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C. Jacquet, R. Erivan, A. Sharma, M. Pithioux, S. Parrattte, J.N. Argenson, M. Ollivier

Preservation Methods Influence Human Lateral Menisci Biomechanical Properties.

An ex-vivo Comparative Study of Three preservation methods (Freezing, Cryo-preservation and Freezing+Irradiation).

26 **ABSTRACT.**

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

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

31 **Methods:** Twenty-four human lateral menisci were collected from patients operated on for
32 total knee arthroplasty. The inclusion criteria were patients under 70 years of age, with
33 primary unilateral (medial) femorotibial knee osteoarthritis. Cross sectionally each meniscus
34 was divided into 2 specimens extending from the end of the central edge peripheral/capsular
35 attachment to obtain 2 similar samples from the same meniscus. One sample was
36 systematically cryopreserved constituting the control group (Cy;-140°C) and the other was
37 used for either the simple frozen group (Fr;-80°C) or the frozen + irradiated group (FrI;-80°C
38 + 25kGy irradiation).

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

43 **Results:** A significant difference of the mean compression elasticity's modulus was observed
44 between Cy group and the Fr group (respectively 28.86 ± 0.77 MPa vs 37.26 ± 1.08 MPa; mean
45 difference 8.40 ± 1.33 MPa; $p < 0.001$) and between the Cy group and the FrI group
46 (respectively 28.86 ± 0.77 MPa vs 45.92 ± 1.09 MPa; mean difference 17.06 ± 1.33 MPa;
47 $p < 0.001$).

48 A significant difference of the mean tensile elasticity's modulus was observed between Cy
49 group and the Fr group (respectively 11.66 ± 0.97 MPa vs 19.97 ± 1.37 MPa; mean difference
50 8.31 ± 1.68 MPa; $p = 0.008$) and between the Cy group and the FrI group (respectively

51 $11.66\pm 0.97\text{MPa}$ vs $45.25\pm 1.39\text{MPa}$; mean difference $33.59\pm 1.59\text{MPa}$; $p < 0,001$).

52 We did not find any significant difference regarding the Tensile Force at failure between the
53 different groups.

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

57 **Conclusion:** We rejected our hypothesis that all preservation methods will result in similar
58 biomechanical properties. Cryopreservation allows to obtain a more elastic and less fragile
59 tissue than the simple freezing or freezing plus irradiation.

60

61 **Key Words: Meniscus; Allograft ; Conservation ; Storage ; Irradiation ;**

62 **Cryopreservation ; Freezing ; Mechanical Properties.**

63

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

68

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

75

76 **What this study adds to existing knowledge:**

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

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

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85 **INTRODUCTION.**

86 The long-term damaging effects of total meniscectomy include: pain, potential
87 instability and osteoarthritis^{12,13,16}. Menisci allografts have been advocated to treat these
88 issues and potentially slow the onset of osteoarthritis. Mid-term results of this procedure
89 demonstrate significant improvement in patient's pain scores^{26,27}, as well as increasing
90 survivorship without failure (85%) of meniscal allografts^{10,28}. To play its biomechanical role,
91 meniscus allograft tissue must resemble the qualities of native fibrocartilage²⁵. As such, graft
92 preservation methods will play a vital role in the biological, mechanical and thus clinical
93 success of menisci allograft techniques⁵. Three main menisci preservation methods have been
94 used over the last decade: freezing, freezing with gamma-irradiation, and cryopreservation²⁵;

95 In a recent comparative study Jacquet et al¹⁴ observed that Cryopreservation does not
96 cause significant histological alterations as compared to fresh tissue. On the other hand,
97 significant differences were only found comparing between freezing and freezing with
98 irradiation processes to fresh tissue or cryopreserved samples.

