CURRENT FRONTIERS IN CRYOPRESERVATION

Edited by **Igor I. Katkov**

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Contents

Preface

Almost a decade has passed since the last textbook on the science of cryobiology and the most common methods of cryopreservation was published [Fuller *et al*, 2007], to which we will refer as *"the previous book"* here and below. When it was published, it became a useful guide for both "seasoned" cryobiologists and those who had just started their journey to this fascinating science.

However, there have been some serious tectonic shifts in cryobiology, which were perhaps not seen on the surface but may have a profound effect on both the future of cryobiology and on the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas and introduce the recently emerged practical methods of cryopreservation. The present books, *Current Frontiers in Cryobiology* [Katkov, 2012A] (referred here as *Book 1*) and *Current Frontiers in Cryopreservation* [Katkov, 2012B] (*Book 2*), will serve the purpose. These two books are not a substitute for *the previous book* but are rather complementary, so we highly recommend to all readers who want to know the background on which *the current books* were written to read *the previous book* as well.

Before we describe the current books, let us first briefly compare them to the previous book in retrospective. First of all, there were some very promising directions a decade ago that unfortunately did not meet the expectations. Molecular biology and genetics, particularly in regards to expression of stress proteins and other pathways related to the cell injury, have not introduced any serious breakthroughs except for the use of ROCK inhibitors for cryopreservation of human embryonic and induced pluripotent stem cells. The latter really was a revolutionary discovery, which however, was not made by cryobiologists; it was just *"*picked up*"* by them from the Watanabe's seminal work [Watanabe *et al*, 2007] (see the Chapter by Martin- Ibáñez in *Book 1* for details). In general, however, all those molecular biology tools have helped the solution but have not solved the cryopreservation problems *per se*. One of the backlashes of this new era is that the "traditional" cryobiologists now have little chances of getting a grant from many funding agencies such as NIH, whose panels are dominated by molecular biologists and geneticists, unless the applicant is willing to study those pathways and use of transcriptomics, proteomics, metabolomics, and other *"*omics*"*. Yet, all those very expensive tools have so far added a little to the science of cryobiology, and

especially to the practices of cryopreservation. Moreover, it is sad to see the how some new publications "rediscover the wheel", repeating many achievements of cryobiologists that had been done one or two decades before but were not referred as full size papers on PubMed, and these novel rediscoveries are often done at a much greater cost. We must agree with the author of *Foreword* of the previous book, who insightfully wrote "*I see now much of the early ground being replowed, often by equally empirical methods, albeit as far greater expenses…The concept of science as a community of colleagues engaged in public service … has been eroded by the cost of research and the emergence of industry as not only a major source of research funding but as the ultimate exploiter of the results, and we have no choice but to play the game"* (Foreword in [Fuller *et al*, 2007] by H. Meryman). However, we hope that might change in the future and that

an alliance between cryobiology and molecular and cellular biology will bring *real* practical fruits.

The slogan *"Let Us Learn from Mother Nature",* while being attractive *per se* (it is actually imbedded in the title of our first Chapter by Katkov *et al* in *Book 1*), must be taken with a grain of salt. Yes, Mother Nature has liquid crystals in biological membranes, but LCD TV screens were invented by man. Yes, there are rotifers and other "molecular motors" in cells, but the wheel was discovered and built by the human race. And finally, there are TV, radio, internal combustion engines, and many other devices and apparatuses that have no close analogy in wildlife. Similarly, while some robust creatures are well adapted to survive for short time at up to -20oC, there is no place on Earth that cools down below the glass transition temperature of water (-136oC), and there is no place on Earth where liquid nitrogen is present. Ergo, practically no one natural biosystem can adapt to such low temperatures just by natural selection, it needs human help to be stabilized for infinite time at -196oC. Thus, while learning something from the natural phenomena, it is our strong opinion that we should not rely on them too much: the money for supplying an Antarctica deep lake drilling or a Mars expedition can be spent much more efficiently and *usefully for humankind* if channeled to the development of a new controllable freezer for cryopreservation of large tissues and organs, similar for instance, to the described in our *Book 1* by Butler and Pegg.

The next large area where the progress has been quite slower than it was expected a decade ago is lyophilization and desiccation of cells of vertebrates. So far, there is no compelling evidence that would convince us that there is a method of freeze-drying or desiccation that has produced *viable* mammalian cells that can be stored for sufficient amount of time (> 2 years) at temperature above +4oC despite the fact that the opposite was claimed many times in the last 50 years. We briefly explored this aspect in our *Chapter 1* of *Book 1*. We think that this field has remained to be trapped in a set of scientific misconceptions, such as the possibility of drying the sample to the glass transition T_g that is above the final temperature or drying $T_{dr,f}$, or the related misleading concept of the possibility of substantial movement of water in a sample below its glass transition. We think that such statements violate the laws of

thermodynamics and the definition of the vitreous phase as the state with enormous viscosity, as well as that it is in contradiction with the Stokes-Einstein Law of diffusion. Numerous reports, which state T_g of the sample as high as + 60oC while drying was stopped at, say, $T_{dr,f}$ = +20oC are *incorrect*. Such overestimation of T_g (which in fact is 20-25 degrees lower than $T_{dr,f}$) lead to unsubstantiated expectations of long stability at supra-zero, even ambient temperatures at relatively *high* water content of the sample, which has never proven the case in thorough experiments (with some reservation to platelets as rather *"cell debris"* than true cells). All these data, if checked properly, are in fact either artifacts - the results of incorrect gravimetrical measurements or the use of inaccurate methods such as DSC. We are confident that the real viscosity, not those *mysterious* high temperature DSC peaks, should be measured for correct determination of the *biostabilization* T_g (defined as the point at which viscosity reaches 10 x $10^{13.6}$ Pa x sec).

And as *the last but not the least in our list* is the notion that all those ice-blockers, freeze and shock proteins, and other promising from a decade ago classes of molecules have not so far shown to be used in cryopreservation protocols alone but always in a concert with *ole good* permeable cryoprotectants and impermeable sugars, and other low molecular weight molecules, which have been around for decades. This is especially true in regards to vitrification, specifically of organs: *"the promise of the 21st century medicine"* has remained as far from the completion as it was 25+ years ago with the report on *equilibrium* vitrification of a kidney by Fahy and colleagues. On the other hand, the assisted reproduction cryobiology is rapidly moving toward *kinetic* vitrifcation, the very method of cryopreservation described by the most prominent pioneer of cryopreservation, Father Basil Luyett, more than 7 decades ago. We specifically dedicated our *Chapter 1* both to the memory of this brilliant scientist and to the detailed analysis of the situation, the difference between the two approaches to vitrifcation (*kinetic* vs. *equilibrium*), and to a quite opposite foreseeable future for them. On the other hand, there are new directions (or the old ones, replowed with deeper and more thorough plowing techniques) on the horizon of cryobiology. Among them, we can mention the attractiveness of cryopreservation of *adherent* cells (often in monolayers) not only for the benefit of the cells *per s*e (by avoiding anoikis triggered apoptosis, etc), but mainly for the convenience of the rapidly emerging field of cell based high throughput and high content analyses, where cells can be frozen, stored, shipped, and *ready-to-go* after thawing directly in multi-well plates (see Chapter by Martín-Ibáñez in *Book 1*).

The other serious breakthrough that was missed by many authors a decade ago is the kinetic vitrifcation of sperm and the emergence of what we call *"Race for the Pace"*, a set of new devices for ultra-fast cooling of samples such as Open Pulled Straws, Microdroplets, Vitrification on the Solid Surface, VitMaster (slush cooling), Cryogenic Oscillating Heat Pipes, Quartz Capillaries, and some others (see Chapter by Cipri *et al* in *Book 1*). We think that many of these devices are rather transient to a new generation

of hyper-fast coolers and warmers, but yet, the rapid ascent of kinetic vitrification is the phenomenon that has been largely missed and often simply ignored by the "classical cryobiologists "at the end of the last century and the beginning of the current one. Our books dedicate a lot of space to those aspects and their future directions.
There are also some other differences between our books and *the previous book* published by CRC, which are mainly determined by the very nature of how the Open Access operates. To begin with, our books are closely related but yet are different. *Book 1* contains mainly reviews that were written by the leaders in the field and were solicited by the Editor. In contrast, *Book 2* (in general, with some exceptions) is dedicated mostly to the reports of concrete methods of cryopreservation, and its chapters are often written by young or emerging scientists who want to make their discoveries public as soon as possible. The Editor is well aware of how discouraging and often devastating the reviewing process in "standard' journals can be just because the reviewer(s) did not share innovative ideas proposed by the author, even though the experimental or mathematical aspects of the manuscript raised no questions. The Editor of these books has reviewed all submitted chapters, about a dozen of them has been rejected, and among 41 published chapters, many were revised one, two, or sometimes three times. But that was always regarding the quality of the manuscripts, not the quality of the author's science; if I sometimes disagreed with the author's opinion, I then *"let the cryo people go"* with their perception, not mine or the one of some external reviewer. In the revolutionary spirit of Open Access, let the common reader, not an elitist reviewer, be the judge in the end!
Another "democratic" aspect in our books in the times of globalization is that the contributions were made by people from 27 countries from *all* continents (except Antarctica). Editor *greatly* appreciates the *invaluable* contribution of the American, Australian, British, Canadian, and New Zealand scientists to the field, and 10 out of 42 Chapters in our books were contributed by authors from those countries. However, cryobiology has long existed in many other languages and cultures. We found the tone of some, especially "historical" reviews written by prominent cryobiologists that may make an impression that the scientists of *that* linguistic domain have predominantly contributed to cryobiology quite uncomfortable; in other words: *"If it is not published in English - it doesn't exist"* so to speak. In contrast to such biases, the seminal works of Luyett, Smirnov, Jahnel, Boutron, Milovanov, Cassou, Ostashko, Sumida, Kopeika, and many other scientists whose first language of publication and/or mother tongue were not English, but whose *pioneering* contribution to the theory and practice of low temperature stabilization has been recognized over the World, is also highly regarded in our books.
Yet another difference is that the chapters of our books are grouped into topics (Sections) that are "subject oriented" rather than loosely flocked to the "Themes" so none would wonder why one chapter on freezing of plants is in one section, while another one ends up in another. The sections are the same for both our *books*, the only

