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Matrix: estudio de las propiedades biológicas de una matriz dérmica humana para su aplicación en cirugía reconstructiva del suelo pélvico

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Tesis doctoral

TESIS DOCTORAL

Matrix: estudio de las propiedades biológicas de una matriz dérmica humana para su aplicación en cirugías reconstructivas del suelo pélvico.

MARTA PERÓ
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MATRIX: ESTUDIO DE LAS PROPIEDADES BIOLÓGICAS DE UNA MATRIZ DÉRMICA DE ORIGEN HUMANO PARA SU APLICACIÓN EN CIRUGÍAS DE RECONSTRUCCIÓN DEL SUELO PÉLVICO.

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Un gran apoyo personal y profesional.

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Al Jordi, als meus fills i als meus pares.

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PRESENTACIÓN

Los artículos científicos que constituyen esta tesis doctoral pertenecen a una misma línea de investigación y derivan de un estudio de experimentación básica que tiene como objetivo la evaluación de una matriz dérmica de origen humano (MDh) desarrollada en el Banco de Sangre y Tejidos (BST) de Barcelona. Los resultados de este estudio aportan información relevante sobre las propiedades biológicas de la MDh aplicada en un modelo animal, y abren el camino de futuras investigaciones que puedan hallar la aplicación clínica de este nuevo material en el campo de la ginecología, concretamente en la reconstrucción quirúrgica de las disfunciones del suelo pélvico, para mejorar así la calidad de vida de las mujeres que sufren esta patología. Los resultados de este proyecto han sido recopilados en dos artículos originales publicados en revistas de amplia difusión internacional.

Esta tesis ha sido realizada en el servicio de Ginecología y Obstetricia del Hospital de la Santa Creu i de Sant Pau, en estrecha colaboración con el servicio de Inmunología del mismo hospital; l'Institut de Recerca del Hospital de Sant Pau-IIB Sant Pau; el departamento de Ingeniería Biomecánica del Institute for Complex Molecular Systems (ICMS) en la Universidad de Tecnología de Eindhoven (Países Bajos); la Unidad de Biofísica y Bioingeniería de la Universidad de Barcelona; y por último, el Banco de Sangre y Tejidos de Barcelona quien además ha financiado el proyecto mediante una convocatoria competitiva (Convenio BST – IIB Sant Pau 2017-3).

CONTENIDO

I. ABREVIATURAS

DSP   disfunciones del suelo pélvico

ECM   extracelular matrix (matriz extracelular)

EEUU   Estados Unidos de América

FDA   *Food and Drug Administration*

FGF-2   *Fibroblast Growth Factor-2*

IU   incontinencia urinaria

LB   linfocitos B

LT   linfocitos T

MA   matrices acelulares

MDh   matriz dérmica humana

NHS   *National Health Service*

NZW   *New Zealand White*

POP   prolapso de órganos pélvicos

PP   polipropileno

SEGO   Sociedad Española de Ginecología y Obstetricia

TGF-b   *Transforming Growth Factor - Beta*

VEGF   *Vascular Endothelial Growth Factor*

1. RESUMEN

Antecedentes:

Las mallas irreabsorbibles de polipropileno (PP) han sido ampliamente utilizadas en el tratamiento quirúrgico de las patologías del suelo pélvico como la incontinencia urinaria (IU) o el prolapso de órganos pélvicos (POP). Sin embargo, las mallas de PP se asocian a complicaciones graves como el dolor pélvico crónico. Las MDh han demostrado su eficacia y seguridad en el campo de la medicina reconstructiva, pero se desconoce su comportamiento a nivel vaginal.

Objetivos:

1. Evaluar al conejo como modelo animal para el estudio de biomateriales en el tratamiento de las disfunciones del suelo pélvico (DSP)

2. Evaluar las propiedades biológicas de las MDh, en comparación con las mallas sintéticas de PP, implantadas a nivel vaginal y abdominal en un modelo animal (conejo).

Diseño del estudio:

20 conejos hembras blancos de la raza *New Zealand White* (NZW) se aleatorizaron en 2 grupos: grupo estudio (injerto de MDh) y grupo control (injerto de PP). En cada animal, los injertos fueron quirúrgicamente implantados sobre la fascia abdominal anterior por vía subcutánea, y en la capa submucosa vaginal. Tras 180 días, los injertos (explantes) fueron extirpados, y se analizaron las características macroscópicas, biomecánicas, inmunohistoquímicas e histológicas.

Resultados:

Las principales dificultades durante la cirugía de implantación de los injertos fueron: (a) conseguir una adecuada exposición vaginal sin perder la integridad de la mucosa vaginal; (b) mantener condiciones de asepsia; (c) localizar y

disecar la vena mamaria durante la cirugía abdominal; y (d) la extracción de las muestras de sangre de la arteria auricular.

Durante la cirugía de explantación, en el grupo control se observó un mayor número de hallazgos patológicos. La tasa de extrusión de la malla vaginal fue del 33% (3/9) en el grupo de PP, mientras que en el grupo de MDh la tasa de extrusión vaginal fue del 0% (p = 0.015). El estudio macroscópico de los explantes también mostró que los injertos vaginales del grupo de estudio se integraron completamente en el tejido receptor con mayor frecuencia, comparado con el grupo control. En el grupo MDh el 40% de los explantes vaginales (4/10) no pudieron ser identificados debido a su completa integración en el tejido receptor. Sin embargo, en el grupo PP se identificaron el 100% de los injertos vaginales (p = 0.014).

El estudio histológico demostró que tanto el grupo PP como el grupo MDh presentaron infiltrados celulares correspondientes a una respuesta inflamatoria de cuerpo extraño, especialmente en la localización vaginal. En cambio, el patrón del infiltrado inflamatorio fue diferente en ambos grupos: en el grupo PP, los infiltrados presentaron una distribución focal y se localizaron principalmente en la parte interna del epitelio, mientras que en el grupo MDh los infiltrados tuvieron una distribución difusa. Además, el grupo MDh presentó una mayor presencia de linfocitos B en comparación con el grupo control.

El estudio biomecánico de los explantes mostró que el grupo MDh obtuvo una menor resistencia en la prueba de tracción, comparado con la malla de PP. Además, los resultados demostraron que tanto la rigidez como la elasticidad de la malla de PP fueron superiores en comparación con la MDh. Por último, el estudio de resistencia del grupo MDh mostró una disminución de las propiedades en relación a los resultados preimplantacionales, lo que es consistente con la completa integración de la ECM a nivel vaginal observada en los hallazgos macroscópicos.

Conclusiones:

El conejo NZW es un modelo que permite el estudio de injertos abdominales y vaginales. El manejo de los animales es sencillo, tanto durante la cirugía como en el seguimiento postoperatorio, incluso para investigadores con escasa experiencia en la manipulación y cuidado de estos animales. Por otro lado, la MDh se asocia a menos complicaciones clínicas, así como a una mayor integración tisular, comparado con las mallas de PP. Sin embargo, debido a esa integración, las propiedades biomecánicas de la MDh no se mantienen estables tras 6 meses de implantación.

2. INTRODUCCIÓN

Las disfunciones del suelo pélvico (DSP) son un problema común que afecta negativamente la calidad de vida de al menos 1 de cada 4 mujeres de la población femenina adulta(1). Debido al envejecimiento de la población y a que la prevalencia de estas disfunciones se incrementa con la edad, se prevé que, en las próximas décadas, la atención a estas patologías constituirá un problema sanitario de primer orden tanto por sus implicaciones psicosociales como por el impacto económico en los sistemas públicos de salud.

Tanto en la IU como en el POP, cuando el tratamiento conservador ha fracasado, el tratamiento quirúrgico ha demostrado tener los mejores resultados(2). En ambas patologías la reparación quirúrgica puede implicar el uso de una prótesis de material sintético no reabsorbible. En el caso de la IU, en el tratamiento quirúrgico estándar se utiliza una malla sintética para reforzar el tejido suburetral. En el caso del POP las mallas permiten la reparación anatómica del defecto proporcionando un soporte adecuado a los órganos pélvicos(3).

El uso de malla sintética, habitualmente de PP, se ha asociado a complicaciones graves como erosión, retracción y dolor. Una de cada treinta mujeres intervenidas de IU con malla de PP deberá ser re-intervenida en los diez años siguientes debido a alguna complicación asociada a la malla(4). En el caso del uso de malla para cirugía correctora del POP, la incidencia de complicaciones es mayor y de más difícil manejo, estimándose que el riesgo de reintervención se sitúa entre un 3 y un 10%, aunque la incidencia real sea probablemente mayor(5).

Debido al riesgo de complicaciones asociado al uso de mallas para la cirugía del POP y la IU, la autoridad competente en el uso de estos dispositivos en Estados Unidos (EEUU), la *Food and Drug Administration (FDA)* emitió una nota en 2008 advirtiendo del riesgo para la seguridad de las pacientes, nota que fue actualizada en 2011(6). Esto supuso una reclasificación de este tipo de mallas, que pasaron de la clase II (riesgo moderado), a la clase III (riesgo

alto), aumentando los requerimientos previos para la comercialización de este tipo de productos. Estas advertencias tuvieron impacto y fueron recogidas por la mayoría de sociedades científicas, incluyendo la propia Sociedad Española de Ginecología y Obstetricia (SEGO) a través de su Sección de Suelo Pélvico(7). Recientemente el sistema de salud de Reino Unido, *National Health Service (NHS)* se ha unido a la FDA y las sociedades científicas especializadas emitiendo un informe en el que se desaconseja el uso de mallas transvaginales para el tratamiento del POP(8).

Por lo tanto, el progreso de la investigación se centra en la búsqueda de nuevos materiales para ser utilizados como prótesis de forma segura en las cirugías reconstructivas de suelo pélvico. En este sentido, el desarrollo de las matrices biológicas acelulares representa una de las últimas aproximaciones propuestas. Se trata de una nueva generación de materiales biocompatibles que proceden de tejidos de diferentes orígenes biológicos. Estos tejidos se someten a procesos de eliminación celular dejando únicamente el entramado de fibras extracelulares (ECM), careciendo por tanto de capacidad antigénica para generar rechazo.

La innovación recae en el mecanismo de actuación de dichas matrices. A diferencia de las prótesis sintéticas, que simplemente actúan como material de sustitución del tejido propio, las matrices acelulares (MA) pretenden ser una base de soporte estructural natural a partir de la cual se estimulará la regeneración tisular, con una total integración a nivel de los tejidos blandos que la rodean. Este mecanismo de acción dota a la matriz de una completa biocompatibilidad con el tejido receptor, minimizando las complicaciones asociadas a las mallas sintéticas, como la extrusión.

Hasta la fecha se han desarrollado numerosos tipos de MA procedentes de fuentes alogénicas o xenogénicas, a partir de tejidos con diferentes propiedades como por ejemplo dermis porcina, pericardio bovino y submucosa del intestino delgado. En función de las características histológicas y de las

propiedades mecánicas de las matrices, éstas pueden tener distintas aplicaciones clínicas, como por ejemplo en el cierre de heridas o úlceras crónicas, en la recuperación de tejido blando perdido como retracción gingival, en los defectos de la duramadre, en la reconstrucción mamaria, en la reparación de la pared abdominal, en la recuperación de desgarros en tendones, o como relleno(9,10).

En los últimos años, se han comercializado numerosos tipos diferentes de mallas y matrices cuya eficiencia está siendo analizada(11,12). Las matrices dérmicas comerciales de origen humano están disponibles en EEUU desde hace más de 15 años (Allo-Derm®, LifeCell Branchburg New Jersey fue la primera), donde cuentan con una larga experiencia con más de 2 millones de implantes y decenas de publicaciones donde se exponen los buenos resultados clínicos alcanzados con su uso en determinadas cirugías(13,14). Sin embargo, la alta tecnología empleada en su producción, en la mayoría de los casos, encarece el producto convirtiéndolo en una opción de disponibilidad limitada. En Europa su disponibilidad es más limitada aún. En España, según la directiva 2004/24/CE del Parlamento Europeo y el Real decreto-Ley 9/2014, AlloDerm, SureDerm, Allocover y Decellularised Dermis son los productos legalmente aceptados.