99 These ex-vivo microscopic findings need to be validated to estimate their clinical implication.

100 This biomechanical study was designed to compare “preserved menisci allografts”

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

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107 **METHODS.**

108 Following local board approval, twenty-four human lateral menisci were collected
109 from patients operated on for total knee arthroplasty from September to October 2017. All
110 patients gave written consent prior to their inclusion into the study. Inclusion criteria were:
111 patients aged <70 years undergoing Total Knee Arthroplasty with isolated medial femoral-
112 tibial arthritis or femoral-patellar and medial femoral-tibial joint degeneration (but with an
113 lateral femoral-tibial compartment graded Kellgren and Lawrence <2¹⁵) and no history of
114 prior surgery, trauma, or developmental disease of the operated knee. An MRI was
115 systematically performed 1 month pre-operatively to verify the absence of radiological
116 meniscal lesion. If a grade <1 lesion was detected, the patients were not included in the study
117 Patient's characteristics are summarized in *Table 1*.

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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

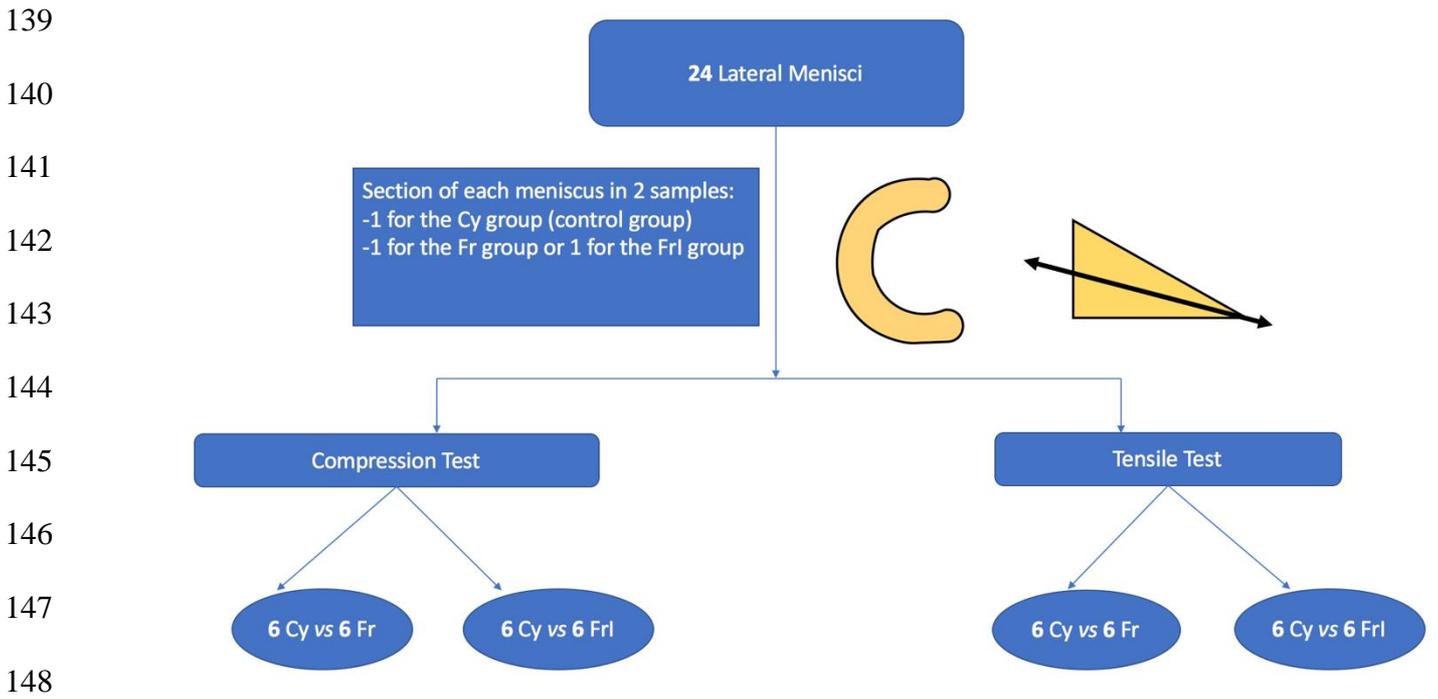
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Table 1 Patient's characteristics