difference is the type of the paper as described earlier, yet in many cases, this difference is rather vague: we do not consider chapters in *Book 2* as "second class" at all: *Books 1* and *2* are inseparable. The sections and chapters of the books are as follows**:**
Section *"Basic Cryobiology and Kinetic Vitrification"* opens *Book 1*. The first, and the two following chapters are dedicated to kinetic vitrification as the re-emerging method of cryopreservation. Chapter 1 by Katkov and colleagues reiterates the idea that basically all methods of long-term stabilization of cells are in fact different ways (the authors identify 5 of them) of vitrifcation of the intracellular milieu. The chapter gives a detailed thermodynamical description and analysis of the methods. The second part of the chapter is dedicated to the kinetic vitrification of human and animal spermatozoa, the concept of the *"Universal Cryopreservation Protocol*" and what the author called *"Race for the Pace"*, though the last one needs a separate chapter and is only mentioned briefly as one of the future directions. The chapter by *Isachenko and colleagues* tells the story of successful vitrification of human and animal spermatozoa, and its emerging as a valuable tool applied to the assisted reproduction technologies. The third chapter, by a Canadian group (Moskovtsev *et al*) is an *independent* report of the success of vitrifcation of human sperm without permeable (and potentially toxic) cryoprotectants (vitrificants) with certain modifications of the Isachenko's method. The chapter by Gao

& Zhou is dedicated to the basic cryobiology of osmotic effects, prevention of the osmotic injury, as well as to the equipment for the optimal addition and elution of osmotically active permeable cryoprotective agents (CPAs).

Section *"Stem Cells and Cryopreservation in Regenerative Medicine"* in *Book 1* is presented by a review by Martín-Ibáñez on cryopreservation of human *pluripotent* stem cells; it is the cutting edge of the contemporary cryobiological science where major discoveries have been made very recently. Cryopreservation of *adult* rat mesenchymal stem cells by vitrification is the theme of the chapter by Bahadori *et al* in *Book 2*. It is the one of the chapters when the Editor disagrees with the evaluation of the convenience of cryopreservation of stem cells by vitrification in small containers such OPS, but as we said before, we judged the experimental science, not the concept, and the former one was self-evidently good. The chapter by Campbell & Brockbank reports very interesting results on cryopreservation of adherent smooth muscle and endothelial cells, a direction that, as we mentioned before, may bring about some interesting practical applications. The other two chapters in *Book 2* are the one by U. Santos and colleagues, dedicated to cryopreservation of musculoskeletal cells and tissues, and the other application of regenerative medicine - cryopreservation of allograft for knee ligament construction is the theme of the Chapter by Bitar *et al*.

Section *"Human Assisted Reproduction Techniques (ART)"* opens with a review by Liebermann on vitrifcation of embryos and oocytes, a fast developing method of ART. Juergen and Michael Tucker have edited an excellent book dedicated to the use of vitrifcation in human ART [Liebermann & Tucker, 2007] that we highly recommend for reading to the specialists in the field. This review in *Book 1* summarizes the latest

achievements in the area. Another chapter in *Book 1* (Bigelow & Copperman) is also dedicated to cryopreservation of human oocytes, and altogether, both chapters provide a good glance at the comparative advantages of slow freezing vs. vitrifcation in cryopreservation of human eggs. As the background in cryopreservation of human spermatozoa is extensively covered by three chapters of the first section in *Book 1*, the third chapter of this section written by Honaramooz discusses the recent advances in cryopreservation of testicular tissues. The chapter in *Book 2* by Criado covers a very "hot" topic of contamination associated with vitrifcation in the so called "open systems", in which there is a direct contact (or a possibility of such) of the vitrified sample with liquid nitrogen. The chapter also provides a comprehensive review of current containers used for vitrifcation in human ART.

The section *"Farm / Pet/ Laboratory Animal ART"* is generically related to the previous section, but with an emphasis on animal reproductive cells and tissues. The first chapter in *Book 1* by Rodriguez-Martinez covers the cryopreservation of porcine (pig) gametes, embryos and genital tissues. It is followed by a chapter on cryopreservation of embryos of model animals, written by Tsang & Chow. *Book 2* contains a series of reports and mini-reviews on cryopreservation of boar (Kaeoket) and rat (Yamashiro & Sato) spermatozoa, cryopreservation of genetic diversity (sperm, oocytes, embryos, somatic cells) of rabbit species by Jolly *et al*, cryopreservation of ovarian tissues of large domestic animals (cow, pig and sheep) and non-primates (macaque) by Milenkovic and colleagues, and cryopreservation of reproductive cells of domestic animals (Neto *et al*). While there is a certain overlap in the coverage among those chapters, we feel that such diversity enriches the *Book 2* as different points of view are considered.

Section titled *"Cryopreservation of Wildlife Genome"*, particularly of terrestrial vertebrate species, is comprehensively covered by Saragusty and is supplemented by a review on cryopreservation of genome of wild *Felidae* by Paz in *Book 1*.

Section *"Cryopreservation of Aquatic Species"* lacks a general review but several aspects are covered in a variety of chapters. Zilli & Vilella (*Book 1*) discuss the effect of cryopreservation on bio-chemical parameters, DNA integrity, protein profile and phosphorylation state of proteins of seawater fish spermatozoa, with a similar topic, but at a different angle and with their own recent experimental results, covered by Li and colleagues in *Book 2*. This book also contains several experimental reports on cryopreservation of sperm of freshwater species, such as European pikeperch and catfish (Bokor *et al*), a variety of Malaysian freshwater species (Chew), brown trout and koi carp (Bozkurt *et al*) and African giant catfish (Omitogun *et al*).

The section titled *"Cryopreservation in Plants"* is the most *populous* and is represented by four chapters in *Book 1* and five chapters in *Book 2*. Two extensive reviews by Kaczmarczyk *et al* and by Kami cover general aspects of plant cryopreservation. Again, while those two chapters overlap in many aspects, they are rather complementary. The third chapter in *Book 1,* written by Babu and colleagues, is

dedicated to cryopreservation of species of spices plants, while the review by Quain *et*

al discusses the current advances in cryopreservation of vegetatively propagated tropical crops. Similar subject (vegetatively propagated crops), but with an emphasis on the thermal analysis of the cryopreservation methods using DSC, is covered by Zámečník *et al* in *Book 2*. In the same book, Martinez-Montero and colleagues cover current frontiers in cryopreservation of sugarcane and pineapple, C. Santos reports the results of cryopreservation of cork oak, and Radha *et al* discuss cryopreservation of a medicinal Indian plant of *Icacinaceae* species. The Chapter by Burritt covers an interesting topic of action of proline as a "natural" multi-functional cryoprotectant that is accumulated in higher plants under stress, and can be considered as an attractive CPA candidate for cryopreservation in general.

Section *"Equipment and Assays"* is the last but definitely not the least important section of these two books, as the entire progress of cryobiology and cryopreservation depends on the development of devices and containers for cryopreservation, and proper and adequate assays of cryopreserved cells after resuscitation. The first chapter in *Book 1*, by Butler & Pegg, covers the precision in and control of cryopreservation, the pivotal components in the modern cryopreservation technologies. While this chapter covers mostly slow (equilibrium) programmed freezing, it is supplemented by a review by Cipri *et al*, which discusses some novel equipment and carriers, particularly for vitrification. To some extent it complements the last sub-section of our chapter 1, but we have to emphasize that none of the devices described in the Cipri's chapter can achieve the very rapid rates of cooling as many of the inventors claim. For example, the notion that *VitMaster* can achieve as high as 135,000 oC/min is largely overestimated even for very small samples, as slush freezing does not completely eliminate the Leidenfrost effect. In regard to assays, Partyka and colleagues review the methods of assessment of viability of cryopreserved sperm, many of which can be adapted to other types of cells as well. Finally, Pérez Campos *et al* present some interesting ideas on using X-ray diffraction for the assessment of quality of cryopreserved tissues in tissue banks in *Book 2***.**

In conclusion, *Books 1* and *2* cover a vast variety of topics regarding the current development of both fundamental cryobiology and practical aspects of cryopreservation, and we hope they will help the researches to grasp the background, state of the art, and future of this captivating and very important field of Life Sciences.

Igor I. Katkov, Ph.D.
Head of Cryobiology and Biostabilization
Stem Cell Center
Burnham-Sanford Institute for Medical Research
and
Chief Scientific Officer of CELLTRONIX
San Diego, California,
USA

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Part 1
Stem Cells and Cryopreservation in Regenerative Medicine

4
Validation of Primary Packaging for

Cryopreserved Musculoskeletal Tissues

Luiz Augusto Ubirajara Santos[1], Rosa Maria Vercelino Alves[2], Alberto T. Croci[1], Fábio Gomes Teixeira[2], Paulo Henrique Kiyataka[2], Marisa Padula[2], Mary Ângela Fávaro Perez[2], Monica Beatriz Mathor[3], Renata Miranda Parca[6], Arlete M.M . Giovani[1], Cesar Augusto Martins Pereira[4], Graziela Guidoni Maragni[1], Thais Queiróz Santolin[1] and Lucas da Silva Pereira[5]

1. Introduction

Bones and tendons are obtained from donors who have been pronounced brain dead after a rigid and extensive screening process. The tissues obtained are sent to a Tissue Bank and submitted to processing steps, packaging and cryopreservation at -80◦C. It is vital to maintain sterility and integrity, so that no tissue is discarded.These precautions also extend to the packaging, which should promote containment and protection. Although minimum technical criteria have already been defined for the food industry, this has still not been regularized in Brazil. We emphasize, therefore, the need to study this subject, focusing on maintaining the quality of the musculoskeletal tissues produced by tissue banks. All procedures developed byTissue Banks currently present in the country have rigid control over the quality and the traceability of tissues made available, based on international standards (EATB, 2004; AATB, 2007) on the legislation (BRASIL, 1997-2006) and in conformity with Good Manufacturing Practices - GMP.