Por otro lado, la utilización de MA de origen humano en la reparación de las disfunciones del suelo pélvico ha sido muy poco evaluada. Se sabe que las mallas sintéticas se comportan de manera distinta cuando se utilizan en cirugía de la pared abdominal (corrección de hernias, por ejemplo) que cuando se utilizan por vía vaginal, ya que, entre otras cosas, las exigencias biomecánicas y el nicho de colocación en ambas localizaciones son distintas. Así pues, no se dispone de suficiente evidencia científica que permita el uso de dichas matrices en el campo de la ginecología, con lo que es necesaria más investigación en este campo.

El BST ha desarrollado una matriz dérmica acelular procedente de tejido cutáneo de donante mediante la eliminación de la epidermis y las células de la dermis y conservando la estructura extracelular tridimensional original. Las matrices dérmicas tienen alto contenido en colágeno tipo I, III y V y su porción no colágena contiene entre otras proteínas, factores de crecimiento, como factor de crecimiento de fibroblastos 2 (FGF-2), el factor de crecimiento transformante beta (TGF-b) y el factor de crecimiento endotelial vascular (VEGF), que actúan como estímulo para la repoblación y la neo-vascularización de la matriz una vez está implantada en el receptor. Por ello, esta MA actúa como un andamio sobre el cual las células del receptor pueden migrar y proliferar y de esta forma regenerar el tejido donde se implanta.

La posibilidad de infección o de reacción de hipersensibilidad o rechazo en el receptor debido al uso de un derivado humano se minimiza gracias a los tratamientos que sufren estos tejidos para su descelularización. Al carecer de células, carecen también del componente antigénico responsable del rechazo inmunitario.

Este proyecto cuenta con la colaboración transversal de un equipo multidisciplinar de excelentes profesionales que incluye: el Banco de Sangre i Tejidos de Barcelona, los Servicios de Ginecología y de Inmunología del Hospital de la Santa Creu i Sant Pau, el Servicio de Experimentación animal del Institut de Recerca del Hospital de Sant Pau, y cuenta además con la colaboración del departamento de Ingeniería Biomecánica del *Institute for Complex Molecular Systems (ICMS)* en la Universidad de Tecnología de Eindhoven (Países Bajos); y de la Unidad de Biofísica y Bioingeniería de la Universidad de Barcelona. Así pues, este trabajo aporta la ventaja de estar representado por un equipo multidisciplinar que evalúa la seguridad y la calidad del tejido desarrollado para su potencial uso en cirugías reconstructivas del suelo pélvico. Desde el BST se controla todo el proceso desde la evaluación del donante y el proceso de obtención del tejido hasta la descelularización y conservación de la MDh. Otra ventaja importante de este

grupo es que la disponibilidad de la MDh desarrollada en el BST no supone un coste tan elevado como el de un producto comercial importado, lo que resulta atractivo para el sistema sanitario público(15). El BST cuenta con una amplia experiencia en la obtención y preservación de tejidos procedentes de donante para uso terapéutico en el campo de los trasplantes permitiendo asegurar la máxima calidad y rigurosidad en los procesos de obtención de la MA.

3. OBJETIVOS

Este proyecto parte de la necesidad de encontrar un material seguro para ser utilizado como refuerzo en el tratamiento quirúrgico de las DSP, ya que el material que se utiliza actualmente -PP- puede asociarse a complicaciones graves.

Las MA han demostrado buenos resultados en términos de eficacia y seguridad en otras aplicaciones clínicas. Por eso se plantea la MDh como una posible alternativa a las mallas de PP en cirugía reconstructiva del suelo pélvico. Sin embargo, se desconoce el comportamiento de las MDh en la localización vaginal, debido a la escasez de evidencia disponible.

La hipótesis inicial es que la MDh desarrollada por el BST constituye una posible alternativa frente a las mallas sintéticas de PP que actualmente se comercializan para el tratamiento quirúrgico de las DSP.

El objetivo principal de este proyecto es evaluar las propiedades biológicas de la MDh, incluyendo su capacidad de integración en el tejido receptor, y su respuesta *in vivo* en comparación con las mallas de PP. Esta información permitirá determinar su seguridad, así como su potencial uso en la cirugía reconstructiva del suelo pélvico. Para ello se recogerán las variables clínicas poniendo especial interés en los signos de complicación, y el estudio de los explantes mediante el análisis histológico, inmunohistoquímico y biomecánico.

El objetivo secundario es evaluar la factibilidad del conejo como modelo para el estudio de nuevos biomateriales en el tratamiento de las DSP, así como la estandarización del modelo. Para ello se describirán las dificultades quirúrgicas durante las cirugías de colocación y retirada de los implantes, así como las dificultades durante el seguimiento, monitorización y estabulación de los animales. Esta información podría orientar futuros trabajos destinados al estudio de nuevos dispositivos de aplicación vaginal.

4. RESULTADOS

Los resultados de esta tesis se organizan en dos capítulos. El capítulo 1 recoge el artículo 1 publicado, donde se evalúa la factibilidad del conejo NZW hembra como modelo para el estudio de biomateriales para la cirugía reconstructiva del suelo pélvico. El Capítulo 2 corresponde al artículo 2 publicado, donde se detallan los resultados globales del uso de la MDh en suelo pélvico comparada con el uso de PP durante 6 meses en el modelo animal, teniendo en cuenta la evaluación macroscópica, histológica, inmunohistoquímica y biomecánica de los explantes. Estos resultados constituyen una base sólida sobre la que desarrollar futuras investigaciones respecto a la idoneidad del uso de una MDh en cirugía del suelo pélvico en un modelo clínico humano.

4.1 CAPÍTULO 1

En este capítulo se evalúa al conejo como modelo animal para estudiar el desarrollo de biomateriales en cirugía reconstructiva del suelo pélvico.

ARTÍCULO 1: El conejo como modelo animal para el estudio de injertos biológicos en el tratamiento de las disfunciones del suelo pélvico.

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Resumen

Los objetivos de este estudio fueron evaluar la viabilidad del conejo NZW para el estudio de biomateriales en la reconstrucción quirúrgica de las DSP, y comparar la evolución clínica entre los grupos de estudio. Veinte conejos NZW hembra se aleatorizaron en dos grupos: experimental (MDh) y control (malla comercial de PP). En cada animal, los injertos fueron quirúrgicamente implantados en la fascia abdominal, y en la submucosa vaginal. Tras 180 días, los injertos fueron extirpados. Los principales desafíos quirúrgicos durante la cirugía de implantación fueron: (a) exposición vaginal adecuada manteniendo la integridad de la capa mucosa vaginal; (b) mantener condiciones asépticas; (c) localizar y disecar la vena mamaria durante la cirugía abdominal; y (d) extraer muestras de sangre de la arteria auricular. En el grupo control se encontraron un número superior de hallazgos patológicos durante la cirugía de explantación, con una tasa de extrusión de la malla vaginal del 33 %. Mientras que en el grupo experimental no se observó ningún caso de extrusión vaginal (p = 0,015). Por otro lado, la integración de los injertos vaginales en el tejido circundante fue más frecuente en el grupo MDh, donde el 40 % de los explantes no eran macroscópicamente identificables. Sin embargo, en el grupo PP, los explantes se identificaron en el 100 % de los animales (p = 0,014). Así pues, podemos concluir que el conejo NZW es un buen modelo para la evaluación de materiales en la localización abdominal y vaginal. Los animales son fácilmente manejables durante los procedimientos, incluida la intervención quirúrgica y el abordaje de la mucosa vaginal. Por último, la MDh se asocia a menos complicaciones quirúrgicas, así como una mejor integración tisular macroscópica, en comparación con la malla de PP.

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Rabbit as an animal model for the study of biological grafts in pelvic floor dysfunctions

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The aims of this study were to evaluate the feasibility of the New Zealand White (NZW) rabbit for studying implanted biomaterials in pelvic reconstructive surgery; and to compare the occurrence of graft-related complications of a commercial polypropylene (PP) mesh and new developed human dermal matrix implanted at vaginal and abdominal level. 20 white female NZW rabbits were randomized into two groups, experimental group (human acellular dermal matrices-hADM-graft) and control group (commercial PP graft). In each animal, grafts were surgically implanted subcutaneously in the abdominal wall and in the vaginal submucosa layer for 180 days. The graft segments were then removed and the surgical and clinical results were analyzed. The main surgical challenges during graft implantation were: (a) an adequate vaginal exposure while maintaining the integrity of the vaginal mucosa layer; (b) to keep aseptic conditions; (c) to locate and dissect the breast vein abdominal surgery; and (d) to withdraw blood samples from the ear artery. The most abnormal findings during the explant surgery were found in the PP group (33% of vaginal mesh extrusion) in comparison with the hADM group (0% of vaginal graft extrusion), $p = 0.015$. Interestingly, macroscopic observation showed that the integration of the vaginal grafts was more common in the hADM group (40%) than in the PP group, in which the vaginal mesh was identified in 100% of the animals ($p = 0.014$). The NZW rabbit is a good model for assessing materials to be used as grafts for pelvic

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reconstructive surgery and vaginal surgery. Animals are easily managed during the procedures, including surgical intervention and vaginal mucosa approach. Additionally, hADM is associated with fewer clinical complications, as well as better macroscopic tissue integration, compared to PP mesh.

Pelvic floor dysfunctions (PFD) such as pelvic organ prolapse (POP), or urinary incontinence (UI) are common conditions that affect a third of the adult female population [1]. In these frequent dysfunctions, surgical treatment has shown good results. The surgical repair may involve the use of non-absorbable, synthetic prostheses, usually polypropylene (PP). PP meshes have been associated with severe complications such as erosion, retraction, and pain[2].

In July 2018, the use of mesh implants to treat stress urinary incontinence was suspended by the National Health Service (NHS) of the United Kingdom [3]. And in April 2019 the United States (US) Food and Drug Administration (FDA) banned vaginally-placed mesh implants for treating pelvic organ prolapse [4]. With this growing concern for safety, there is a worldwide agreement on the need for research and innovation to find alternative materials to be used in pelvic reconstructive surgery.

This need of new materials with an effective and safe mesh design has approached the development of acellular matrices (AM). AMs represent a new generation of biocompatible materials processed to obtain a decellularized scaffold of fibers whose architecture and extracellular matrix remain intact[5–7].

In recent years, many different types of biological meshes have been marketed and their efficacy evaluated [8,9]. Specifically, human acellular dermal matrices (hADM), available in the US for more than 15 years, have been used in more than 2 million implant procedures and information is available on its clinical safety and efficacy in different clinical applications[10,11]. Reconstructive surgeries such as chronic wounds closure, immediate breast reconstruction, abdominal wall and hernia repair, and tendon reinforcement are likely to use dermal matrices[10,12,13].

However, the gynecological application of dermal matrices has been poorly evaluated[14], and there is limited information about the behaviour of vaginally applied hADM. Therefore, it is required to carry out preclinical studies to assess hADM in the repair of PFD. In this way, international associations such as the *International Urogynecological Association* (IUGA) have presented a consensus document that specify the steps to follow for the introduction of new devices to be used in prolapse surgery, and they recommend to previously perform preclinical studies in animals for the evaluation of the host's inflammatory response[15].