BMI : body mass index

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150 **Figure 1 : Series' flow-chart**

151 *Cy : cryopreservation*

152 *Fr : Frozen*

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154 **Samples Preparation**

155 Cross sectionally each meniscus was divided into 2 specimens extending from the end of the

156 central edge peripheral/capsular attachment to obtain 2 similar segments, one superior and

157 one inferior (*Figure 1*). One sample was systematically cryopreserved constituting the control

158 group (Cy) and the other was used for either the simple frozen group (Fr) or the Frozen +

159 Irradiated group (FrI), *Figure 1*. The choice of the sample among the superior and inferior

160 fragments was done randomly for each group.

161 For compression testing a parallelepiped specimen was harvested from each sample to obtain

162 parallel flat surfaces at the central region of the meniscus (*Figure 2*). Tensile testing did not

163 require further preparation. Each sample was measured with a digital caliber (Absolute

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

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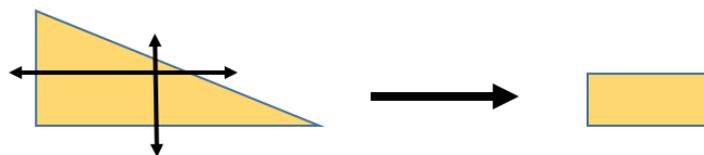
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Figure 2 Sample's preparation for the compression tests

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

189 freezing process was used, progressively decreasing the temperature (starting at -4°C then
190 decreasing at -2°C per minute to -40°C and then -5°C per minute to -80°C). 3) For the frozen
191 + irradiated group, a simple congelation with a progressive decrease in temperature (starting
192 at -4°C then decreasing at -2°C per minute to -40°C and then -5°C per minute to -80°C) was
193 performed. The grafts were then transported in a dry ice-controlled container (stored at -80°C)
194 to be irradiated by gamma-rays by IONISOS factory ©. The doses received ranged between
195 22.7 and 27.8 kGy (2.2-2.7 Mrad). After this treatment, the samples were again stored at -
196 80°C until analysis was undertaken. All samples were Stored at least 1 month prior to
197 biomechanical testing

198

199 **Biomechanical Testing**

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

203

204 *Compression test (Figure 3).*

205 Each sample was subjected to 5 relaxation compression cycles with a maximum load of 50 N.

206 The speed of progression was 3mm / min.

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

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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.

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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 $\Delta: 5\pm 3$ 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 $\backslash.05$. Statistical analysis was performed using SPSS/JMP software (version 13; Microsoft software).

Results :

264 *Compression test (Table 2)*

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		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

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

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

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

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287 Tensile test (Table 3-4)

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		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

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Table 3 Tensile Elasticity's modulus

N: Newton

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292 A significant difference of the mean tensile elasticity's modulus was observed between Cy
293 group and the Fr group (respectively 11.66 ± 0.97 MPa vs 19.97 ± 1.37 MPa; mean difference
294 8.31 ± 1.68 MPa with $p = 0.008$)

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

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		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