2. Cryopreservation of musculoskeletal tissues

In the cryopreservation room, the tissues are stored according to their status in the process. Thus, there are designated areas for tissues under analysis or in quarantine (where they remain for around 60 days before the result of all the exams) and for those that have been liberated for use. both areas are equipped with ultra-low temperature freezers, with temperatures ranging from minus 80 to minus 100 degrees Celsius.

[1]*Institute of Orthopedics and Traumatology, Hospital das Clínicas of the University of São Paulo School of Medicine, Sao Paulo, SP, Brazil*
[2]*Packaging Technology Center – Institute of Food Technology, Campinas, SP, Brazil*
[3]*Nuclear and Energy Research Institute - IPEN/CNEN-SP – São Paulo, Brazil*
[4]*Biomechanics Laboratory - Institute of Orthopedics and Traumatology, Hospital das Clínicas of the University of São Paulo school of Medicine, Brazil*
[5]*Dental Student Institute of Orthopedics and Traumatology, Hospital das Clínicas of the University of São Paulo school of Medicine, São Paulo, SP, Brazil*
[6]*National Health Surveillance Agency- ANVISA*

The room is also equipped with an air conditioning system, its own energy generator, and carbon gas backup as protection against defrosting, as well as a rigorous temperature monitoring system that generates 24-hour printed temperature recordings, and a satellite alarm system, which ensures adequate maintenance of the temperature and early detection of any flaws.

Depending on the results of the analyses, the tissues are transferred to the room designated for materials liberated for use. The maximum cryopreservation period is 5 years for bone tissue and 2 years for soft or tendinous tissues.

It is vital to maintain sterility and integrity, in order to avoid disposal of any material. This is the purpose of **packaging**, which is aimed at containment and protection. In Brazil, there are no specific regulations for packaging of sterile, cryopreserved tissues for transplants, and in this chapter, we present our experience in the definition and validation of a type of packaging used for this purpose. Our proposal is to characterize a coextruded plastic film structure used for packaging of musculoskeletal tissues at low temperatures, in regard to the aspects sterility, cytotoxicity, migration, mechanical resistance and oxygen permeability.

Fig. 1. Cryopreservation of tissues at -80 ◦C in drawers, separated by batches.

3. Validation of packaging for use in tissue cryopreservation.

3.1 Description of our sample

A roll of transparent, unprinted seven layers, coextruded plastic:

Layer 01: LLDPE - 1-octene comonomer Linear Low-Density Polyethylene
Layer 02: LLDPE - 1-octene comonomer Linear Low-Density Polyethylene and 1-octene comonomer Linear Low-Density Polyethylene modified with anhydride maleic
Layer 03: PA - hexamethylenediamine, adipic acid e caprolactam copolyamide

Layer 04: LLDPE - 1-octene comonomer Linear Low-Density Polyethylene and 1-octene comonomer Linear Low-Density Polyethylene modified with anhydride maleic
Layer 05: PA - hexamethylenediamine, adipic acid e caprolactamcopolyamide
Layer 06: LLDPE - 1-octene comonomer Linear Low-Density Polyethylene and 1-octene comonomer Linear Low-Density Polyethylene modified with anhydride maleic
Layer 07: LLDPE - 1-octene comonomer Linear Low-Density Polyethylene
In relation to the physicomechanical and barrier properties characteristics, we investigated the alterations that occurred in the packages after 30, 60, 90, 120, and 150 days of cryopreservation at -80◦C. For the cytotoxicity and sterility analysis, two groups (before and after sterilization by ethylene oxide) were analysed.

3.2 Physicomechanical and barrier properties tests

A) Characterization of coex film in relation to its thickness and water vapor transmission rate (WVTR)

The thickness of each layer of plastic material in the film sample was determined through images captured by a Metaval inverted microscope operating at a magnification of 200x, using the image analysis system Axio Vision (Zeiss®). The cross-section of the sample was obtained using a Leica microtome, model RM2245, with section thickness set to 40 μm. To facilitate visualization of the barrier layer, 2% iodine solution was used as a contrasting agent. Five cross-section samples of the material were obtained; five measurements were performed for each sample, totalling 25 thickness measurements. The test was carried out at a temperature of around 23° C, after packaging of the sample in a controlled environment at 23° C ± 2° C and (50 ± 3)% relative humidity for a minimum period of 48 hours.

The water vapor transmission rate was determined using a MOCON PERMATRAN-W 3/31 device, following the procedure described in regulation ASTM F1249-06 - Standard test methods for water vapor transmission rate through plastic film and sheeting using a modulated infrared sensor. In this test, the water vapor that passes through the film is carried to the infrared sensor by ultra-dry nitrogen flow. The sensor measures the fraction of energy absorbed by the water vapor, and emits an electrical signal with amplitude proportional to the concentration of water vapor. The range of this signal is compared with that of the signal produced by the water vapor that passes through a calibration film with a known water vapour transmission rate. The effective permeation area of each sample was 50cm2. The assay was performed at 38◦C/100%RH and in this condition, the calibration standard showed water vapour transmission rate of 4.54g water.m-2.day-1. The water vapor transmission rate of the sample was corrected for the condition 38◦C/90%RH, multiplying the results by a factor of 0.9.

The total thickness of each layer of coextruded film is shown in Table 1. Figure 10 shows an example of a cross-section image of the sample obtained for the assay.

Determinations	**Thickness (μm)**		
Mean	**Variation Interval**	**Coefficient of variation (%)**	
Total	91.1	90.0 - 92.9	0.9
LLDPE (A)	27.3	26.2 - 28.5	2.4
LLDPE blend (B)	7.6	7.0 - 8.0	3.8
PA (C)	5.6	5.1 - 5.9	4.8
LLDPE blend (D)	6.8	5.3 - 7.8	11
PA (C)	5.6	5.0 - 6.0	5.6
LLDPE blend (F)	7.7	7.1 - 8.6	5.9
LLDPE (G)	31.0	30.1 - 32.1	1.9

Values for 25 measurements
A-G: Text for visualization in Figure 10

Table 1. Total thickness of each layer of sample of coextruded plastic film.

Thus, by optical microscopy, it was observed that it is a coextruded material with seven layers, with two intermediate layers of PA of approximately 6 μm each. The other LLDPE layers totalled 80 μm.

Fig. 2. Example of cross-section image of the sample obtained with the microscope operating at a magnification of 200x.

The characterization of the film in relation to water vapor transmission rate is shown in Table 2.

Sample	WVTR (g water . m-2.day-1) Mean	VI	CV (%)
Coextruded film LLDPE / LLDPE / PA / LLDPE / PA / LLDPE / LLDPE	3.30	3.2 - 3.4	4.4

Values relate to four measurements
VI - variation interval; CV - coefficient of variation
Table 2. Water vapor transmission rate (WVTR) of the coextruded plastic film at 38°C/90%RH - Permatran-W 3/31 method.
The WVTR depends on the thickness of the LLDPE layers of the coex film (80 μm).

B) Penetration resistance

The penetration resistance of the coextruded film was determined based on Standard ASTM F 1306-90 (2008) e1 - Standard test method for slow rate penetration resistance of flexible barrier films and laminates, on an Instron 5500R universal testing machine, using load cells of 100 N. The speed of penetration, performed with a spherical-tipped metal probe with diameter of approximately 3.2 mm, was 25 mm/min. The penetration was performed from the inner surface to the outer surface of the material. The test was conducted in an environment of 23° C ± 2° C and (50 ± 3)% relative humidity, after leaving the packaged samples for a minimum period of 48 hours in this environment.
The evaluation of penetration resistance was carried out on samples in their original or nonfrozen
condition (0) and 30, 60, 120 and 150 days after freezing at -80 ◦C. The data are shown in Table 3 and Figure 3.

Penetration resistance	Evaluation periods (days) 0	30	60	90	120	150
Force at break (N)						
Mean	12.1 ^{ab}	12.6 ^{bc}	12.9 ^{c}	12.6 ^{bc}	11.1 ^{d}	11.8 ^{a}
VI	11.5-12.7	11.8- 13.5	11.9- 14.2	11.2- 14.1	9.4 - 12.3	10.9- 12.4
CV (%)	3.1	3.6	4.7	6.9	9.1	3.7
Deformation at break (mm)						
Mean	9.4 ^{a}	11.0 ^{b}	10.0 ^{c}	9.8 ^{c}	8.3 ^{d}	9.0 ^{e}
VI	9.2 - 9.8	10.7- 11.4	9.5 - 10.5	8.9 - 10.6	7.4 - 9.1	8.2 - 9.5
CV (%)	2.3	2.3	2.6	5.2	5.5	3.7
Energy at break (mJ)						
Mean	70 ^{ad}	76 ^{b}	78 ^{b}	73 ^{ab}	62 ^{c}	69 ^{d}
VI	64 - 74	70 - 84	72 - 85	64 - 86	52 - 73	62 - 73
CV (%)	4.6	5.5	5.4	8.6	11	5.6

Values relate to 10 measurements: 1 N = 0.102 kgf
VI/CV: Variation interval/Coefficient of variation
a,b,c,d: for an analyzed property, mean values on the same line accompanied by the same superscript letter did not show any difference between them in the least significant difference (LSD) test, at a level of error of 5%.
Table 3. Penetration resistance of the sample during storage at -80° C.