The election of the most suitable animal model to obtain clinical results after placing the hADM at the vaginal level requires to fulfill different objectives and conditions. The most studied animals are mice, rat, rabbit, sheep, pig, and non-human primates (NHP). Published studies show that

several models can be used, and there is no animal that is perfect for this purpose. Each one has its own benefits and weakness [16–18].

We selected the New Zealand White (NZW) rabbit as animal model because of its adequate life expectancy for the duration of the study, it has perineal musculature associated with the urogenital tract similar to humans[19,20], and has appropriate vaginal size to perform a vaginal graft placement. Additionally, the rabbits are economical and easily housed, handled and anaesthetized.

Another advantage of this model is that it allows the study of implants in the vaginal and abdominal location in the same animal concomitantly, with the aim of evaluating whether clinical changes appear depending on the implant location.

Other animals have a reproductive and urinary pathophysiology more similar to humans than rabbits, such as ewes or NHP[16–18]. These models would probably be better for studying the physiology of POP or UI. However, this research does not pretend to study the therapeutic efficacy of hADM. Instead, it is dedicated to demonstrating the occurrence of local complications, such as exposure or infection, especially at vaginal location. Additionally, the scientific literature shows many examples of experimental studies using NZW rabbits for the evaluation of new biomaterials for the treatment of PFD[21–31].

This study aims to describe the surgical complexity of the NZW rabbit model, their clinical monitoring, as well as the standardization of the model. It includes the description of the surgical difficulties of implanting prostheses at the vaginal and abdominal level in rabbits, as well as the difficulties in housing them, and the occurrence of graft-related complications in different locations.

This information could guide future works designed to test devices for vaginal application and will help other groups that focus their research in the urogynecology area.

Material and methods

Experimental design and subjects of study. The study was performed by Barcelona Tissue Bank (BTB), the Hospital de la Santa Creu i Sant Pau, and at the Research Institute of the Hospital de Sant Pau-IIB Sant Pau.

The study protocol was approved by the Internal Animal Care and Use Committee (CEEA-IRHSCSP) and the competent government authority (Generalitat de Catalunya, Animal Experimentation Commission, project number 9669). All animal procedures were carried out in strict accordance with the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. In addition, we followed the ARRIVE guidelines and committed ourselves to the 3Rs of laboratory animal research. The animal experimental project was performed in the Animal Experimental Service of the accredited IRHSCSP, ISO 9001:2015 accredited.

This study followed the ethical precepts of the Declaration of Helsinki and was approved by local ethics committee. Human tissue was processed according to guidance for clinical use (EEC regulations 2004/23/CE and 2006/17/CE) and to the legal requirements for the use of biological samples for research in Spain (Law 14/2007 and RD 1716/2011). Ethics institutional review board (IRB) approval was obtained (CEIm Hospital Valle Hebrón, Barcelona; PR (BST)314/2019). In all cases, informed consent was obtained from the donors' relatives.

A total of 20 female multiparous NZW rabbits were randomly allocated to receive control (PP mesh) or experimental (hADM) grafts.

Each rabbit received 4 grafts: 2 grafts in the vaginal submucosa layer and two in the subcutaneous tissue of the abdominal wall, over the muscular fascia.

Regarding the vaginal grafts, one (5×5 mm) was placed in the anterior vaginal wall and used for histological and immunohistochemical studies. The other one (10×5 mm) was placed in the submucosa of the posterior vaginal wall and was used to perform the biomechanical study.

The size of the abdominal grafts was the same as that of the vaginal grafts, but both were stitched together in the right caudal quarter of the abdominal wall.

The implants were removed 180 days later, at which time the animals were also humanely euthanized.

Graft preparation. *Preparation of hADM samples.* hADM was obtained from skin tissue procured from the back and lower limbs of a human cadaveric donor by manual dermatome. The tissue was processed in clean rooms in accordance with Good Manufacturing Practices (GMP) regulations in the BTB. The processing consisted of, first, the selection of the homogeneous fragments in thickness and their decontamination in antibiotic solution for 16–24 h, and then their decellularization. Decellularization was achieved by chemical, biological and mechanical treatment as follows: the skin was incubated in hypertonic solution, which led to the cellular lysis, then an incubation in a proteolytic enzyme, resulting in the removal of the genetic material, and a final incubation in anionic surfactant for the washing out of the cellular debris. In order to remove any reagent, 10 washes were carried out in 0.9% NaCl. The 10×5 mm samples were prepared and stored in glycerol solution in a double
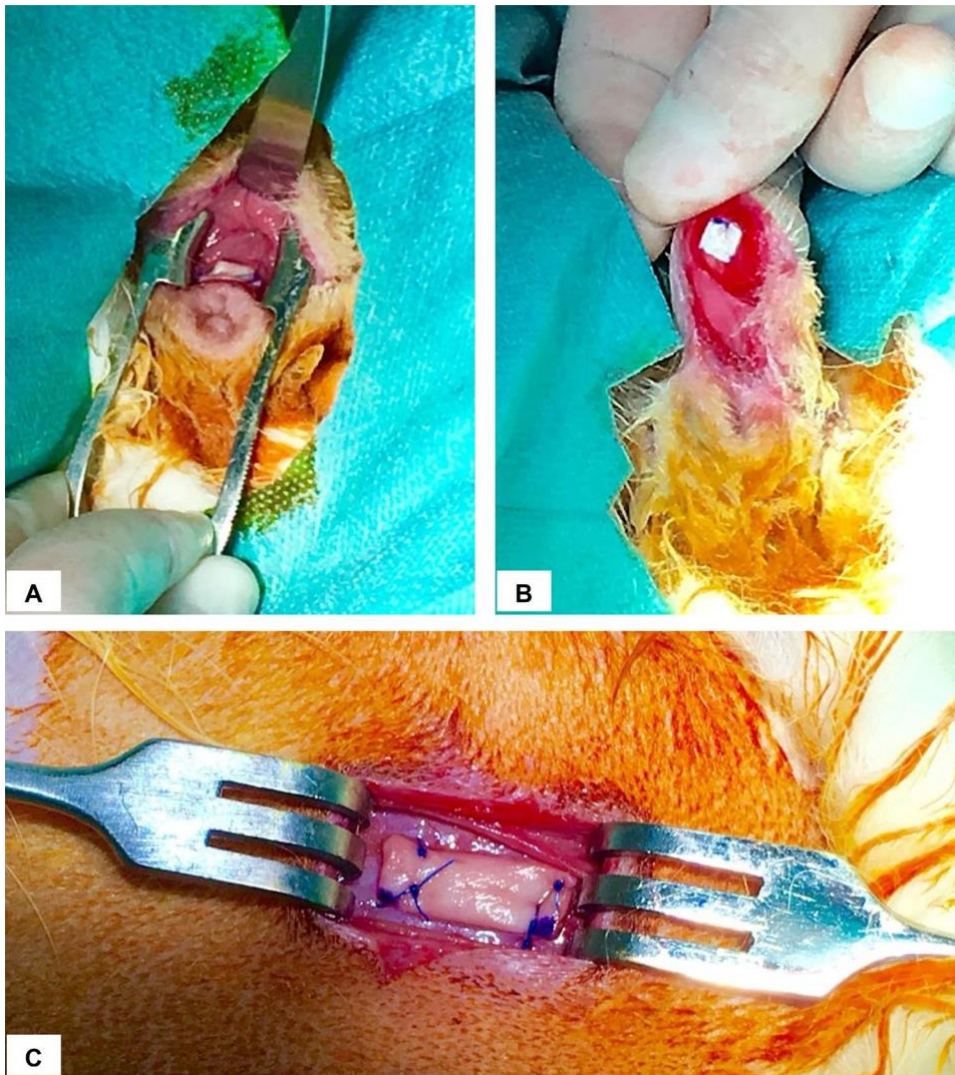
Figure 1. Vaginal and abdominal implant placement in NSW rabbit model. (**A**) hADM fixed in posterior vaginal submucosal layer. (**B**) hADM fixed in anterior vaginal submucosal layer. (**C**) hADM fixed in the subcutaneous tissue of the abdominal wall.

bag at room temperature until use. To ensure strict microbiological control, several microbiological controls were performed throughout the graft processing.

PP graft preparation. The material (Gyneband, Mallanets, Spain) was delivered in a commercial sterile container, ready for medical use in humans. Under conditions of surgical asepsis, it was removed from the container and cut into 10×5 mm and 5×5 mm pieces immediately before proceeding to the implant surgery.

Surgical procedure. Animals were anesthetized with ketamine (15 mg/kg subcutaneous; sc) and medetomidine (0.5 mg/kg sc). Each rabbit received a prophylactic antibiotic dose (Ceftiofur 50 mg/kg sc) and nonsteroidal anti-inflammatory drug (meloxicam 1 mg/kg intramuscular -im-).

Before starting the surgery, the areas of surgical incision were shaved and disinfected, and blood samples (6 cc) were obtained from the ear artery to study inflammatory markers. Serial blood samples were obtained on days: 0 (day of implantation surgery, 7, 30 and 180 (day of euthanasia).

Abdominal implants. A transverse incision was made in the abdominal midline, at the level of the intermammary line of the last two nipples on the right side of the rabbit, to expose the anterior abdominal fascia. Both fragments of the graft (hADM or PP) were positioned and fixed with prolene (Ethicon) 5/0 discontinuous suture (Fig. 1C). The abdominal wall was closed with 4/0 vicryl rapide (Ethicon) thread in two layers: continuous suture for subcutaneous tissue, and continuous intradermal suture for skin tissue.

Vaginal implants. A transverse incision was made in the anterior vaginal wall, approximately 1 cm from the vaginal entrance. The vaginal mucosa layer was dissected and the 5×5 mm graft was implanted and fixed with the same procedure as in the abdominal implant (Fig. 1B). The same procedure was repeated on the posterior vaginal wall, using the 10×5 mm graft (Fig. 1A).

The vaginal mucosa was closed with a 4/0 vicryl thread using an interrupted suture.

Once the implants had been placed, a preventive dose of buprenorphine (0.01 mg/kg, sc) was administered. To avoid licking and infection of the wound, rabbits wore a protective collar for 7 days after surgery. Animals could move freely in their pens and were under a strict veterinary control. During the entire period of study animals were supervised daily and weighted weekly and complications related to the implant were closely monitored.

After 180 days, rabbits were anesthetized as described before and the grafts were explanted, removing the prosthesis together with surrounding tissue.

Animals were euthanized under deep anesthesia according to the protocol by administration of 150 mg/kg intravenous pentobarbital.

Variables and parameters investigated at surgery and at follow-up. *Surgical variables.* Surgical time duration, complications and difficulties were collected by the investigator during the implant surgery.

Clinical complications during animal follow-up. Signs of pain/stress in the animals were evaluated by the Grimace Scale. This scale allows an objective evaluation of animals' pain and distress through their facial expression, especially orbital closure, the flattening of the cheeks, angulation of the nostrils, stiffness of the whiskers and subsequent

rectification of the ears. Each item was scored from 0 to 3, as follows: 0 = not present, 1 = moderately present and 2 = obviously present.

Pain/discomfort (any value > 1) was treated with an additional painkiller dose (buprenorphine, 0.01 mg/kg, sc).

Signs of loss of well-being: anxiety, depression, inactivity, restlessness, shrieks, or groans, grinding of teeth, tonic immobility, rejection of water and/or food, weight loss were also surveyed. Clinical signs of surgical site complication were equally inspected and registered.

Macroscopic observation of explants. During explant surgery, the macroscopic aspect of the explants was evaluated with particular interest in the presence of: (a) evidence of seroma (accumulation of serous fluid around the graft); (b) signs of local infection (erythema or purulent suppuration); and, (c) evidence of extrusion of the graft (skin necrosis or dehiscence of the surgical wound with exposure of the graft).