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Table 4 Force at Failure

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N: Newton

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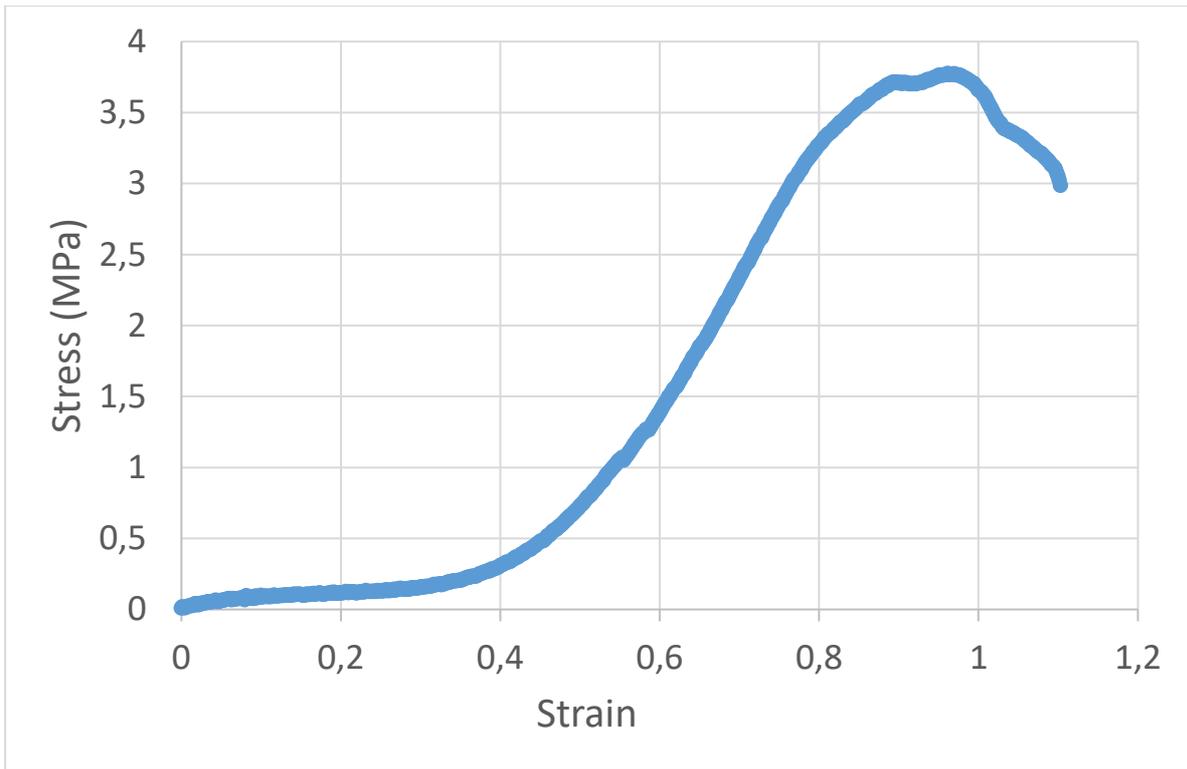
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With the number available we did not find any significant difference regarding Force at failure between the different groups, the mean difference being 78.3N IC95% [16.02-131.33] between cryopreserved and frozen specimens (p=0.186) and 40.5 IC95% [28.95-107.25]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



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Figure 5A Stress-strain curve of a cryopreserved sample