Fig. 3. Penetration resistance of the sample during the period of storage at -80° C (days).
The results shown in Table 3 and Figure 3 demonstrate that the process of sterilization and packaging of human bone, and storage for 150 days at -80° C, did not alter the penetration properties of the coextruded film. Variations were observed in the results of the three properties evaluated, including statistical differences in some periods/properties, but as these variations were small, without any clearly-defined trend, no alteration is expected in penetration resistance of the film during packaging and storage of human tissue for a period of 150 days at -80 ° C.

C) Seal strength

The seal strength of the packaging was determined according to ASTM F 88/F 88M-09 - Standard test method for seal strength of flexible barrier materials. Samples of 25.4 mm in

width were submitted to tensile test in a 5500R Instron universal testing machine, operating with load cells of 50N and 100N, at a speed of 300mm/min. The distance between the fixing clamps and the sample was 25mm. The test was conducted in an environment of 23 ° C ± 2 ° C and (50 ± 3)% relative humidity, after leaving the pre-prepared, packaged samples in this same environment for at least 48 hours.

The strength of the side seal is different from that of the top seal, because the top seal of the
packaging was made after the bone tissue packaging process, while the side seals were made by the package manufacturer.

Despite this, there was no statistical difference between the results obtained for both seals. Thus, the sterilization and packaging process of human tissue, and storage for 150 days at -80° C did not alter the seal strength.

Maximum seal strength (kgf/25.4 mm)						
Evaluation periods (days)	**0**	**30**	**60**	**90**	**120**	**150**
Side seal						
Mean	3.60 ab	3.65 b	3.60 ab	3.73 b	3.73 b	3.44 a
VI	3.36 - 3.95	3.41 - 3.83	3.38 - 3.80	3.34 - 3.97	3.43 - 3.96	3.12 - 3.78
CV (%)	4.4	**4.0**	**4.4**	**5.7**	**4.5**	7.0
Top seal						
Mean	- 4.69 a	4.75 a	5.07 a	5.09 a	4.84 a	
VI	- 3.65 - 5.20	3.20 - 5.85	4.31 - 5.95	4.68 - 5.54	4.48 - 5.44	
CV (%)	- 9.6	15	9.6	5.1	6.6	

Values relate to ten determinations: 1 kgf/25.4 mm = 386.1 N/m

VI/CV: Variation interval/Coefficient of variation

a,b: for a seal type, mean values on the same line accompanied by the same superscript letter did not show any difference between them in the least significant difference (LSD) test, at a level of error of 5%.

Table 4. Seal strength during storage at -80° C.

Fig. 4. Maximum seal strength during the period of storage at -80° C (days).

D) Oxygen transmission rate

The oxygen transmission rate at humid was determined by the coulometric method, according to the procedure described in ASTM F 1927 - Standard test method for determination of oxygen gas transmission rate, permeability and permeance at controlled relative humidity through barrier materials using a coulometric detector, on MOCON OXTRAN device, model 2/20, operating with pure oxygen as permanent gas. The tests were carried out at 23ºC to 75%RH, with the samples packaged for 88 to 112 hours in a temperature-controlled room at 25°C and 75%RH. The effective area of permeation of each sample was 50cm2. The result obtained was corrected for 1 atm of partial pressure gradient

of oxygen.

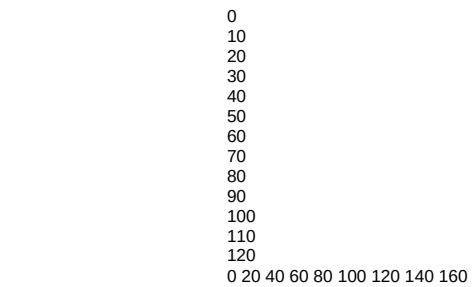
O_2TR mL (STP).m-2.day-1 at 23° C/75%RH	**Evaluation periods (days)**					
	0	**30**	**60**	**90**	**120**	**150**
Mean	69.75 a	75.95 a	74.97 a	82.57 a	76.98 a	113.67 b
VI	68.63-70.87	71.77-80.14	74.48-75.46	79.34-85.80	73.83-80.13	105.05-122.29
CV (%)	2.3	7.8	0.9	5.5	5.8	10.7

values relate to two determinations
VI - variation interval; CV - coefficient of variation
a,b: for a seal type, mean values on the same line accompanied by the same superscript letter did not show any difference between them in the least significant difference (LSD) test, at a level of error of 5%.

Table 5. Oxygen transmission rates (O_2TR) during storage at -80° C.

Fig. 5. Oxygen transmission rates (O_2TR) during storage at -80° C.

The results of the oxygen permeability rates shown in Table 5 and Figure 5 indicate a mean increase of 10% in permeability after the process of sterilization, vacuum packaging of the bone, and storage at -80° C, which was observed after 30 days of storage. This tendency to increase, probably due to humidification of the PA, led to a small loss of barrier which was maintained throughout the storage period of 120 days. Meanwhile, in the analysis of samples from 30 days of storage at -80° C, a high oxygen transmission rate of was observed, which was not expected, and as we did not have any more stored samples, it was not possible to re-evaluate this result. This higher O2TR may be the result of some variation in thickness of the PA in the samples evaluated in this period. In any case, the level of oxygen transmission rate of the film did not lead to loss of vacuum in the samples stored in the Tissue Bank for 150 days at -80° C.



E) Overall Migration

The evaluations of overall migration were performed according to Resolution 51 of 26 November 2010 published by *Agência Nacional de Vigilância Sanitária do Ministério da Saúde* (National Agency of Sanitary Surveillance of Health Ministry) in Diário Oficial da União (official journal of the Brazilian Government) on 30 November 2010. This Resolution internalizes Mercosul Technical Regulation GMC 32/10.

The methodology to quantify the overall migration was according to the method of the European Standard EN 1186-1: materials and articles in contact with foodstuffs. Plastics. Part 1: guide to the selection of conditions and test methods for overall migration, EN 1186-

3: Materials and articles in contact with foodstuffs. Plastics. Test methods for overall migration into aqueous food simulants by total immersion and EN 1186-14: materials and articles in contact with foodstuffs. Plastics. Part 1: Part 14: test methods for "substitute tests"
for overall migration from plastics intended to come into contact with fatty foodstuffs using test media isooctane and 95% ethanol and consists of the sample contact with extraction solutions in certain periods and temperatures that simulate their actual condition of use. The residues of overall migration were determined by the weight difference after the contact and evaporation of the solutions through an analytical scale with 0.01mg of accuracy. The sample was evaluated under the contact conditions shown in Table 6.

Model Solution Contact condition

Ultra purified water 40ºC/10 days
Acetic acid solution in Ultra purified water at 3% (w/v) 40ºC/10 days
Isooctane 40ºC/10 days

Table 6. Conditions of time and temperature used in the overall migration.

The results of the overall migration tests performed on the transparent coextruded plastic film,
obtained using the model solutions and the specific contact conditions are shown in Table 7.

Model Solution/Contact Condition | **Maximum limit of overall migration** | **Sample Mean Standard Deviation** | **Variation interval**

Ultra purified water/ 40ºC/10 days | 8.0 | Before EtO ≤0.72 | 0.21 ≤0.50(2) - 1.00
After EtO ≤0.51 | 0.02 ≤0.50(2) - 0.55
3% Acetic acid solution (w/v)/ 40ºC/10 days | 8.0 | Before EtO ≤0.54 | 0.09 ≤0.50(2) - 0.67
After EtO 0.92 | 0.19 | 0.72 - 1.12
Isooctane/ 20ºC/48 hours | 8.0 | Before EtO ≤1.21 | 0.64 ≤0.50(2) - 1.85
After EtO ≤1.50 | 0.72 ≤0.50(2) - 2.05

(1) Result of four determinations.
(2) Limit of quantification of the method in the analytical conditions used.
(3) Not applicable.

Table 7. Residues of overall migration obtained for the transparent coextruded plastic film, before and after the application of EtO, in mg/dm2 (1).

The maximum limit of overall migration provided by Resolution n°105/99 is of 8mg of residue per dm2 of contact plastic material, with an analytical tolerance of 10%. Therefore 8.8mg/dm2 is the maximum tolerable value.
The overall migration values found in the samples analyzed, in the analytical conditions used, were below the established limit. There was no statistical significance with sterilization with ethylene oxide (EtO).

E 1.) Physicochemical tests – According to USP 33

The physicochemical tests were conducted based in the methodology describe in the Chapter <661> Containers - Plastics - Physicochemical Tests of the **United States Pharmacopeia (USP 33)**.

In accordance to the United States Pharmacopeia, physicochemical tests are designed to determine physical and chemical properties of plastic materials. The extracts methodology consists of the sample contact with a extraction solution (deionized water) at 70°C during 24 hours, maintaining the ratio area / volume of 120 cm^2 total surface area of plastic material for each 20 mL of extraction solution.
The analyzed sample was received in the form of the film cut into strips with dimensions of 5.0 cm long, 0.3 cm wide and thickness less than 0.1 cm. In this case, the thickness of the material to determine the total area was not considered and was maintained the ratio of 120 cm^2 for each 20 mL of extraction solution. Water was used as extraction solution.
After the contact, the extraction solution and blank reagent were analyzed by the following tests:
Buffering Capacity: Titrate 20 mL of the extraction solution potentiometrically to a pH of 7.0, using 0.01 N sodium hydroxide. Treat a 20.0 mL portion of the blank reagent similarly. The difference between the two volumes can not be greater than 10.0 mL.
Nonvolatile Residue: 50 mL of the extraction solution were evaporated on a hot plate, after the residue was dried at 105°C for 1 hour on a oven and finally the nonvolatile residue was weighted through an analytical balance with 0.01mg of accuracy. Treat a 50.0 mL portion of the blank reagent similarly. The difference between the two volumes can not be greater than 15.0 mg.
Residue on Ignition: in the residues obtained in nonvolatile residues test, add sulfuric acid and burn on the muffle furnace until constant weight. Treat the blank reagent similarly. The difference between the two volumes should not be greater than 5.0 mg. It is not necessary to perform this test when the nonvolatile residue test result does not exceed 5.0 mg.
Heavy Metal, as lead: an aliquot of extraction solution has been transferred to a volumetric flask and acidified with nitric acid and the volume was completed with the extraction solution. After treating the sample, the lead content was quantified by atomic emission spectrometry induced by plasma, with an optical detector, in a Perkin Elmer equipment, model OPTIMA 2000DV, using appropriate calibration curves for the analyses. This test was conducted in replacement to the heavy metal test stablished by American Pharmacopeia, whose result is expressed as lead (and is based on the colour comparison among the test solution and a solution of lead with concentration of 1.0 mg / kg).