Statistical analysis. As descriptive data analysis we used the median, and also the mean with standard deviation. The relationship between categorical variables was analyzed using the corresponding contingency tables, calculating the percentage in each group and application of chi-square test with the approximation of the probability ratio. In the ordinal variables, the comparison between two groups was made with non-parametric Mann–Whitney test. In all cases, the usual level of significance was 5% (alpha = 0.05). All analyses were performed with the statistical IBM-SPSS package (V25).

Results

Twenty animals were included, 10 in the experimental group (hADM), and 10 in the control group (PP). During the study (83 days after surgery), one rabbit in the control group died due to causes not related to the grafts.

Surgical challenges during surgical graft implantation. *Exposure of the vaginal surgical field.* Due to the small size of the surgical field, a recurring difficulty was the vaginal exposure. This challenge was overcome by placing an eyelid retractor in the vaginal introitus. The rest of the instruments used were the standard microsurgical devices.

Integrity of the vaginal mucosa layer. Grafts were implanted at the level of the vaginal submucosa layer, so a meticulous vaginal dissection was needed. Because it is an extremely thin layer, another difficulty in most of the surgeries performed was to maintain the integrity of the vaginal mucosa layer during the graft implantation. The maintenance of this layer is crucial to reduce the risk of future implant extrusions. Despite these difficulties, the preservation of the mucosa was successfully obtained in all animals.

Aseptic conditions. It was difficult to keep aseptic conditions due to the large amount of hair in this animal model. The methods used to achieve adequate asepsis in the surgical field were an extensive shaving of the NZW's abdomen and external genitalia, and a careful and precise handling of the animals during surgeries.

Location and dissection of the breast vein during abdominal surgery. During abdominal surgery, the last two nipples on the right side of the animal were used as anatomical reference to locate the explant position during its surgical removal.

The breast vein is located at the intermammary line. This situation required a careful dissection to avoid accidental damage during implantation surgery. In one case, the vein was damaged and resulted in an extensive bleeding that was resolved with a hemostatic stitch; however, the animal presented postoperatively an abdominal wall hematoma that was resolved spontaneously after few days.

	Control (PP) group (N = 10)	Experimental (hADM) group (N = 10)	*p*
Surgical time of implant surgery	75′ (SD* = 37)	80′ (SD* = 35)	0.760
Surgical complications of implant surgery	10%	0	0.305
Clinical findings during the animal monitoring			
Abdominal wound infection	30%	10%	0.582
Dirty genitalia	10%	30%	0.582
Stereotypes harm lesions	20%	20%	1.000
Abdominal mesh extrusion	20%	0	0.474
Accidental facial injury	10%	0	1.000
Death (normal autopsy)	10%	0	1.000
Abdominal wound hematoma	10%	0	1.000
Average weight gain	884 g	714.5 g	0.641
Vaginal mesh extrusion	33%	0	0.024
Abdominal mesh extrusion	11%	0	0.474
Chronic infection signs in abdomen location (Fig. 2)	33%	10%	0.303
Chronic infection signs in vaginal location	11%	10%	1.000
Vaginal graft not visible	0	40%	0.014

Table 1. Surgical variables, clinical findings during the animal follow-up and macroscopic study of explants. **SD* Standard deviation.

Figure Multiple purulent collections arranged around the mesh, during explant removal surgery.

Blood extraction from the ear artery. Blood withdrawal from the ear artery may be a difficult procedure. Blood was obtained in 79 occasions. In 6 cases (4.74%) we experienced difficulties that led to the collection of insufficient blood volume to complete the studies. These difficulties occurred in both groups.

Surgical variables, clinical findings during the animal follow-up and macroscopic study of explants are described in Table 1.

Surgical complications of implant surgery. Only one animal suffered a mild hemorrhage in the subcutaneous tissue during abdominal surgery, which was resolved with a hemostatic suture.

Control group
A. Three cases (30%) of abdominal surgical wound infection. Resolved with antibiotic treatment
B. One case (10%) of filthiness in the genital area. No intervention needed
C. Two cases (20%) of minor self-inflicted damage. Resolved with antiseptic and environmental enrichment measures
D. Two cases (20%) of extrusion of the abdominal mesh. In one there was spontaneous resolution (correct subcutaneous location of the mesh at the time of euthanasia). In the second case the extrusion persisted
E. One case (10%) of minor facial injuries due to an accidental incorrect position of the protective collar on the first postoperative day. Resolved favourably with antiseptic measures
F. One case (10%) of death in the control group, 58 days after surgery. The necropsy showed no complications at the mesh level or other pathological findings of interest
G. One case (10%) of abdominal wound hematoma which was spontaneously resolved
Experimental group
A. One case (10%) of abdominal wound infection. Resolved spontaneously

B. Three cases (30%) of filthiness in the genital area. Resolved in one case by shaving and extensive washing under sedation
C. Two cases (20%) of minor self-inflicted damage. Resolved with antiseptic and environmental enrichment measures

Table 2. Incidences at follow-up.

Clinical complications during follow-up. Clinical complications occurred, and actions taken during follow-up and are described in Table 2. The Grimace scale was 0 in all animals and in all evaluations during follow-up.

Discussion

This research has evaluated the feasibility of the rabbit as an animal model for the study of biological grafts placed in the abdominal and vaginal locations, and it shows that the NZW is a good model for studying the behaviour of biomaterials in either locations.

The hADM used in this study is an acellular biological matrix, obtained from human dermis, produced to improve the biocompatibility of grafts over that of current synthetic alternatives. hADM is free of allergens, DNA and other pathogens. In this study, the hADMs were implanted in the abdominal fascia and in the vaginal submucosa layer of rabbits. The aim was to evaluate clinical complications during and after graft implantation surgery and macroscopic findings after graft explantation surgery, in different in vivo settings. We used the rabbit as a model on the basis of previous publications, as well as on the characteristics of the animal: adequate life expectancy for the duration of the study; perineal musculature associated with the urogenital tract like humans; enough vaginal ability to perform a vaginal and abdominal implant at the same time; easy and economical animal accommodation; and the availability of investigators trained in handling these animal species.

Other animal models besides the rabbit have been used to study biomaterials in urogynecology[17]. Rats[32–40] were used; however, due to its small body size studies at vaginal level are very difficult to be performed. The same for mice[18], where most studied materials are implanted subcutaneously, rather than vaginally[41–43].

Reviewing studies with large animal models, authors such as Endo et al.[44], Tayrac et al.[45], or Feola et al.[46] studied biological prostheses at vaginal level in sheep. Endo M. compared a cross-linked acellular collagen matrix with a PP mesh, placed simultaneously at vagina and abdomen, also demonstrating greater degradation of vaginal implants (70%). Tayrac compared a noncoated PP mesh against a coated PP mesh with an absorbable hydrophilic film, placed vaginally. In this case, an increase in vaginal exposure rate was also demonstrated in the noncoated PP group (33.3%). Finally, Feola A. compared a PP mesh, a collagen-coated mesh and the native tissue implanted in the vagina and abdomen. This study also showed 22% vaginal erosion rate associated with PP mesh group. These results are consistent with our findings. However, ewes are a more

complicated and expensive model that many research centers cannot afford.

Regarding the pig model[47], the drawbacks are similar to those of sheep: although it has enough size to perform vaginal surgery, and its anatomy is appropriated to the human being, the time required to perform the explants (180 days) makes the sows to increase their weight over 150 kg, which means that the handling of these animals and the costs of the study, even using minipigs, preclude their use in some groups. In the case of the dogs [48] and NHP, one should add the ethical and legal conflicts concerns.

There are several authors that have used the rabbit model to study different biomaterials in gynecology [21–31]; therefore, we strongly believe that the rabbit is a good model for the study of biomaterials for abdominal and vaginal application.

Graft implantation at the level of the abdominal subcutaneous tissue and in the anterior and posterior vaginal submucosa layer of rabbits was technically simple, and it was associated with very minor surgical complications. However, adequate exposure of the vaginal field is difficult due to its small size. Hence, appropriated training of an assistant and the specific surgical material (suitable for microsurgery) are needed.

Ear blood extraction was also challenging, especially after successive extractions in the same animal because of the narrowing of the vascular lumen secondary to consecutive punctures. Therefore, it is advisable to have the help of trained personnel to perform this technique. Another cause of difficulty in blood withdrawal is the arterial vasoconstriction associated with the decrease in the body temperature of the animals, as well as pain at the puncture site if adequate anesthesia is not achieved.

Complications during the clinical monitoring consisted in minor facial injuries due to accidental incorrect position of the protective collar. To avoid other similar types of injury, protective collars were removed after observing there were no self-inflicted injuries in the surgical wound area. Animals did not show signs of pain during follow-up, so we concluded that a quick, low-invasive, and uncomplicated surgical manipulation is associated with low postoperative pain allowing the avoidance of protective collar placement.

In both groups, stereotypical self-injuries appeared, so it is very important to add environmental enrichment measures in these animals. It is also very important to maintain strict hygiene measures to avoid complications derived from dirtiness.

The occurrence of graft-related complications of implants showed a very different behavior between two groups, especially in the vaginal location.

The clinical complications associated with the graft (wound infection and exposure) were more common in the control (PP synthetic mesh) group, especially in the vaginal location where mesh exposure occurred in 33% of cases ($p = 0.024$). Conversely, in the experimental group, macroscopic hADM degradation at the vaginal level occurred in 40% of cases as compared with 0% in the PP group ($p = 0.014$) whereas in the abdominal location the macroscopic characteristics of the hADM graft remained

intact in all cases. These results are consistent with the publications of: Hilger et al.[25], Pierce et al.[27] and Higgins et al.[49]. Hilger compared human dermis, porcine dermis, porcine collagen-coated PP mesh, and autologous fascia, implanted in the abdomen and the vagina of a rabbit, also demonstrating greater degradation of the implants in the vaginal location. Pierce compared PP mesh with porcine dermis placed in the vagina and abdomen in a rabbit and observed a 30% degradation rate of the biological graft in the vaginal location. Higgins studied the behavior of PP mesh at the vaginal level in relation to estrogenic levels of a rabbit, demostrating 18% erosion rate in hypoestrogenic group.

Therefore, these latter results show that the rabbit model mimics what is actually observed in humans: graft materials behave differently when implanted in the abdominal wall (i.e., for hernia repair) or in the vaginal submucosa (i.e., for pelvic reconstructive surgery)[50,51]. More extrusion of PP was observed in the vaginal location, whereas more degradation of the hADM was observed in the same location. Higher extrusion suggests a greater inflammatory response after PP implants; while high degradation of hADM suggests better biocompatibility but questions the long-term efficacy for pelvic surgery. The rabbit model allows the study of potential reasons that lead to these differences. Further analysis based on the inflammatory response to different materials in different locations observed in the present study will follow in the future to enable a better understanding of the whole process and to help guiding the development of biomaterials to be used in a human clinical scenario.

The main limitation of the project is the translation of the results from an animal model to a human situation. In this specific case hADM is a heterologous matrix to the rabbit, since is prepared from human material; therefore studies are needed to verify cross-species effects.

None withstanding this is the first experimental model approximation, subsequent clinical studies in women with this hADM will be necessary to verify the results obtained.

Conclusions

The NZW rabbit is a good model for assessing materials to be used as grafts for pelvic reconstructive surgery and vaginal surgery. The hADM is associated with fewer clinical complications, as well as better macroscopic tissue integration, compared to PP mesh. Additional research is needed to investigate the long-term safety and efficacy of hADM used in women for pelvic reconstructive surgery.

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4.2 CAPÍTULO 2

En este capítulo se exponen los resultados del comportamiento a los 6 meses de una MDh, en comparación con PP, en cirugía vaginal en un modelo animal.