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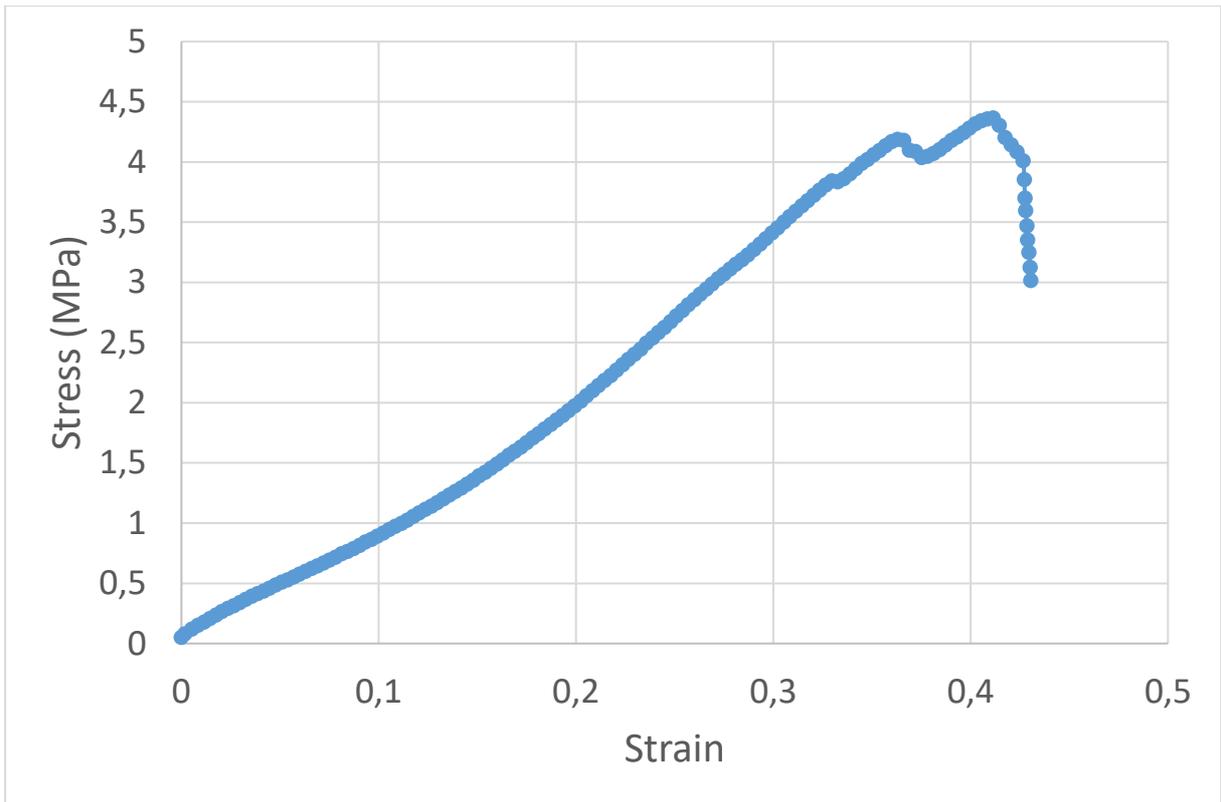
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Figure 5B Stress-strain curve of a frozen sample

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383 **DISCUSSION**

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386 The key finding of this study is that Cryopreservation allows **for** more elastic and less fragile

387 tissue than the simple freezing or freezing plus irradiation. We rejected our hypothesis that all

388 preservation methods will result in similar biomechanical properties. We observed a

389 significant change in the Young's modulus in both compression and traction testing when

390 comparing Cryopreserved and Frozen specimens. These findings were more obvious when

391 comparing differences between Cryopreserved and Frozen + irradiated specimens. All of our

392 findings might be explained by an increased rigidity of the meniscal tissue related to the

393 freezing and/or irradiation procedures.

394 The relatively large variability in tensile and compression stiffness amongst different

395 preservation processes is multifactorial. In general, the tensile mechanical properties of

396 biological materials depend on the relative contents of major extracellular matrix constituents,

397 the organization of the matrix constituents and the interactions of these constituents meniscal.

398 Prior studies have reported that different preservation methods can alter meniscal

399 ultrastructure^{8,9}, which corroborates the differences we saw between cryopreservation,

400 freezing and freezing + irradiation.

401 Whilst conducting this study we were also able to examine the meniscal Tensile Force at

402 Failure and rupture profile of the tensile strain- curve. This is also defined as the ability of

403 collagen tissue to absorb energy until it fractures. The Tensile Force at Failure of the Frozen

404 and Frozen + irradiated samples were lower than for cryopreserved samples even if this

405 difference was not statistically significant. This decrease in Tensile Force at failure could

406 lead to more frequent lesions of Frozen and Frozen + irradiated grafts during transverse

407 stresses occurring during flexion-extension movements.¹⁷.