Physicochemical Assay	**Limit based on USP**	**Sample Mean**	**Standard deviation**	**Variation interval**
Buffering capacity (mL)	10.0 mL			
Before EtO	≤ 0.5	(2)	(3)	(3)
After EtO	≤ 0.5	(2)	(3)	(3)
Non-volatile residue: (mg)	15 mg			
Before EtO	≤ 1.0	(2)	(3)	(3)
After EtO	≤ 1.0	(2)	(3)	(3)
Heavy Metals (as lead) (mg/kg (ppm))	1 mg/kg (ppm)			
Before EtO	≤ 0.05	(2)	(3)	(3)
After EtO	≤ 0.05	(2)	(3)	(3)

(1) Results of three determinations.
(2) Corresponds to the limit of quantification of the method in the analytical conditions used.
(3) Values no applicable

Table 8. Physicochemical assays of the analyzed samples(1).

E 2.) Assays According to the European Pharmacopoeia

The tests acidity or alkalinity, sulfated ash, absorbance, extractable aluminum, chromium, titanium, vanadium, zinc, zirconium and extractables from heavy metals, expressed as

lead were conducted based in the methodology described in the European Pharmacopoeia, Chapters “3.1.3 Polyolefines”, “3.1.4 Polyethylene without Additives for Containers for Parenteral Preparations and for Ophthalmic Preparations” and “3.1.5 Polyethylene with Additives for Containers for Parenteral Preparations and for Ophthalmic Preparations”.

Extractables of aluminium, chromium, titanium, vanadium, zinc and zirconium

The methodology for quantification of extractables aluminum, chromium, titanium, vanadium, zinc and zirconium involved contact of 100 grams of sample with a solution of 0.1 M hydrochloric acid for one hour at the reflux temperature. After treating the sample, the metals contents were quantified by atomic emission spectrometry induced by plasma, with an optical detector, in a Perkin Elmer equipment, model OPTIMA 2000DV, using appropriate calibration curves for the analyses.

Extractables of Heavy Metals, expressed as lead

The same procedure described for quantification of extractable aluminum, chromium, titanium, vanadium, zinc and zirconium was used. The method for quantification of extractable lead was used instead of the colorimetric method of determination of heavy metals established by the European Pharmacopoeia, which is based on color comparison between the extracting solution and a solution of lead at a concentration of 2.5 mg/kg.

Alkalinity or Acidity, and Absorbance

The method for quantification of alkalinity or acidity and absorbance involved contact of 12.5 grams of sample with 250 mL of deionized water for five hours at the reflux temperature.

After the extraction time, both the extracting solution in contact with the samples and a blank solution (reference) were assessed as to the following tests:

Alkalinity and Acidity: measurement of pH in a Micronal pHmeter, model B 474 and titration of 100 mL of extracting solution with sodium hydroxide 0.01M or hydrochloric acid 0.01M up to pH 7.0.

Absorbance: absorbance of the extracting solution was measured in the 220 nm to 340 nm wavelength range, using a quartz cuvette with 10 mm pathlenght in a UV / VIS spectrophotometer, using an Analytik Jena instrument, model Specord 210.

Sulphated Ash

Sulfated ash were assessed per requirements in European Pharmacopoeia 6.0, Chapter 2.4.14 - Sulfated Ash. The method for quantification of sulfated ash consisted in weighing 5.00 g ± 0.01 g of sample on an analytical scale with 10^{-5} g resolution and incineration at a temperature of 600 ° C ±20 °C using a Milestone microwave heating furnace, model Pyro. After incinerating the sample, the ash were determined gravimetrically, using an analytical scale with 10^{-5} g resolution. There was not need to use sulfuric acid.

Extractable aluminum, chromium, titanium, vanadium, zinc and zirconium

The results of the extractable aluminum, chromium, titanium, vanadium, zinc and zirconium tests for the analyzed sample are shown in Table 9.

Extractables	**Limit based on European Pharmacopeia 6.0**	**Sample Mean**	**Standard Deviation**	**Variation interval**
Al	1.0			
Before EtO		0.71	0.08	0.62 - 0.78
After EtO		0.74	0.06	0.70 - 0.81
Cr	0.05			
Before EtO		0.03	0.00	0.02 - 0.03
After EtO		0.33	0.05	0.27 - 0.37
Ti	1.0			
Before EtO		≤0.10(2)	(3)	(3)
After EtO		≤0.10(2)	(3)	(3)
V	0.10			
Before EtO		≤0.10(2)	(3)	(3)
After EtO		≤0.10(2)	(3)	(3)

Zn 1.0
Before EtO 0.05 0.00 0.05 - 0.06
After EtO 0.05 0.01 0.05 - 0.06
Zr 0.10
Before EtO ≤0.05(2) (3) (3)
After EtO ≤0.05(2) (3) (3)
(1) Result of four determinations.
(2) Corresponds to the limit of quantification of the equipment in the analytical conditions used.
(3) Not applicable.
Table 9. Extractables of the metals aluminum (Al), chromium (Cr), titanium (Ti), vanadium (V), zinc (Zn) and zirconium (Zr), in mg/kg(1).

Extractable Heavy Metal, Expressed as Lead

The results of the extractable heavy metals, expressed as lead, acidity, absorbance and sulphated ash tests, for the analyzed sample are shown in Table 10.

Physicochemical
Assay
Limit based on
European
Pharmacopeia 6.0
Sample Mean Standard
Deviation
Variation
interval
Extractables of Heavy
Metals, expressed as
lead
(ppm)
2.5mg/kg (ppm)
Before EtO ≤0.1(2) (3) (3)
After EtO ≤0.1(2) (3) (3)
Acidity
(mL of NaOH 0.01 M)
1.5 mL de sodium
hydroxide 0.01 M
Before EtO ≤0.5(2) (3) (3)
After EtO ≤0.5(2) (3) (3)
Absorbance
(UA) 0.2 UA
Before EtO 0.23 0.01 0.22 - 0.23
After EtO 0.24 0.04 0.22 - 0.26
Sulphated Ash
(%) 0.02% and 1.00%(4)
Before EtO 0.29 0.01 0.28 - 0.30
After EtO 0.25 0.12 0.11 - 0.33
UA- Unit of Absorbance.
(1) Results of three determinations.
(2) Corresponds to the limit of quantification of the method in the analytical conditions used.
(3) Values no applicable
(4) Varies according to the presence of additives
Table 10. Results for heavy metals, expressed as lead, acidity, absorbance and ash of the samples analyzed (1).

The lead values, volume of 0.1 M sodium hydroxide used, and sulphated ash values found in the samples analyzed were below the maximum limits established in the European Pharmacopeia 6.0. In relation to absorbance, the values obtained in the two samples analyzed, before and after the application of sterilization with ethylene oxide (EtO), were slightly higher than the maximum limits established.

A038-2/11 - Packaging for human tissue - Final Report 17/20 This means that some substance of the coextruded film may have migrated to the extraction solution in contact with the sample (deionized water), a fact that requires further investigation.

It should be emphasized that the methodology of the American and European Pharmacopeias apply to single-layer packaging, and that substances from internal layers of the film analyzed

may have been extracted, slightly increasing the absorbance of the extraction solution.

E 3.) Specific migration

The extruded film before and after sterilization with ethylene oxide was evaluated in relation to specific migration of 1-octene and ε-caprolactam. The evaluations of specific migrations of 1-octene, ε-caprolactam and hexamethylenediamine were carried out according to the Brazilian legislation.

E 3.1) Specific migration of 1-octene

The quantification of specific migration of 1-octene was evaluated based on Standard **CEN/ TS 13130-26**: materials and articles in contact with foodstuffs - Plastics substances subject to
limitation - Part 26:

Determination of 1-octene and tetrahydrofuran in food simulants, and consists of contact of the sample with solutions of extraction with times and temperatures that simulate its real condition of use.

The internal sides of the samples were placed in contact with the simulants, obeying an area:volume ratio of 600 cm2 to 1000 mL. The same was evaluated in the contact conditions shown in Table 11.

Simulants/Contact Condition	**Maximum limit of specific migration of 1-octene**	**Sample Mean (1)**	**Standard Deviation**	**Variation Interval**
Ultra purified water/ 40ºC/10 days	15	Before EtO	≤2.8(2) (3) (3)	
		After EtO	≤2.8(2) (3) (3)	
3% Acetic acid solution in ultra purified water (w/v)/ 40ºC/10 days	15	Before EtO	≤1.6(2) (3) (3)	
		After EtO	≤1.6(2) (3) (3)	
Olive oil/ 40ºC/10 days	15	Before EtO	≤8.7(2) (3) (3)	
		After EtO	≤8.7(2) (3) (3)	

(1) Result of three determinations.
(2) Quantification Limit of the method under the analytical conditions.
(3) Not applicable.

Table 11. Specific migration of 1-octene obtained for transparent coextruded plastic film, before and after the application of EtO, in mg/dm2.

