. This means that cryopreserved samples have the ability to deform without breaking at higher absorbed energy levels than frozen samples and frozen + irradiated samples during extreme traction [20].

No data was found in the literature with regards to estimating the elasticity’s modulus of fresh meniscus (in compression or traction), or the force at failure.

Regarding tensile elasticity modulus, the available data is summarized in the *table 5.*

	Mean tensile elasticity’s modulus (MPa)
Bursac et Al 2009 [2] Frozen specimen from deceased donor Storage time: not disclosed	80.9 ± 24.6 [20.3-129.1]
Tissackh et Al [24] *1995* Frozen specimen from deceased donor Storage time: not disclosed	72.85 ± 22.91 [3.59-151.80]
Ahmad et Al 2017 [1] Frozen specimen from living donor Storage time: 6 weeks	54.17 ± 19.54 [NC]

Table 5 summary of available data for the tensile elasticity modulus

Our values are slightly lower than elastic moduli presented in similar published literature. Those differences can be explained by the fact that most of the studies [2,24], utilized samples harvested from deceased donors without any information on the sampling sequences and the storage time. In our study, all samples were from living donors. In order to limit the deleterious effects of prolonged exposure to ambient temperature, the samples were immediately placed in a Cryo-kit at 8 ° C and the preservation process was carried out in less

than 6 hours [7]. Using tissue from living donors instead of cadaveric tissue avoids bias related to death-induced hypoxia which could adversely affect the biomechanical tissue properties [19]. In Ahmad et Al Study [1], meniscus samples came from a patient with a tumor near the knee whom required a prosthetic replacement. No information was disclosed regarding possible radiotherapy treatment received, which would likely modify the biomechanical properties of the meniscus. In these three studies [1,2,24] no information was provided on the freezing process utilized, in particular the rate of descent of temperature, which has been described as a factor that may cause tissue damage [22].

For compression testing, the only data identified from the literature comes from Chia et Al'.s study[3] which described a highly variable Young's Modulus (between 0,135 and 1,130 MPa) according to the preconditioning strain level (3%, 6%, 9% or 12% strain). In this study only ten cadaveric medial menisci were studied (in our study we only considered lateral menisci). The authors did not indicate the time between death and freezing, the existence of degenerative or traumatic pathology, or the freezing process used. These differences may contribute and explain the greater variability of these published results in comparison to our conducted study.

One of the limitations of our study is the lack of fresh tissue group. However, it was impossible to obtain three different samples from the same meniscus because the amount of material was insufficient to perform the mechanical tests. More, testing fresh tissue suppose to be able to create and attach specimens into the loading device before tissue's ischemia. We did not found solutions in the actual literature to avoid this limitation. Most of the authors freezed their specimens before testing and do not estimate fresh tissue properties.

We recognize another limitation of our study, the mean age of our patients in which specimens were harvested were in comparison older than donors in others studies (average age 63.8 years in our study versus 53,5 in the register [4]). Because of this, menisci evaluated

during our analyses might have been altered by aging and degenerative processes. We tried to avoid limitation related to this methodological bias by excluding menisci with significant MIR's lesion and studying only non-arthritic joints (lateral compartment) from subjects suffering from only medial femoral-tibial degeneration. It's also described by Bursac et Al[2] that there are no significant correlations, between either the biochemical composition or the tensile mechanical properties and donor age of lateral or medial menisci. . Another difficulty encountered in this study was the creation of 2 samples from the same meniscus. Although there is no data in the literature that asserts that the superior and inferior parts of a meniscus have different biomechanical properties, we have randomly assigned each fragment (superior or inferior) in each group to limit this potential bias.

Finally, our study only approximates the physiological biomechanical environment of the meniscus. The compression tests simulate the loading of the meniscus during walking and thus its ability to absorb axial shocks during several loading cycles [6,11]. But the compression forces are not distributed uniformly over the entire surface of the meniscus and essentially only concerns the middle segment[18]. Our tensile tests simulate the transverse stresses applied to the horn-root junction of the meniscus during flexion-extension movements [29]. But in-vivo tensile strains are predominantly located at the root-horn junction, where the meniscus is adherent to the tibial plate[29]. We tried to reproduce this anatomical representation by placing the fixed point of the jaws at the ends of the menisci, near the insertion of the roots. During weightbearing and movement, the menisci are normally subjected to a combination of tension, compression, and shear forces. Shear forces could not be evaluated in this study because no device allowed to reproduce in vitro the impact of these forces. Thus, the ability of a meniscal allograft to withstand these forces after transplantation would appear to be a key element in the successful outcome of such a procedure.

CONCLUSION

Cryopreserved meniscal sections demonstrated superior stress-strain, tension, and compression biomechanics compared to frozen and frozen+ irradiated specimens. Cryopreservation allows preservation of an elastic and less fragile meniscal allograft than freezing and the freezing + irradiation process.

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