ARTÍCULO 2: Comparison of a human acellular dermal matrix and a polypropylene mesh for pelvic floor reconstruction: a randomized trial study in a rabbit model

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Resumen:

Las mallas de PP han sido ampliamente utilizadas en la reconstrucción quirúrgica de las DSP. Sin embargo, se asocian con complicaciones graves. Las MDh han demostrado seguridad y eficacia en medicina reconstructiva, pero se desconoce su idoneidad y eficacia a nivel vaginal. Este estudio compara las propiedades biológicas de la malla de PP y una MDh recientemente desarrollada. Se aleatorizaron 20 conejos para recibir el injerto de MDh, o la malla de PP. Los injertos se implantaron quirúrgicamente en la pared abdominal y la vagina. Después de 180 días, se explantaron y evaluaron los injertos.

La tasa de extrusión de la malla vaginal fue mayor en el grupo PP (33 % frente a 0 %, p=0,015). La integración completa de los injertos vaginales fue más frecuente en el grupo MDh, donde el 40 % de los injertos fueron macroscópicamente no visibles a los 6 meses. En contraste, en el grupo PP la malla vaginal fue identificada en el 100% de los animales (p=0,014). Respecto el estudio histológico e inmunohistoquímico, en el grupo PP, los infiltrados tenían una distribución focal y se localizaban mayoritariamente en la parte interna del epitelio, mientras que en el grupo MDh, los infiltrados presentaban una distribución difusa. Respecto la representación celular, en el grupo MDh se observó una mayor presencia de linfocitos B (y menor de linfocitos T), en comparación con el grupo control. El análisis biomecánico mostró que la MDh presentaba una menor resistencia al estrés. Además, la rigidez y la elasticidad de la malla de PP fueron superiores al grupo experimental.

Así pues, podemos concluir que la MDh se asocia a menos complicaciones clínicas, y presenta una mejor integración tisular en el tejido nativo circundante, especialmente en la zona vaginal. Sin embargo, las propiedades biomecánicas no se mantienen estables 6 meses después de su implantación.

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Comparison of a human acellular dermal matrix and a polypropylene mesh for pelvic floor reconstruction: a randomized trial study in a rabbit model

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Non-absorbable polypropylene (PP) meshes have been widely used in surgical reconstruction of the pelvic floor disorders. However, they are associated with serious complications. Human acellular dermal matrices (hADM) have demonstrated safety and efficacy in reconstructive medicine, but their suitability and efficacy at vaginal level is not known. This study compares the biological performance of PP mesh and a newly developed hADM. 20 rabbits were randomized to receive the hADM graft or the PP mesh. Grafts were surgically implanted in the abdominal wall and vagina. After 180 days, grafts

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| (2022) 12:20698 |

were explanted and evaluated. The vaginal mesh extrusion rate was higher in the PP group (33% vs. 0%, p = 0.015). Full integration of the vaginal grafts was more frequent in the hADM group, where 35% of the grafts were difficult to recognize. In the PP group, the vaginal mesh was identified in 100% of the animals (*p* = 0.014). In PP group, the infiltrates had a focal distribution and were mostly located in the internal part of the epithelium, while in the hADM group, the infiltrates had a diffuse distribution.

Additionally, the hADM group also presented more B-lymphocytes and less T-lymphocytes. Biomechanical analysis showed that hADM had lower resistance to stress. Moreover, PP mesh stiffness and elasticity were higher. Then, hADM is associated with fewer clinical complications, as well as better tissue integration. However, it shows greater incorporation into the surrounding native tissue, especially in the vaginal location, undergoing a reduction in its biomechanical properties 6 months after implantation.

Pelvic floor dysfunction is a very common condition that affects 30–50% of the adult female population[1]. Surgical repair may involve the use of a non-absorbable synthetic macroporous prosthesis (polypropylene [PP] meshes). However, synthetic meshes may induce a severe chronic inflammatory response, and have been associated with severe complications such as erosion, retraction, and pain[2]. Due to the risk of complications associated with their use in pelvic organ prolapse (POP) and urinary incontinence (UI) surgery, some international regulatory authorities[3,4] have banned vaginal mesh implants for pelvic surgeries since July 2018. Accordingly, there is a need to investigate safer and more biocompatible materials to improve the current situation with PP meshes.

Tissue engineering is emerging as a new technology for tissue regeneration when native tissues are compromised. Application of these novel approaches may overcome the problem of weakened pelvic floor that occurs in women with POP or UI and offer personalized therapy for patients. This research will help to solve an as yet unmet clinical need.

Acellular matrices (AM)—decellularized scaffolds of biological origin—have emerged as good candidates to replace synthetic meshes. In acellular dermal matrices (ADM), the architecture and components of the extracellular matrix are mostly preserved, making them useful for clinical applications where tissues need to be replaced or reinforced, such as rotator cuff repair, and breast or abdominal wall reconstructions[5–7]. Dermal allografts have demonstrated their safety and clinical efficacy in different clinical applications [8,9], but their application in the field of gynecology has been scarcely evaluated [10–15].

Our group has previously reported the development of an acellular dermal matrix from human cadaveric donors (hADM)[16], an allograft with a set of mechanical, structural, biochemical and storage properties suitable for a broad range of clinical applications, including gynecology.

We have also previously reported the development of an animal model to study PP and hADM grafting in the vaginal position[17], showing that the New Zealand White (NZW) rabbit is a suitable model for assessing engrafting of biomaterials in vaginal and pelvic reconstructive surgery. The hADM developed was associated with fewer clinical complications, as well as better macroscopic tissue integration than PP meshes.

Therefore, the aim of the present study was to fully characterize the biological properties of hADM, its integration capacity and response in an in vivo animal study, in order to determine the safety and potential use of this graft in pelvic floor reconstructive surgeries.

Materials and methods

Preparation of hADM samples. The study was performed according to European and Spanish Directives for Tissues and Cells for tissue donation, retrieval, processing and preservation and clinical use.

Ethics institutional review board (IRB) approval was obtained (CEIm Hospital Valle Hebrón, Barcelona; PR (BST)314/2019) for human cadaveric donors. Also research purposes of the tissue was obtained through signed informed consent. No tissues were obtained from the prisoners.

The skin decellularization protocol was previously defined to remove the cellular content while maintaining the structure and mechanical properties of the native tissue. 16 Skin fragments were decontaminated and decellularized by hypertonic and hypotonic solutions, followed by an enzymatic treatment and a detergent-soaking step. Finally, hADM fragments were trimmed to 20×10 mm and preserved in a glycerol solution.

Animal model. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Research Institute at the Hospital de la Santa Creu i Sant Pau and authorized by the Animal Experimental Committee of the local government authority (Generalitat de Catalunya, authorization No. 9669) in accordance to the Spanish law (RD 53/2013) and European Directive 2010/63/EU. In addition, the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985), follows the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), and is committed to the 3Rs of laboratory animal research and consequently used the minimal number of animals to reach statistical significance.

An in vivo study based on a NZW rabbit model was previously performed to evaluate its feasibility [17]. hADM was compared to PP mesh in abdominal and vaginal locations. Briefly, a total of 20 female multiparous NZW rabbits were randomly assigned to receive a PP mesh (control group) or hADM graft (experimental group). Each rabbit received four grafts: two grafts in the vaginal submucosa layer and two grafts in the subcutaneous tissue of the abdominal wall. For the vaginal implants, one

graft (5 × 5 mm) was placed in the anterior vaginal wall and was used for histological and immunohistochemical studies. The second graft (10 × 5 mm) was placed in the submucosa of the posterior vaginal wall and was used to perform the biomechanical study. Both abdominal grafts were implanted in the right caudal quarter of the abdominal wall. The surgical procedure and animal housing were performed following the protocol previously published by Pero et al.[17].

Explant retrieval. After 180 days of veterinarian monitoring, the rabbits were euthanized and the grafts explanted, removing the surrounding tissue from the prosthesis (explants).

Histological study. Explants (5 × 5 mm) from the abdomen and vagina were immersed in fixative solution (4% paraformaldehyde) and embedded in paraffin with an automatic tissue processer (Thermo Shandon, Citadel 2000). Paraffin blocks were cut into 5 μm-thick serial sections with a microtome (Leica, JUNG RM2055) and placed on poly-L-lysine coated glass slides. Histological slides were observed using a binocular transmitted light microscope (Nikon Eclipse 80i) at 8X, 40X and 100X magnifications. Images were acquired using a digital camera (R3 Retiga, Qimaging) and processed with the software Image-Pro 10 (Media Cybernetics). Morphological, histological and immunohistochemical properties were analyzed.

The following staining was performed: hematoxylin–eosin (H&E) to evaluate the structure and grade of inflammatory reaction, Masson's Trichrome to evaluate the collagen fiber organization, and Sirius Red to evaluate the organization of collagen fibers (type I and III) of the explants. A macroscopic image at 100X magnification of each explant was performed ($n = 10$ hADM; $n = 9$ PP) and thereafter the images were quantified using ImageJ software (Launcher). The percentage of fibrosis was calculated as the stained area (SA) with respect to the total area (TA): (SA/TA)*100.
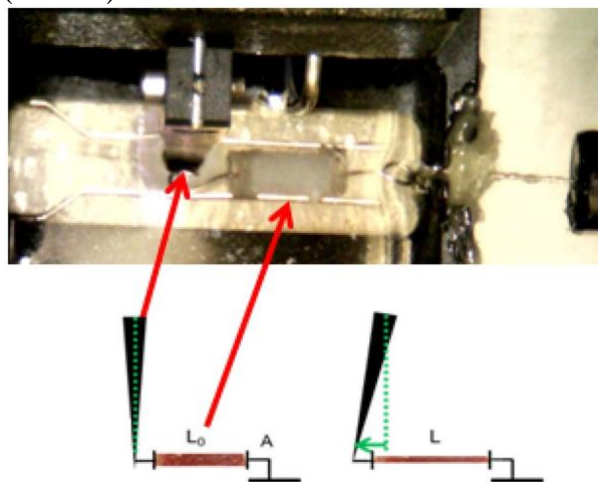

Figure Photograph and diagram of the experimental setup for biomechanical testing of the imp

Immunohistochemical study. Anti-CD3 (T-cell marker), anti-CD79a (B-cell marker) and anti-RAM11 (macrophage marker) antibodies were used to assess the cellular inflammatory profile in the sections with cellular infiltrates observed in the H&E staining. Sections were deparaffined and the antigen was recovered. The sections were washed with phosphate buffered saline (PBS) and incubated with 5% hydrogen peroxide for 30 min. Subsequently, 1 h of blocking was performed using a blocking solution composed of 0.2% albumin, 1% mouse serum and 0.5% goat serum. The sections were then incubated for 1 h with mouse primary antibodies against CD3 markers (CD3 epsilon antibody PC3/188A, Novusbio), CD79a (CD79a HM57, Bio-Rad) and macrophages (RAM11, Dako). This was followed by incubation with peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). Finally, the reaction was revealed by incubation with the DAB substrate (Vector Laboratories) for 10 min and hematoxylin counterstaining. The slides were fixed and dehydrated on an ethanol-xylene gradient and covered with Entellan® mounting medium.

The presence of macrophages, B cells and T cells present in the inflammatory infiltrates (100X magnification) was interpreted using a 0 to 4 + scale: 0 indicates absence of cells; 1 + indicates scattered presence of isolated cells; 2 + indicates slight presence; 3 + indicates moderate presence; and 4 + indicates severe presence. The results were subsequently grouped into 2 categories: mild presence (1 + , 2 +) and severe presence (3 + , 4 +).