The specific limit of monomer migration of 1-octene established in Resolution 105/99 of the National
Health Surveillance Agency - ANVISA of 19 May 1999 is 15 mg/kg of simulant. The values for specific migration of 1-octene found in the samples analyzed, in the analytical conditions used, were below the established limit. Sterilization with ethylene oxide (EtO) did not affect the monomer migration potential of 1-octene.

E 3.2) Specific migration of ε-caprolactam

The quantification of specific ε-caprolactam was evaluated based on Standard **CEN/ TS 13130-16**: materials and articles in contact with foodstuffs - Plastics substances subject to limitation - Part 16: Determination of caprolactam and caprolactam salt in food simulants.

The internal surfaces of the samples were placed in contact with the simulants, obeying an area:volume ratio of 600 cm2/1000 mL. The samples were evaluated under the contact conditions shown in Table 12.

The specific limit of monomer migration of ε-caprolactam established in Resolution 105/99 of the National Health Surveillance Agency - ANVISA of 19 May 1999 is 15 mg/kg of simulant. The values for specific migration of ε-caprolactam found in the samples analyzed, in the analytical conditions used, were below the established limit. Sterilization with ethylene oxide (EtO) did not affect the potential monomer migration of ε-caprolactam for the fatty simulant, but was significantly lower (probability of 95% confidence - Tukey Test)

for the ultrapure water simulants and 3% acetic acid solution (m/v) in ultrapure water after sterilization with ethylene oxide.

Simulants/Contact Condition	**Maximum limit of specific migration of** ε-caprolactam	**Sample Mean** (1)	**Standard Deviation**	**Variation Interval**
Ultra purified water/ 40ºC/10 days	15			
Before EtO		2.4	0.3	1.9 - 2.7
After EtO		1.7	0.2	1.3 - 1.9
3% Acetic acid solution in ultra purified water (w/v)/ 40ºC/10 days	15			
Before EtO		2.7	0.1	2.6 - 2.8
After EtO		2.2	0.3	1.8 - 2.6
Olive oil/ 40ºC/10 days	15			
Before EtO		3.4	0.4	3.0 - 4.0
After EtO		3.2	0.2	2.9 - 3.5

(1) Result of three determinations.

Table 12. Specific migration of ε-caprolactam obtained for transparent coextruded plastic film, before and after the application of EtO, in mg/dm2.

E 3.3) Specific migration of hexamethylenediamine

The quantification of specific hexamethylenediamine migration was evaluated based on Standard **CEN/TS 13130-21**: materials and articles in contact with foodstuffs - Plastics substances subject to limitation - Part 21: Determination of ethylenediamine and hexamethylenediamine in food simulants. The sample of hexamethylenediamine, adipic acid e caprolactam copolyamide in the form of a film, was placed in contact with the simulants, obeying an area:volume ratio of 600 cm2 to 1000 mL. The sample was evaluated under the contact conditions shown in Table 13.

Simulants/Contact Condition	**Maximum limit of specific migration of** hexamethylenediamine	**Mean** (1)	**Standard Deviation**	**Variation Interval**
Ultra purified water/ 100 °C/30 minutes + 40 ºC/10 days	2,4	≤1.2(2)	(3)	(3)
3% Acetic acid solution in ultra purified water (w/v)/ 100 °C/30 minutes + 40 ºC/10 days	2,4	≤1.1(2)	(3)	(3)
Olive oil/ 100 °C/30 minutes + 40 ºC/10 days	2,4	≤2.0(2)	(3)	(3)

(1) Result of three determinations.

(2) Quantification Limit of the method under the analytical conditions.
(3) Not applicable.
Table 13. Specific migration of hexamethylenediamine obtained for a 42 μm film copolyamide, in mg/kg.

The specific limit of monomer migration of hexamethylenediamine established in Resolution 105/99 of the National Health Surveillance Agency - ANVISA of 19 May 1999 is 2.4 mg/kg of simulant. The values for specific migration of hexamethylenediamine found in the analysis of the 42 μm film copolyamide, under the analytical conditions used, were below the established limit.

3.3 Cytotoxicity assay

Assay performed in accordance with Standard ISO 10993-5: Biological evaluation of medical devices - Part 5: Cytotoxicity Assays: *in vitro* methods in samples of coextruded plastic film.

3.3.1 Cytotoxicity

Definitions of cytotoxicity vary, depending on the nature of the study and whether cells are killed or simply have their metabolism altered. Cytotoxicity is the toxicological effect that a
substance can cause *in vitro*, at cellular level (Freshney, 2000).
As defined in ISO 10993, "the numerous methods used and end-points measured in cytotoxicity determination can be grouped into categories of evaluation type, like assessments of cell damage by morphological means; measurements of cell damage; measurements of cell growth and measurements of specific aspects of cellular metabolism" [IOS, 2010]. The cells can be exposed to the samples or their extracts.

3.3.2 The protocols

Cell culture

Chinese hamster ovary cell line (CHO-k1) was standardized for cytotoxicity and genotoxicity tests. Cells were maintained in RPMI medium supplemented with antibiotics and antimycotics (100 units/mL penicillin, 100 μg/mL streptomycin and 0.025 μg/mL amphotericin), 2mM glutamine, and 10% calf serum, at 37°C in a humidified 5% CO_2 atmosphere until they reached confluence. For subculturing and for experiments, cells were harvested using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4.

Extract preparation

Samples of packaging, before and after sterilization, were submitted to this assay. The samples were immersed separately in RPMI culture medium at a final concentration of 1 cm^2 / mL and left in an incubator at 37°C for 72 hours to fulfil the extraction condition. The first concentration was sterilized by filtration and the subsequent dilutions were performed in sterile RPMI medium at a ratio of 1:2.

Cytotoxicity test

A colorimetric method that uses the tetrazolium compound MTS was used to determine the number of viable cells in proliferation (Cory et al, 1991). 96-well microplates were prepared with 50 μL of extract diluted from 100 to 6.25% in RPMI medium in quadruplicates. The positive control was a phenol solution (0.5% v/v) as 100% concentration and the negative control was a high density polyethylene (HDPE) extract. The 100% concentration was the

non-extract well A suspension of CHO-k1 (from second to fourth passages after thawing) with 6 x 10^4 cell/mL was prepared and 50 μL/well was pipetted into the microplates. The microplates were incubated for 72 hours at 37°C in a humidified 5% CO_2 atmosphere. Blank and controls of the cells were also prepared. Cell viability was measured by adding 20 μL of MTS/PMS (20:1) solution to the humidified 5% CO_2 incubator, followed by incubation for 2 hours at 37°C. The microplates were read in a spectrophotometer reader at 490 nm.
Cell viability was calculated by the equation:

$$CV\% = \frac{OD_{sample}}{OD_{nonextract}} \times 100$$

Where: CV% = cell viability, OD sample = optical density at 490 nm of the extract dilution, OD non extract = optical density at 490 nm of the well without extract.
The results consider the following parameters:
a. Controls (positive and negative)
Positive control: 0.5% Phenol solution
Negative control: HDPE (high-density polyethylene) extract.
b. Observations: definitions of some terms.

- Positive control: material which, when tested according to Standard ISO 10993-5, promotes a cytotoxic response.
- Negative control: material which, when tested according to Standard ISO 10993-5, does not promote a cytotoxic response.
- $IC_{50(\%)}$: cytotoxicity index 50%, concentration of extract that kills 50% of the viable cell population.

The results of the assay showed that the samples of packaging material, before and after sterilization by ethylene oxide (EtO), resulted in viability of over 90%, and therefore do not become cytotoxic.

3.4 Sterility assay and ethylene oxide residues

The samples were classified according to their position (0 to 5a; 0 to 5b) during exposure to EtO, for the penetration analysis. The outermost or surface position corresponds to the number 5, and the innermost position to the number 0. (Figure 6)

Sterility tests were carried out through the analyses of two biological indicators (bi 3M - ATTEST -™ Bacillus atrophaeus and Terragene - Bionova BT40 - Bacillus atrophaeus). Incubation time was 48 h at a temperature of 35° C ± 1.5.

Samples of packaging were also submitted to direct incubation for 7 days with TSB liquid culture at a temperature of 35° C ± 1.5. The methodology used is in accordance with the Brazilian Pharmacopoeia. The analyses of the three sterility tests (biological indicator Bionova BT40, 3Mattest and direct incubation) confirm the sterility of the packaging material after being submitted to ethylene oxide gas.

Fig. 6. Samples of packaging positioned from 0 to 5 for EtO penetration analysis.

Fig. 7 and 8. Performance of the sterility test by direct incubation in samples submitted to ethylene oxide.

The analysis of ethylene oxide residues was performed by the Gas Chromatography test, determining the levels of Ethylene chlorohydrin and Ethylene glycol. The data are shown in **Figure 9** and show that the levels are within the limits accepted by our legislation.

Note. Maximum limits according to the Brazilian legislation. [ETO up to 25/ ETCH up to 25/ETG up to 250]

Fig. 9. Residues (ETO, ETCH, ETG) found in the samples submitted to sterilization.

4. Final considerations

Analyses of the packaging used in this study demonstrated that it is a good option for cryopreservation of tissues at a temperature of - 80°C.

Our experience with assays to validate coextruded polyethylene and polyamide plastic film shows that the mechanical properties of this material are not altered by cryopreservation and sterilization. Penetration resistance of the thermal seal remained unaltered after all the processes carried out in a tissue bank, such as sterilization and cryopreservation.

We found a loss of barrier due to increased oxygen permeability of around 10% after sterilization and cryopreservation, which can be explained by the humidification of the polyamide. However, this slight alteration in oxygen permeability does not compromise the inner vacuum of the packaging, and does not place at risk the tissue packaged in it.