408 Our analysis of the stress-strain curves demonstrates that the cryopreserved meniscal tissue

409 has a very gradual rupture profile reflecting a "ductile material", where Frozen and Frozen +

410 irradiated samples, present an acute rupture often found in “fragile material”. This means that
 411 cryopreserved samples have the ability to deform without breaking at higher absorbed energy
 412 levels than frozen samples and frozen + irradiated samples during extreme traction ²⁰.
 413 No data was found in the literature with regards to estimating the elasticity’s modulus of fresh
 414 meniscus (in compression or traction), or the force at failure.
 415 Regarding tensile elasticity modulus, the available data is summarized in the *table 5*.
 416

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

417 **Table 5 summary of available data for the tensile elasticity modulus**

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

425 than 6 hours ⁷. Using tissue from living donors instead of cadaveric tissue avoids bias related
426 to death-induced hypoxia which could adversely affect the biomechanical tissue properties ¹⁹.

427 In Ahmad et Al Study ¹, meniscus samples came from a patient with a tumor near the knee
428 whom required a prosthetic replacement. No information was disclosed regarding possible
429 radiotherapy treatment received, which would likely modify the biomechanical properties of
430 the meniscus. In these three studies ^{1,2,24} no information was provided on the freezing process
431 utilized, in particular the rate of descent of temperature, which has been described as a factor
432 that may cause tissue damage ²².

433 For compression testing, the only data identified from the literature comes from Chia et Al'.s
434 study³ which described a highly variable Young's Modulus (between 0,135 and 1,130 MPa)
435 according to the preconditioning strain level (3%, 6%, 9% or 12% strain). In this study only
436 ten cadaveric medial menisci were studied (in our study we only considered lateral menisci).
437 The authors did not indicate the time between death and freezing, the existence of
438 degenerative or traumatic pathology, or the freezing process used. These differences may
439 contribute and explain the greater variability of these published results in comparison to our
440 conducted study.

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

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

450 during our analyses might have been altered by aging and degenerative processes. We tried to
451 avoid limitation related to this methodological bias by excluding menisci with significant
452 MIR's lesion and studying only non-arthritic joints (lateral compartment) from subjects
453 suffering from only medial femoral-tibial degeneration. It's also described by Bursac et Al²
454 that there are no significant correlations, between either the biochemical composition or the
455 tensile mechanical properties and donor age of lateral or medial menisci. . Another difficulty
456 encountered in this study was the creation of 2 samples from the same meniscus. Although
457 there is no data in the literature that asserts that the superior and inferior parts of a meniscus
458 have different biomechanical properties, we have randomly assigned each fragment (superior
459 or inferior) in each group to limit this potential bias.

460 Finally, our study only approximates the physiological biomechanical environment of the
461 meniscus. The compression tests simulate the loading of the meniscus during walking and
462 thus its ability to absorb axial shocks during several loading cycles ^{6,11}. But the compression
463 forces are not distributed uniformly over the entire surface of the meniscus and essentially
464 only concerns the middle segment¹⁸. Our tensile tests simulate the transverse stresses applied
465 to the horn-root junction of the meniscus during flexion-extension movements ²⁹. But in-vivo
466 tensile strains are predominantly located at the root-horn junction, where the meniscus is
467 adherent to the tibial plate²⁹. We tried to reproduce this anatomical representation by placing
468 the fixed point of the jaws at the ends of the menisci, near the insertion of the roots. During
469 weightbearing and movement, the menisci are normally subjected to a combination of
470 tension, compression, and shear forces. Shear forces could not be evaluated in this study
471 because no device allowed to reproduce in vitro the impact of these forces. Thus, the ability of
472 a meniscal allograft to withstand these forces after transplantation would appear to be a key
473 element in the successful outcome of such a procedure.

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476 **CONCLUSION**

477 Cryopreserved meniscal sections demonstrated superior stress-strain, tension, and

478 compression biomechanics compared to frozen and frozen+ irradiated specimens.

479 Cryopreservation allows preservation of an elastic and less fragile meniscal allograft than

480 freezing and the freezing + irradiation process.

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