Biomechanical assay. Biomechanical properties were analyzed using the uniaxial tensile stretch test. The analyzed samples were native human skin and baseline hADM (not implanted sample), and the explanted grafts (both hADM and PP mesh). Each sample of native human skin, baseline hADM, or explanted hADM was cut into three different strips (replicates) of ~ 5 mm × 1 mm × 0.7 mm. For each strip, one end was attached with cyanoacrylate glue to a fixed hook while the other was glued to a hook attached to the lever of a servo-controlled displacement actuator with an integrated 1 N force sensor (300C-LR, Aurora Scientific, Aurora, Canada) (see Fig. 1). In the case of PP explanted meshes, the complete fragment was measured using a 5 N force sensor (305C, Aurora Scientific, Aurora, Canada). The stress–strain (σ-ε) curves were calculated from the force–displacement curves using geometrical measurements of the sample made by a calibrated CCD camera. Biomechanics of the samples were characterized as the stress measured at the 20% stretch point [18].

Statistical analysis. A descriptive analysis was performed, determining the median and also the mean with standard deviation (SD). The relationship between categorical variables was analyzed using the corresponding contingency tables, calculating the percentage in each group and applying the chi-square test with approximation of the probability ratio. For the ordinal variables, the comparison between two groups was made using the

non-parametric Mann–Whitney test. In all cases, the usual level of significance was 5% (alpha = 0.05). In the case of the biomechanical study, the Student t-test was performed to compare differences in σ and E_M in the different groups at $\varepsilon = 0.2$. All analyses were performed with the statistical package IBM-SPSS (V25).

Results

Preparation of hADM samples. Twenty-five pieces of hADM measuring 20×10 mm were prepared. Each decellularized piece had a low DNA content, specifically 0.55 ± 0.68 ng/mg dry tissue and 1.03 ± 0.71 ng/ mg dry tissue, while maintaining the extracellular matrix (ECM) structure and major proteins [16].

Explant retrieval. Twenty animals were included, ten in the experimental group (hADM) and ten in the control group (PP mesh). One rabbit from the control group died due to causes unrelated to the surgeries or graft. After six months of housing in the animal facilities, both abdominal and vaginal implants were explanted. All PP meshes in both the abdominal and vaginal location ($n = 36$) and all hADM in the abdominal location

	PP mesh ($n = 9$, $N =$ 36)	hADM ($n = 10$, $N =$ 26)	p value*
Vaginal mesh extrusion	3 (33%)	0	0.024
Abdominal mesh extrusion	1 (11%)	0	0.474
Chronic infection signs in abdomen location	3 (33%)	1 (10%)	0.303
Chronic infection signs in vaginal location	1 (11%)	1 (10%)	1
Vaginal graft recovered	9 (100%)	6 (60%)	0.014

Table 1. Macroscopic study of explants. *N* number of animals explanted, *n* number of analyzed samples. *$p < 0.05$.

	PP mesh ($n = 9$)	hADM ($n = 9$)	p value
Infiltrates	4	9	0.040
Vagina	4	5	0.630
Abdomen	0	5	0.008

Table 2. Number of inflammatory infiltrates observed. Statistical analysis was performed using chi-square test. *n* = number of analyzed samples.

($n = 20$) were recovered. However, only 13 of the vaginal hADM implants (65%) were recovered; the other seven matrices were integrated in the rabbit vaginal mucosa and could not be explanted.

Macroscopic study. Table 1 describes the pathological findings observed during explantation surgery. PP implants showed mesh extrusion at both the vaginal (33%; $p < 0.05$) and abdominal (11%) location, compared to

hADM implants (0% mesh extrusion). The PP group presented a higher incidence of chronic infection such as abscess formation and wound dehiscence than the hADM group. Chronic infection was 33% vs. 10% in the abdominal position and 11% versus 10% in the vaginal position. All PP meshes were recovered from both the vaginal and abdominal sites. Of ten hADM vaginally implanted, six were fully integrated with the surrounding tissue, but two were able to be identified through the non-absorbable prolene stitches used to attach them to the host during the implant surgery. Therefore, six hADM were recovered and further analyzed.

Histological study. Eighteen PP explanted meshes and eighteen explanted hADM samples were analyzed both histologically and immunohistochemically. The H&E study (Table 2) showed that 4/9 animals (44%) treated with PP mesh (control group) presented inflammatory infiltrates in the vaginal explants. These infiltrates had a predominantly focal distribution and were located in the internal part of the epithelium (Fig. 2F,G,H,I,J). No animal treated with the PP mesh presented inflammatory infiltrates at the abdominal level (Fig. 2P,Q). Additionally, 100% of the animals treated with the hADM presented inflammatory infiltrates. A large number of foci of infiltrates were identified at both the vaginal and abdominal sites (compared with the control group, showing diffuse distribution of the infiltrates, Figs. 2A,B,C,D,E,K,L,M,N,O, respectively). One animal showed infiltrates in both the vaginal and abdominal positions.

Sirius Red staining showed the percentage of fibrosis of each type of explant and location (Figs. 2B,G,L,Q). The percentage of fibrosis was higher in the control group. The arrangement of collagen fibers was more compact in this group, especially in the abdominal location (Fig. 2Q). Unlike in the experimental group, the collagen structure remained stable (Figs. 2B,L).

Immunohistochemical study. Immunohistochemical analysis (Table 3) showed that in the experimental group, 7/10 infiltrates (70%) had a slight T lymphocyte (TL) expression and high macrophage expression. The presence of B lymphocytes (BL) depended on the location, with high expression being observed at the vaginal level and mild expression at the abdominal level. Regarding the control group, the presence of BL was low in all cases, variable in macrophages and high in TL. No animal in the control group presented infiltrates in the abdomen.

Biomechanical assay. A total of ten samples of native human skin, ten samples of baseline hADM, sixteen PP explanted meshes (nine from the abdominal area and seven from the vaginal area) and twelve explanted hADM samples (eight from the abdominal area and four from the vaginal area) were measured, as detailed in Table 4.

Three vaginal hADM explants could not be identified due to their integration into the host tissue, while three vaginal hADM explants were not measured due to difficulties during sample dissection. The hADM placed in the abdominal position were clearly identified, but two of them were not measured due to technical problems during sample preparation for the experiment. With regards to PP meshes, two vaginal samples with PP explants could not be measured due to similar technical problems. One PP mesh subject died during the follow-up.
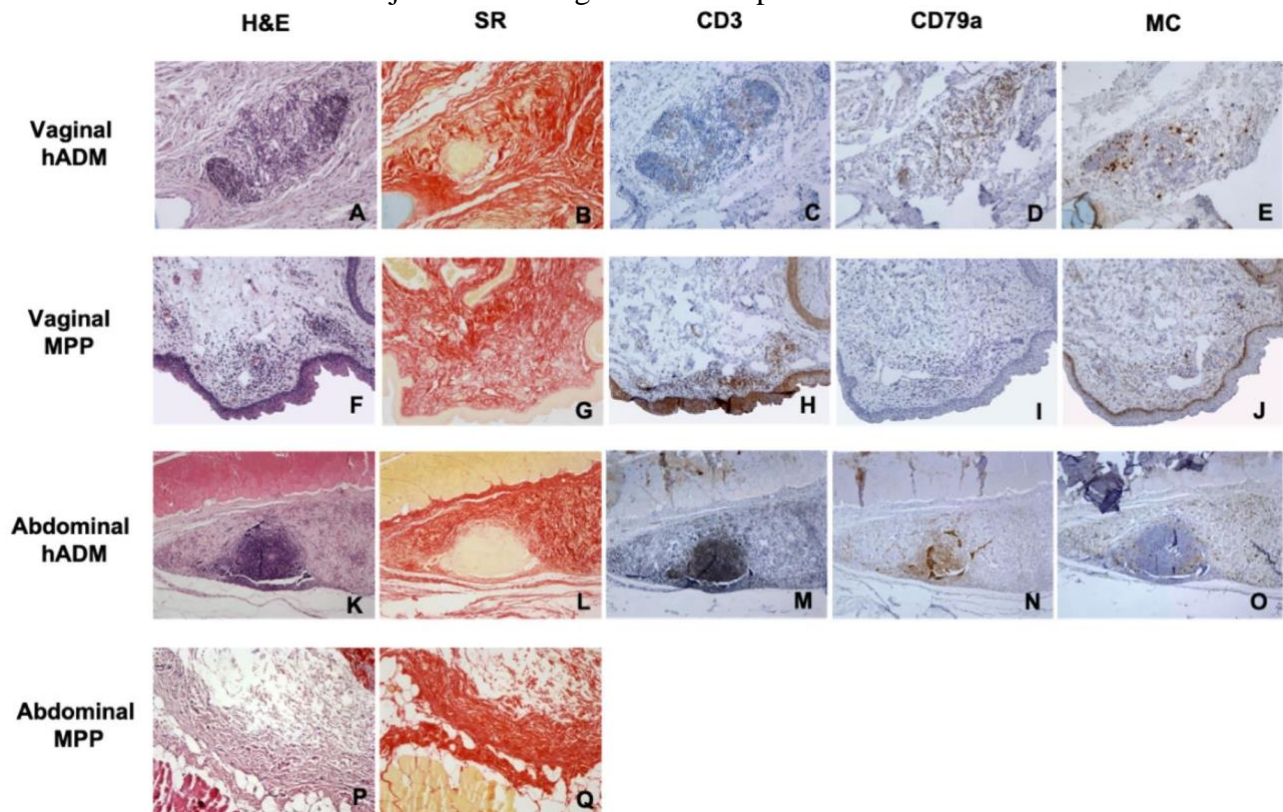
Figure 2. Representative histology and immunohistochemical staining of serial vaginal (**A**–**J**) and abdominal
(**K**–**Q**) sections: Hematoxylin–Eosin (**H&E**), (**A**, **F**, **K**, **P**), Sirius Red (SR), (**B,G,L,Q**), T lymphocytes (CD3), (C,H,M), B lymphocytes (CD79a), (**D,I,N**) and macrophages (MC), (**E,J,O**), (X100).

		hADM		PP mesh	
		Vaginal ($n = 5$)	Abdominal ($n = 5$)	Vaginal ($n = 4$)	Abdominal ($n = 0$)
CD3 (TL)	MP	4	3	1	0
	SP	1	2	3	0
CD79a (BL)	MP	0	4	4	0
	SP	4	1	0	0
Macrophages	MP	2	2	1	0
	SP	3	3	2	0

Table 3. Presence of CD3 + cells, CD79 + cells and macrophages in the hADM group and PP mesh group biopsies. Cellular presence was quantified using a 0–4 + scale and these data were grouped into 2 categories: mild (1 + , 2 +) and severe (3 + , 4 +). *MP* mild presence; *SP* severe presence. *n* = number of analyzed samples.

Location	PP mesh (*n* = 9)	hADM (*n* = 10)
Vagina	7	4
Abdominal wall	9	8

Table 4. Description of the analyzed explants during the mechanical test. *n* = number of analyzed samples.

Results in Fig. 3 show that the stress at 20% of stretch of the explants in the experimental group decreased, especially at the vaginal location, compared to the pre-implanted biomechanical properties. PP mesh explants were found to have a higher stiffness compared with hADM explants. Explanted hADM showed significantly lower stiffness than hADM prior to the in vivo implantation.