In relation to total migration, we did not observe any alterations in the assays, i.e. once again, sterilization and cryopreservation did not lead to monomer migration at levels above

0
5
10
15
20
25
30
35
40
5A 4A 3A 2A 1A 0 1B 2B 3B 4B 5B
ETO
ETCH
ETG

those required by our legislation. This is also valid for specific migrations of 1-octene, ε -

caprolactam and hexamethylenediamine.
The application of ethylene oxide is safe for sterilization of this type of packaging, as it results
in good penetrability and safe levels of Eto, Etch and Etg residues at the end of the procedure.
In the cytotoxicity test, we observed levels of cell viability of over 90%, therefore they do not
become cytotoxic.
Thus, analyses of coextruded plastic polyethylene and polyamide film used in this study proved to be a good option for cryopreservation of tissues at temperatures of -80°C, even for prolonged periods of 150 days.

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5

Cryopreservation – A Viable Alternative in Preparation for Use of Allografts in Knee Ligament Reconstruction

Alexandre C. Bitar et al.*
Vita Institute
Brazil

1. Introduction

The use of allogenic tissues is growing in orthopedic practice, as well as the number of studies on methods for processing, sterilization and cryopreservation that interfere as little as possible with the original physiological properties of the tissues (Nutton et al., 1999).

In addition to bone tissue, other tissues of the locomotor system can be captured, processed and stored in tissue banks with the purpose of transplantation. Therefore a strict quality control must be implemented and set after discussions compiled by international organizations such as AATB e EATB.

The first report of the use of allografts in humans dates back to 1881. The first tissue bank of bone grafts was created in 1940 in the United States and the initial clinical results were published in 1942 by Inclan, 1942. Since then a series of regulations and studies has emerged relating to the use of grafts in orthopedic practice19. Currently, tendon allografts are used in knee surgeries, in elbow ligament reconstructions and in revisions of the acromioclavicular joint (Costic et al., 2004).

In our country we have few tissue banks. The tissue bank (BTME) from the Institute of

Orthopedics and Traumatology (IOT), Hospital das Clínicas da Faculdade de Medicina da Universidade de Sao Paulo was the first and is the biggest bank in activity nowadays. It thas been in operation since 1999 and is governed by local legislation (Amatuzzi et al., 2000). With the restructuring of our service in 2005, we initiated a new technique aimed at the provision of the tendon with a well-structured quality program in line with other centers of excellence nationally and internationally. Today our service is provided by a series of tendons (tibial tendon, Achilles, patellar and peroneal) taken from different regions with very specific applications. Thus, we can then follow the technological trend in the use of

*Caio Oliveira D' Elia[1], Antônio Guilherme P. Garofo[1], Wagner Castropil[1], Luiz Augusto U. Santos[2], Marco K. Demange[2], José Ricardo Pécora[2] and Alberto T. Crocci[2]
[1]*Vita Institute, Brazil*
[2]*Universidade de São Paulo; Institute of Orthopedics and Traumatology, Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo, SP, Brazil*

tissues already practiced in other global centers of excellence in orthopedics and traumatology.

In our medical service, Vita Institute (private use), and in the Institute of Orthopedics and Traumatology (public and private use), allografts are used mainly in knee surgeries, ACL reconstruction, multiple ligament reconstructions, ligament surgery in skeletally immature patients and with double bundle reconstruction.

2. Importance of allografts in knee ligament reconstructions

Grafts are used in various procedures in different branches of orthopedics, including ligament reconstruction. The literature shows the importance of the use of allografts in knee surgery, especially in revision surgeries, multiple ligament reconstructions and, more recently, surgery for primary reconstruction of the anterior cruciate ligament (ACL) in active patients and in those aged over 40 years (Marrale et al., 2007; Sherman & Banffy, 2004). There have been at least 11 clinical studies comparing the use of auto and allografts in the reconstruction of the ACL (Chang et al., 2003; Marrale et al., 2007). Most of these show little
difference between the two techniques with respect to long-term results. However, there has been few prospective randomized studies , and the comparison methods (scores), types of graft, as well as methods of preparing and fixing the graft are highly variable. Furthermore, most studies use the patellar tendon graft; therefore, it may not be possible to generalize the conclusions of these studies to the flexor tendon. Some authors, such as Lawhorn and Howell, suggest the use of allografts without a bone plug because of the potential for slower incorporation of the bone due to immunogenicity and smaller crosssectional
area of transplants with bone plugs (Lawhorn & Howell, 2003). Recently, Sun et al. (2011) published a prospective randomized study comparing non-irradiated allograft with flexors autograft showing similar results between groups in terms of subjective clinical scores, goals, rate of return to sports and incidence of complications (Sun et al., 2011). However, compared with autologous transplants, allografts do have some advantages. For example, they do not increase morbidity for the donor, they require a shorter surgery time, and they are available without restriction on size and morphology. In ligament reconstruction surgeries, the possibility exists of an immune response from the recipient tunnel enlargement, and delayed incorporation of the allograft (Marrale et al., 2007). The risk of disease transmission and the potential for immunogenicity are the major disadvantages of allografts, but these complications can be controlled (Albert et al., 2006; Barrios et al., 1994, Urabe et al., 2007).

3. Controversy of methods of preparation of allografts

The increased frequency of the use of allografts in traumato-orthopedics requires the adoption of storage techniques that interfere as little as possible in the quality of the parts (Vangsness et
al., 2003). Allografts can be stored in different ways; they can be chilled in residential mechanical freezers at temperatures of + 2°C to - 4°C for up to five days. In freezers with temperatures of -20°C to -40°C, they can be stored for up to six months[14]. At these temperatures, the enzymes present in the tissue are still active and can destroy the tissue. Therefore, storage periods of longer than a few months are not recommended. The methods of sterilization used at low temperatures are effective against fungi and do not seem to change

the mechanical characteristics of the grafts. The period of 40 days chosen in our service for cryopreservation coincides with the period of incubation necessary for microbiological investigations for bacteria and fungi (Vangsness et al., 2003). The deep-freezing process enables storage for up to five years, and this is the method we use in our service19.
Many services prefer to carry out the manipulation of tissues under aseptic conditions from acquisition through clinical use, and the samples are discarded when microbiological assays show positive bacterial cultures (20 to 30%) (Zimmerman et al., 1994). Sterilization methods, therefore, are not completely safe. They can alter the biomechanical characteristics of tissues
or fail to penetrate tissue layers, resulting in the protection of microorganisms rather than their destruction. Irradiation with gamma rays is the most common method of sterilization (Sterling et al., 1995). However, to achieve safer sterilization in frozen tissues, high-dose irradiation is necessary, which can alter the biomechanical properties of the tissue in a dosedependent
manner (Curran et al., 2004; Fideler et al., 1995).
Doses as low as 2 Mrad resulted in a statistically significant reduction in biomechanical properties, outcomes, or physical examination measures. Rappe et al. (2007) studied the effect of irradiation on clinical outcomes of ACL reconstruction, they found the irradiated group had an unacceptable higher rate of failure (33%) than the non- irradiated group (2.4%). Fideler et al. (1995) found that the dose of 2.5 Mrad, which was a dose commonly used by tissue banks for sterilization, was just bacteriocidal but ineffective in eliminating viruses such as human immunodeficiency virus (HIV) (Sterling et al., 1995). Doses of 3 to 4 Mrad were necessary to inactivate the virus. Grieb et al. also proved that lower levels of radiation may be inadequate to kill hepatitis and HIV viruses, with a dose of 5 Mrad being necessary (Grieb et al., 2006). When dosage is increased, its clinical implications increase correspondingly. We must question the use of gamma irradiation as there are so many adverse effects and it fails to sterilize the allograft as required.
Also, the sterilization effectiveness against viruses is low (Vangsness et al. 2003). Ethylene oxide sterilization requires strict control of the levels of waste gas in contact with the allograft and is no longer used by tissue banks, due to the possibility of toxic effects for the
recipient (dissolution of the graft and articular inflammatory reactions) (Vangsness et al. 2003). The processing techniques used in the preparation and preservation of grafts have been questioned as potentially altering the initial resistance and mechanical properties of the
graft prior to implantation.
Two studies carried out in Brazil address the biomechanical properties of patellar and calcaneus tendons of cadavers with the same preparation method as that used in our study, comparing fresh and cryopreserved allografts (Giovani et al., 2006; Reiff et al., 2007). They found no differences. A study on metric measurements and attachment levels of the medial patellofemoral ligament shows this to be a distinct structure (Zimmerman et al., 1994).
Although there have been studies on the biomechanical behavior of tendons, the literature does not address histological changes of tendons cryopreserved at -80°C under aseptic conditions (Pearsall et al., 2003). During cryopreservation at -80°C, thedestruction of the allograft enzyme appears to be minimal and at least one enzyme, collagenase, which can destroy the tissue, is inactive (Tomford, 1997). Furthermore, with cryopreservation there is no
intracellular free water, which is thought to be necessary for enzymatic activity, bacterial proliferation and lipid oxidation (Galea & Keamey, 2005; Laitinien et al., 2006). Lipid oxidation inside the tissues induces apoptosis and inhibits cell differentiation; such oxidation

can be minimized or avoided with cryopreservation at temperatures of at least -70° C (Laitinien
et al., 2006). The literature refers to histological changes due to cryopreservation only in cartilage (one of the most commonly used grafts in surgical practice), concluding that during freezing, the vitality of the cells is threatened（Schchar & McGann, 1986). Other injuries may also occur, such as the formation of extracellular ice crystals, intracellular ice nucleation, collapse of the matrix, and breakage of intercellular bridges.
In our study, the histological study of one tendon (not cartilage) was carried out, and none

of these histological phenomena were observed with cryopreservation at -80°C. Freezing with liquid nitrogen at -179°C has also been used as a storage method with similar results but higher cost (Zimmerman et al., 1994). Another widespread storage method is lyophilization. Cryopreservation and lyophilization have been related to a reduction in allograft antigenicity (Jackson et al., 1990). The use of chilled saline solution is not a guaranteed method because the stock can only be kept safely for short periods (Zimmerman et al., 1994). Treatment with paraformaldehyde and fixation with glutaraldehyde are no longer recommended because of the toxicity of these solutions to the recipient tissue. We recently published a study in which we proved the histological properties of the flexor tendons of the knee from cadavers subjected to cryopreservation and experience with the use of allografts of the Knee Group from IOT (Bitar et al., 2010; Damasceno et al., 2009).