No differences were observed between the stiffness of PP meshes explanted from the vaginal or the abdominal zone. However, a significant decrease in the stiffness of hADM explants from the vagina was observed compared to the stiffness of the hADM explants from the abdominal area ($p < 0.05$).
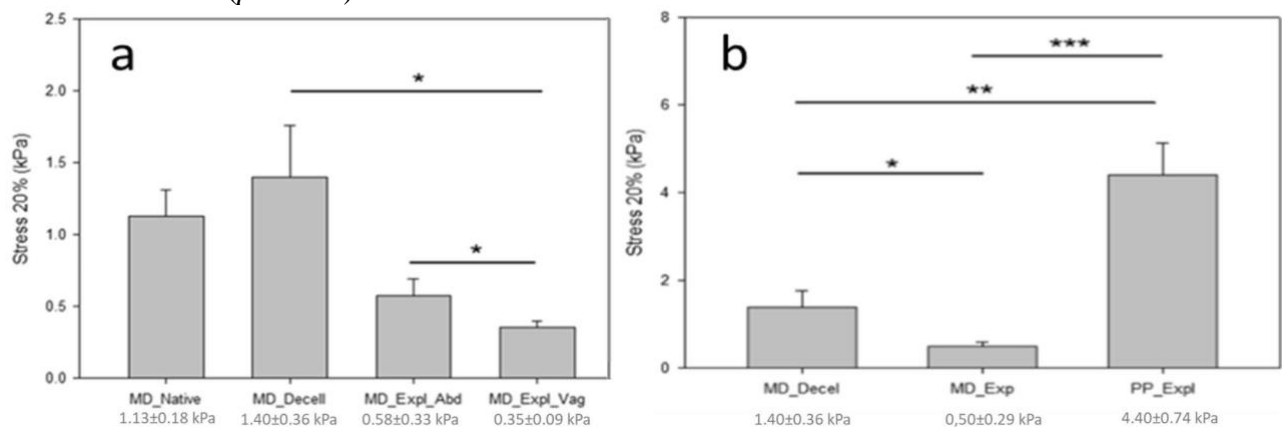

Figure 3. (**a**) Mechanical properties of the hADM grafts before and after their implantation. (**b**) Comparison of mechanical properties between explanted hADM and explanted PP mesh in the abdominal location. *p value < 0.05, **p value < 0.01, ***p value < 0.001.

Discussion

The most important finding of this study was that human dermal matrices implanted in both the abdominal and vaginal site in rabbits were safe. The complete histological characterization of the explants confirmed that new tissue was regenerated in the vaginal site, with maintenance of a well-organized collagen fiber structure that supported the repopulation.

The macroscopic findings of the explants showed very different behavior between the two groups, and these differences were especially relevant in the vaginal location. The control (PP) group showed a statistically significant increase in vaginal extrusion, compared to hADM. These results were expected, since vaginal extrusion of PP meshes is a known common complication. On the other hand, the hADM graft was completely integrated into the surrounding tissue in a significant percentage of cases, and in some subjects, it was impossible to identify and remove the explants, especially at the vaginal level. These results are consistent with previous publications showing 70% and 100% graft degradation in the vaginal location by Pierce et al. and Claerhout et al., respectively[19,20]. The macroscopic findings in our study are therefore consistent with excellent biocompatibility of hADM grafts at the vaginal level.

The inflammatory response is a physiological process that occurs as a consequence of the immunological interaction with a foreign body. Nevertheless, the nature and intensity of this response depend on the characteristics of the implanted material. The results of this study reveal that the immune responses are different in both groups. Six months after implantation, the control (PP) group showed an inflammatory response located mainly at the epithelial level (focal response), and mainly mediated by TL; however, in the experimental group, a heterogeneous chronic rejection response with scattered invasion of inflammatory cells was observed. Our results coincide with those previously reported by Ying Yao et al. with heterogenic meshes [21].

The immunological response differed depending on the implant location (vaginal or abdominal), in line with previous reports[19]. Our study shows a greater immune response to foreign bodies in the vaginal location compared to the abdominal area in both groups; 9/18 animals studied presented an inflammatory response at the vaginal level and only 5 at the abdominal level. This different response depending on the location was more evident in the control group, where no subject had an inflammatory infiltrate at the abdominal level. The results of this study are thus consistent with observations in the real-world setting, where mesh complications are more common when the implant is placed at the vaginal level as compared with the abdominal location. The underlying mechanisms that explain this different behavior according to the location have yet to be determined, but could be influenced by the degree of host tissue vascularization.

The histological study provides information about the arrangement of collagen fibers, showing differences between the two groups. In the control group, collagen fibers were more compact, with a higher percentage of fibrosis both in the vaginal and abdominal locations, which results in a rejection response, as previously shown[19]. Along these lines, it has been described that, in PP implants, the density of collagen increases over time, unlike the inflammatory reaction that disappears over time in the abdominal location[22]. In the study group, the collagen fibers were more structured and organized, which translates into better tissue integration. This maintenance of the collagen structure was previously observed by Ying Yao et al. in heterogenic meshes [21]. In terms of biomechanical properties, this study shows that stiffness of explants diminished 6 months after implantation, especially in the vaginal location, for the study group. These results are probably due to the integration and partial degradation of the explants, predominantly in the vaginal location. These findings are in agreement with previous reports[19,23–26].

In summary, the excellent biocompatibility of hADM grafts suggesting a good safety profile needs to be counterbalanced by the early degradation process that challenges its efficacy profile.

In terms of study limitations, the results demonstrate the safety of hADM grafts, but the animal model used does not allow us to fully evaluate graft functionality, since quadruped versus biped standing position may affect results. Another limitation of the study is the limited number of samples analyzed, as well as the size (5 × 5 mm and 5 × 10 mm) and thickness of the implants (1 mm) used. Although this size is adequate for the animal model used (rabbit), it has caused technical difficulties for the biomechanical study of the explants. In turn, the reduced thickness of the samples also makes it difficult to extrapolate the results in the human model. To better understand these limitations and comprise the potential therapeutic effect of hADM in women, our experience using samples of different thicknesses (up to 4–5 mm) and their correlation with biomechanical properties is added in the following lines. The results obtained from the preliminary in vitro studies showed that the biomechanical properties were proportional to the thickness of the sample, so that the greater the thickness, the greater the stiffness and greater resistance. However, the decision to use 1-mm matrices for this study was based on the idea of using thicknesses appropriate to the subject (in this case, small animals, thin thicknesses).

Despite the animal model limitations, the findings of our work provide a putative therapeutic alternative with relevance and applicability in the field of urogynecology.

Clinical studies in women are needed to assess the functionality of the graft. The immunologic and inflammatory response to the hADM may also be different when implanted in humans, and the biocompatibility and biomechanical properties will need to be assessed.

Unlike synthetic meshes that simply act to reinforce damaged tissue, hADM acts as a natural scaffold with full integration with the surrounding soft tissue, inducing tissue regeneration. This tentative function is

compatible with the hypothesis that hADM sustains biocompatibility with the recipient tissue, and is able to abrogate the complications associated with synthetic meshes that induce local inflammation. This acellular matrix seems to act as a scaffold where the host cells can migrate and proliferate and thus regenerate the tissue where it is implanted; however, biomechanical properties may need to be improved to increase host tissue support. As the previous study conducted by our group concluded, the NZW rabbit is a suitable model for assessing materials to be used as grafts for pelvic reconstructive surgery and vaginal surgery [17]. This study provides further information on the biological properties of a new biomaterial—hADM—surgically implanted in the subcutaneous abdominal wall and in the vaginal submucosa layer of a NZW rabbit.

Conclusions

We have investigated an alternative therapeutic option based on the use of human decellularized dermis for clinical practice in the field of urogynecology, improving current suboptimal therapies. Compared to commercial PP macroporous mesh, hADM is associated with fewer clinical complications, including vaginal mesh extrusion; as well as better incorporation into the surrounding native tissue, especially in the vaginal location. Additionally, the immunological response found in the hADM group shows a diffusely distributed cellular infiltrate, with a greater representation of B-lymphocytes. However, biomechanical analysis showed that hADM had lower resistance to stress, compared to PP mesh.

Data availability

All data analysed during this study are included in this published article. However, there are data collected that are not digitized, but are collected by hand in the notes of each author. Nevertheless, the datasets analysed during the current study available from the corresponding author on reasonable request.

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5. DISCUSIÓN

Este trabajo parte de la conciencia médica de que existe un grupo de pacientes que actualmente no sabemos cómo tratar. Este trabajo nace de la convicción que desde la comunidad científica debemos encontrar una alternativa terapéutica para las mujeres con disfunciones del suelo pélvico, ya que en estos momentos las mujeres con POP severos o recurrentes se encuentran con opciones terapéuticas limitadas. Esta tesis surge de la búsqueda de la solución a un problema: ¿qué material podemos utilizar en la cirugía reconstructiva del suelo pélvico, ahora que sabemos que las mallas sintéticas pueden asociarse a complicaciones graves?

La hipótesis de este grupo es que las mallas biológicas, y concretamente las matrices dérmicas de origen humano, pueden constituir una alternativa terapéutica segura y eficaz en el tratamiento de las DSP. Esta teoría se basa en el conocimiento previo que ha demostrado que las matrices biológicas son materiales biocompatibles con una gran capacidad de integración tisular, y muy baja respuesta inmunogénica, disminuyendo así el riesgo de rechazo(10,12,16–18)

El gran inconveniente de las matrices dérmicas es que existe muy poca información disponible acerca de su comportamiento en la localización vaginal. Por esta razón, este grupo propone el desarrollo de un estudio experimental preclínico utilizando el conejo como modelo animal.

En este contexto surge el primer artículo, que tiene como objetivo: 1) evaluar la seguridad clínica de las matrices dérmicas, y 2) estandarizar el conejo como modelo animal en el estudio de biomateriales para ser aplicados a nivel vaginal.

El primer reto de este trabajo fue seleccionar el modelo animal más adecuado para lograr los propósitos del estudio. Los modelos más comúnmente utilizados en el estudio de prótesis biológicas son los mamíferos, concretamente: rata, ratón, conejo, oveja, perro y primates no humanos. Los

objetivos del estudio requerían un animal con un tamaño corporal suficiente como para permitir la implantación quirúrgica de prótesis a nivel vaginal y abdominal. Por ello, los animales de menor tamaño como rata y ratón fueron excluidos. Todas las especies restantes cumplían los requisitos de tamaño necesarios para participar en el estudio. Sin embargo, se tuvieron en cuenta otros criterios como: la experiencia del equipo veterinario; la disponibilidad de espacio y de recursos personales y materiales; la curva de aprendizaje necesaria para el manejo de los animales en investigadores con poca experiencia; y la esperanza de vida, paridad y anatomía genitourinaria del animal utilizado.

El perro y los primates no humanos también fueron excluidos debido a la controversia ética y moral intrínsecas al modelo.

La oveja es una de las especies más utilizadas en el estudio de MA(19–21). Junto con el cerdo(22), ambos constituyen un modelo con tamaño suficiente para permitir la cirugía vaginal, y su anatomía resulta adecuada para la posible extrapolación de resultados al modelo humano. Sin embargo, el principal inconveniente de estos dos modelos, se asienta en la superior demanda de recursos necesaria para su manejo. En el caso de las cerdas, son animales que, tras los 6 meses de estabulación necesarios para la realización del estudio, pueden alcanzar pesos superiores a los 150kg. Si este dato lo multiplicamos por los 20 individuos necesarios, la estimación de costes referentes al mantenimiento de los sujetos es muy superior en comparación con una posible alternativa de menor tamaño como las conejas.

El conejo es un modelo que ya ha sido ampliamente utilizado en el estudio de biomateriales(23,24,33,25–32); su tamaño es adecuado para permitir la cirugía abdominal y vaginal de forma simultáneas; presenta una esperanza de vida compatible con la duración del estudio; posee una musculatura perineal asociada al tracto urogenital como en el caso de los humanos; su estabulación es sencilla y económica; y en el caso de este equipo, de disponía de un

equipo humano altamente especializado en el manejo de esta especie. Por todas estas razones, finalmente fue el conejo el animal escogido para protagonizar este estudio.

20 animales fueron incluidos en el estudio, 10 conejos en el grupo experimental (MDH), y 10 en el grupo control (PP). Se pudieron completar con éxito todas las cirugías de implantación. 1 sujeto falleció (a los 83 días tras la cirugía) por causas ajenas a complicaciones relacionadas con la cirugía o el implante.