4. Cryopreservation: Our method

4.1 Tendinous tissue removal

The attainment of the musculoskeletal tissues has as its source deceased donors with brain death reported by the Committees Intra-hospital - CIHDOTs, Organ Procurement Organizations - OPOS and by the twenty-three central of notification and collection of organs and tissues - CNCDOs, logistically spread throughout country. Notifications for teams pickups are made after the execution of a series of procedures and tests that aim beyond the evidence of brain death, family consent of the donation of organs and tissues. The donor selection follows a rigorous research with control antigen and antibody serology for HIV, Hepatitis A, B and C, HTLV-1 and 2, syphilis, Chagas disease, toxoplasmosis and cytomegalovirus in addition to testing of last generation for evidencing of DNA (Nucleic Acid Amplification - NAT) for HIV and Hepatitis B and C, required of musculoskeletal tissues. The capture of musculoskeletal tissues (bone and tendons) is performed after the initial screening of donors of multiple organs and tissues (heart, kidney, liver, pancreas, lung, cornea, etc.). In our specific field, we follow a protocol of evaluation of the donor that counts with a written anamnesis of a term to capture and physical examination. Are excluded donors with orthopedic disorders such as osteoporosis, osteonecrosis, rheumatoid arthritis, lupus erythematosus, malignancy, age that compromises characteristic of tissues, blood transfusion, tattoos or adornments (piercings) within the window period, users of illicit drugs, permanence in endemic areas, generalized or localized infections, fractures, bruises on the limbs which are absorbed in the musculoskeletal tissues or any other situation that would call into doubt the quality of these tissues, as arranged in the existing laws. The tissues removed are immediately packed in triple enclosures, hermetically sealed and sent under refrigeration (- 4 ° C) to the Tissue Bank.

A very important step of the process of capture is the reconstruction of the donor and for this matter we use prosthesis specially designed using plaster, wire suture, gauze. This reconstruction is done rigorously and is characterized as the most laborious phase of the procedure. All anatomical parameters are respected, and therefore the deformation of the donor does not occur (Figures 1 and 2).

Fig. 1. Pre-operative preparation of the potential musculoskeletal tissues doner.

Fig. 2. Tissue removal: bone and tendon dissecation under asseptic conditions.

4.2 Processing and cryopreservation of musculoskeletal tissues

At the end of the uptake, the tissues are sent to BTME chilled in coolers with temperature monitoring throughout the period of transportation. The processing step is preceded by a

planning of activities needed for its implementation, such as provision of materials and instruments, convocation of the processing team, definition of preparation and dimensioning according to the need for service (queue) requests from orthopedic and dental surgeons. This step is performed in the operating room properly rated (class 100 or ISO 5) equipped with integrated laminar flow (Figure 3). The room also has an antechamber and pass-through and all environments have strict control of air particles and positive pressure for quality assurance of tissues processed there.

Fig. 3. Tissue cryopreservation área. Ultrafreezer with a temperature of -80°C.

In addition, specific attire is required of the professional team that should only use nonwoven

clothes to avoid dispersion of particles that emit the cotton clothes (Figure 4). Not only the non-woven attire is required but the team's behavior should be differentiated.

Thus, sudden movements, use of cosmetics and hair exposure should be avoided during the permanence in this room. Ensuring an appropriate approach is not only a result of training

of the nursing staff that performs the procedure, but also other professionals who access the environment (cleaning maintenance).
Fig. 4. Processing team in activity in the controlled área (ISO 5: Class 100).
The BTME conducts various types of processing of these tissues with the purpose to use in orthopedic and dental surgeries, each of which requires a specific plan. For the processing of fresh frozen tissues it is performed what we call mechanical processing, ie removal of

adventitial tissue such as blood, periosteum, subcutaneous tissue, muscle, fascia and fibrotic tissue. Then, these tissues are immersed in emulsifying solutions based on hydrogen peroxide and alcohol under ultrasonic agitation (Figure 5).
Fig. 5. Tissue' s chemical processing. Ultrasonic clining with emulsifying solution.
Then, a sampling of these resultant solutions, of bone marrow of long bones and fragments of each tissue processing are subjected to microbiological examination (General Knowledge, and Culture of Anaerobic Fungi). Furthermore, it is also obtained samples to histopathological analysis.
Finally starts the procedure of packaging of all the processed grafts which are measured (length, height, weight, volume, perimeter) and kept in sterile triple wrappers, vacuum sealed and properly identified as tissue in analysis. The label contains information from the donor, examination, lot number, item, expiration date, type of conservation and barcode. Once all the tissues are identified, they are x-rayed at the very BTME and referred to cryopreservation.
The bones can also be processed in its lyophilized form, where all water is removed with the tissue still frozen. The process involves placing the tissue in a lyophilizer chamber where ice
crystals sublimate by the action of the high pressure, not passing through the liquid phase and thus maintaining the viability of bone matrix. The result is a dry tissue, conservable at room temperature that must receive final sterilization by irradiation.
At the end of the processing it is performed the documentation of the procedure in the Processing and archived in the donor's chart. The stock of tissues can be kept either frozen or dried, if necessary, according to the same standards used by the Global Association of Tissue Banks . Other forms of processing have been investigated in order to reduce costs related to storage and maintenance. The glicerolization of bone tissue is presented as a processing methodology capable of maintaining the viability of the matrix and prevent bacterial growth, and allows storage at room temperature (Giovani et al., 2006).

4.3 Tissue cryopreservation

In the room of cryopreservation tissues are stored according to their status in the process. Thus, there is a space for tissues in analysis or in quarantine (where they remain for about 60
days until the results of all examinations) and those already released for use. Both rooms are equipped with ultrafreezers with temperatures ranging from 85 to 110 degrees below zero (Amatuzzi et al., 2000).
The room is also equipped with air-conditioning system, own power generator and the unfreezing protection of carbon dioxide (CO2 Backup), and a rigorous system of monitoring the temperature, with printed record of temperature for 24 hours and alarm system via satellite, which guarantees the right temperature and early detection of complications. Depending on the outcome of the analysis, the tissues are transferred to the room of material released for use. The maximum period of cryopreservation is five years to bone tissue and two years for soft tissues and tendons.

4.4 Quality control and distribution

By the time of transplantation, all tissues processed are subjected to rigorous quality assurance criteria. It requires the evaluation of all data pertinent to the donor, test results,
maintenance and control of equipment, materials and instruments used in all phases of each procedure (Figures 6 and 7).

Fig. 6. and 7. Patellar tendon allograft transplant.

All processes are computerized through the System Manager of the Tissue Bank, a program designed to record all the steps which allows the rescue and traceability of each graft processed and delivered. Through a coding is possible to identify the donor, lot, expiration, and status of the tissue examination (under review, released, deleted and used).

Given the need of retrieval of information, as the evidence of an adverse effect, you can quickly and safely obtain the information and implementation of corrective and preventive actions.

For a lot of graft in analysis to be released for use nurses must analyze the results of all tests

performed: NAT serology or PCR for HIV, HBC and HCV, General Culture, Anaerobic Culture and Culture of Fungi, pathology reports and radiographic findings. These reports of

examinations are assessed and ultimately released by the Technical Director of the Tissue Bank.

Besides examinations, evaluation of the printed record of temperature during the storage period is considered. The temperature oscillations are quickly detected and reported to the team members from the BTME even remotely by cell phones. In addition, audible alarms at strategic points in the hospital and the presence of CO2 backups, ensure system reliability.

After the release of each lot, the nurses carry a detailed examination of integrity of each tissue during the replacement of tissues in analysis labels to released labels and posterior transfer of the sector. The logistics of storage of tissues in ultrafreezers considers the type of

tissue and speed up the search.

We emphasize that for a rigorous quality control all steps of each procedure are carried out through check-lists with double checking and approval.All relevant data of the donor or lot records are filed in a single file and stored at the BTME for a minimum of 25 years.

A serum bank with plasma samples of donors are offered by BTME if necessary examination of counterproof.

5. Our experience

In the last five years, we have performed 35 knee ligament reconstructions, including multiple ligament, and isolated ACL and PCL reconstructions with single and double bundle techniques, and ACL reviews. Twenty seven men and eight women underwent surgery with the employment of the following grafts from the tissue bank: anterior tibial tendon (48 units), patellar tendon (4 units), quadriceptal tendon (5 units), semitendinosus tendon (4 units), calcaneus tendon (1 units) and fibular tendon (1 units). The patients followup

range from 6 to 57 months and we are still collecting data from this cases. Our first results showed that there no viral or bacterial infection associated to the use of allografts in

any of the cases or other complications, and clinical outcome of these patients has shown good results with the use of tendons from the tissue bank.

A study conducted in another service, in which we colaborate, revised the records of 46 patients who were submitted to ligament reconstructions between 1999 and 2007 using grafts supplied by the same tissue bank (Damasceno et al., 2009). Thirty-four male patients and 12 female patients were reviewed, with follow-up time ranging from 10 months to 9 years (mean: 3.1 years).The surgical procedures used 9 units of patellar tendons, 9 units of anterior tibial tendons, 8 units of calcaneal tendons, 6 units quadriceptal tendons and 1 unit of fibular tendon, mainly for multiple ligamentar reconstructions and ACL reviews41. There were also no viral or bacterial infection cases41.

The decrease in morbidity and postoperative complications allied to good results obtained in our samples reinforces the idea that the use of allografts is a good and safe option in knee

ligament reconstruction.

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