El proceso de implantación quirúrgica de las prótesis en el tejido subcutáneo abdominal, y en la submucosa de la cara anterior y posterior vaginales, es técnicamente sencillo. Sin embargo, se deben tener en cuenta una serie de consideraciones: 1) la adecuada exposición del campo quirúrgico vaginal es dificultosa debido a su reducido tamaño; 2) se requiere de material quirúrgico específico (apropiado para microcirugía); 3) la extracción de muestras sanguíneas a través de la arteria auricular puede ser dificultosa, especialmente tras la realización de extracciones sucesivas en el mismo animal (debido al estrechamiento de la luz vascular). Por esta razón es útil la ayuda de personal entrenado en este procedimiento, así como mantener los animales a una temperatura fisiológica (para evitar la vasoconstricción arterial), y con un adecuado plano anestésico que evite el dolor y asegure la relajación de los individuos; 4) los animales no han mostrado signos de dolor durante el seguimiento postquirúrgico, por lo que el uso de collares de protección no añade beneficios, mientras que puede ser causa de lesiones faciales en caso de malposición del mismo; y 5) en ambos grupos aparecieron lesiones causadas por estereotipias, por lo que las medidas de enriquecimiento ambiental, así como mantener estrictas condiciones de higiene, son muy importantes para prevenir posibles complicaciones.

El estudio macroscópico de los explantes evidenció un comportamiento muy distinto entre ambos grupos, y estas diferencias fueron especialmente

relevantes en la localización vaginal. El grupo de las mallas de PP mostró un incremento estadísticamente significativo de extrusión vaginal comparado con el grupo de MDh, con una tasa de exposición vaginal en el grupo PP del 33% (p=0.024). Sin embargo la tasa de extrusión abdominal no mostró diferencias estadísticamente significativas entre ambos grupos (11% vs 0%, p=0.474). Por otro lado, el 40% de los explantes vaginales del grupo MDh presentaron una integración tisular completa en el tejido receptor circundante, donde los explantes eran macroscópicamente indistinguibles del tejido animal. En cambio, en el grupo control, el aspecto macroscópico de los explantes de PP permaneció intacto en todos los casos (p=0.014). Estos resultados son consistentes con las publicaciones de Hilger et al.(32), Pierce et al.(28), Higgins et al.(34) y Claerhout et al.(30). Todos ellos usaron también el conejo como modelo. Hilger comparó la dermis humana, la dermis porcina, una malla de PP cubierta de colágeno porcino, y fascia autóloga demostrando también una degradación superior de los implantes en la localización vaginal. Pierce comparó una malla de PP con dermis porcina, observando un 30% de degradación de los implantes biológicos a nivel vaginal. Higgins estudió el comportamiento de las mallas de PP vaginales en relación a los niveles estrogénicos, demostrando un 18% de erosión en el grupo con hipoestrogenismo. Por último, Claerhout mostró un 100% de degradación del implante biológico a nivel vaginal.

Así pues, estos estudios demuestran que el modelo animal mimetiza aquello que actualmente observamos en humanos: las mallas se comportan de forma distintas cuando son implantadas a nivel abdominal (ej. en la reparación de hernias), vs la localización vaginal (ej. cirugía pélvica reconstructiva). En este estudio se observa que en la localización vaginal se produce una mayor exposición de las mallas de PPl, y al mismo tiempo una mayor degradación de las MDh. Mayor extrusión sugiere mayor respuesta inflamatoria; mientras que una mayor degradación se traduce en una mejor biocompatibilidad.

En resumen, tras evaluar la factibilidad del conejo como posible modelo para el estudio de biomateriales implantados a nivel abdominal y vaginal, se puede concluir que el conejo es un buen modelo para el estudio del comportamiento de biomateriales en pared abdominal y vagina. Además, en este documento se concretan los retos específicos que aparecen tanto en el procedimiento quirúrgico, como en la monitorización y seguimiento clínico de los sujetos. Esta información permitirá guiar otros investigadores que consideren al conejo como sujeto de estudio para la evaluación de prótesis vaginales.

El segundo artículo se centra en la evaluación del comportamiento y de las propiedades biológicas de los implantes, y cómo el entorno (la localización abdominal vs vaginal) puede modificar estas respuestas. Para ello se realiza un exhaustivo análisis que incluye el estudio histológico e inmunohistoquímico, y la evaluación de las propiedades biomecánicas de los explantes. Con este estudio se pretende ofrecer respuestas a las cuestiones relativas a la respuesta inmunológica provocada por los injertos, así como estudiar las modificaciones en la dureza, la rigidez y la elasticidad de los explantes. Este trabajo evalúa las características de la MDh desde la perspectiva de considerar este biomaterial como un posible recurso terapéutico en el campo de la uro-ginecología.

El hallazgo más importante de este estudio es que las MDh implantadas en abdomen y vagina de conejas, son seguras, puesto que no se observa ninguna complicación clínica moderada-severa. El estudio histológico también demuestra que las MDh asocian un proceso de regeneración tisular y de repoblación celular a nivel vaginal.

La respuesta inflamatoria es un proceso fisiológico que ocurre como consecuencia de la respuesta inmunológica ante cuerpos extraños. Sin embargo, la naturaleza e intensidad de esta respuesta depende, en gran medida, de las características del cuerpo extraño (los implantes). Dicho de otro modo, las características del injerto van a condicionar el tipo y la

intensidad de la respuesta inflamatoria del sujeto. Los resultados de este estudio evidencian que la respuesta inmunológica es distinta en ambos grupos. Pasados 6 meses tras la cirugía de implantación, el grupo control (PP) muestra una respuesta inflamatoria focal limitada fundamentalmente a nivel epitelial y principalmente mediada por linfocitos T (LT). Esta respuesta corresponde a una inflamación crónica con presencia de macrófagos y LT, ya que el desencadenante de la inflamación está presente y favorece que se promueva una respuesta inmune celular adaptativa. En cambio, en el grupo experimental (MDh), se produce una respuesta de rechazo crónico heterogénea asociada a una infiltración difusa de las células inflamatorias. En este grupo observamos una gran presencia de macrófagos y la presencia de células B, células importantes en los rechazos crónicos alogénicos por su gran papel en la presentación antigénica y activación celular. Estos resultados coinciden con los resultados publicados por Ying Yao et al(35).

La respuesta inmunológica también difirió dependiendo de las condiciones ambientales. Es decir, los resultados inmunohistoquímicos mostraron diferencias en la localización vaginal vs la localización abdominal. El estudio muestra una respuesta inmunológica a cuerpo extraño más intensa en la localización vaginal, comparado con la ubicación abdominal en ambos grupos. 9/18 animales presentaron una marcada respuesta inflamatoria a nivel vaginal, mientras que tan sólo 5/18 individuos lo hicieron a nivel abdominal. Esta respuesta ubicación-dependiente fue más marcada en el grupo control (PP), ya que ningún sujeto presentó infiltrados inflamatorios en los explantes abdominales. Estos resultados son consistentes con los resultados clínicos observados en pacientes, donde las complicaciones asociadas a las mallas sintéticas son mucho más frecuentes en la localización vaginal, comparado con las mallas abdominales.

El estudio histológico también proporciona información sobre la disposición de las fibras de colágeno, mostrando también diferencias en ambos grupos. En el grupo control las fibras de colágeno fueron más compactas, y con un

porcentaje de fibrosis superior tanto en vagina como en abdomen. Éstos hallazgos se asocian a mecanismo de rechazo crónico(28,36). En cambio en el grupo experimental, las disposición de las fibras de colágeno presentó un patrón más estructurado y organizado, respuesta que se traduce en una mejor integración tisular(35).

El estudio de las propiedades biomecánicas demuestra que en el grupo de MDh, los explantes presentaron menor rigidez que las piezas evaluadas antes de implante, especialmente en la localización vaginal. Estos resultados coinciden con otros trabajos publicados(27–29,31,32), y probablemente se explican por la integración y parcial degradación de las matrices, sobre todo en la localización vaginal.

En resumen, la excelente biocompatibilidad demostrada de las MDh asociada a una menor incidencia de complicaciones clínicas sugiere un adecuado perfil de seguridad. Sin embargo, la integración tisular conlleva un proceso de degradación precoz que altera las propiedades biomecánicas en este grupo a medio plazo.

Las limitaciones de este estudio se basan en la dificultad en la extrapolación de resultados desde un modelo animal hasta el modelo humano. Además, el modelo animal utilizado tampoco permite evaluar eficacia terapéutica. Otra limitación del estudio es el reducido número de muestras analizadas, así como el tamaño y grosor de los implantes (1mm) utilizados. Si bien este tamaño es adecuado para el modelo animal utilizado (conejo), ha provocado algunas dificultades técnicas en el análisis de las propiedades biomecánicas. A su vez, el reducido grosor de las muestras también dificulta la extrapolación de los resultados en el modelo humano. Para comprender mejor estas limitaciones y entender el potencial efecto terapéutico de las MDh en mujeres, se añade a continuación nuestra experiencia previa con muestras de MDh de diferentes grosores (hasta 4-5 mm) y su correlación con las propiedades biomecánicas. (Anexo 1). Los resultados obtenidos de los estudios preliminares in vitro

demostraron que las propiedades biomecánicas de la MDh eran proporcionales al grosor de la pieza, de modo que a mayor grosor, mayor rigidez y mayor resistencia. Sin embargo, la decisión de utilizar MDh de 1 mm se explica por la necesidad de utilizar espesores adecuados al modelo (en este caso, animales pequeños, grosores más finos). Sin embargo, esta limitación a la vez ofrece la oportunidad de mejorar los resultados biomecánicos con la utilización de MDh más gruesas. Así pues, los resultados de este estudio proporcionan sobre las MDh un hipotético efecto terapéutico que, de confirmarse, implicaría un cambio muy importante en el paradigma uro-ginecológico actual.

Se necesitan más estudios clínicos que permitan confirmar las propiedades biológicas halladas, así como su eficacia terapéutica.

6. CONCLUSIONES

Se investiga un nuevo biomaterial desarrollado a partir de dermis humana descelularizada, y se presenta como una hipotética alternativa terapéutica en el tratamiento quirúrgico de las DSP.

En relación al modelo utilizado, se puede concluir que el conejo NZW es un buen modelo para el estudio de materiales para ser utilizados en la cirugía vaginal reconstructiva.

En comparación con la malla macroporosa de PP utilizada en la actualidad, la MDh se asocia a menos complicaciones clínicas, incluida la extrusión vaginal. La respuesta inmunológica hallada en el grupo MDh muestra un infiltrado celular de distribución difusa, con una mayor representación de linfocitos B; y la disposición de las fibras de colágeno presenta un patrón más estructurado y organizado. Estos hallazgos se correlacionan con una mejor integración al tejido nativo circundante, así como una mejor biocompatibilidad. Sin embargo, las propiedades biomecánicas de la MDh no se mantienen estables tras 6 meses de implantación.

7. BIBLIOGRAFÍA

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8. ANEXOS

ANEXO I: Estudio de las propiedades biomecánicos de las matrices biológicas con distintos grosores

Se realiza una recopilación de datos de diferentes MDh. Aunque el ensayo de tracción uniaxial es uno de los que más se realizan, también se ha encontrado en bibliografía el comportamiento de las matrices dérmicas ante la retención de la sutura. En este apartado se recogen los resultados bibliográficos de las propiedades biomecánicas de diferentes productos comerciales, según el tipo de ensayo realizado:

1. **Ensayo tensión-elongación**

Se muestra en la Figura 1, la carga a rotura de ArthoFLEX® 3mm, ArthoFLEX® 2mm, GraftJacket 2mm i AlloPatch 2mm. El tejido más grueso (ArthoFLEX® 3mm) presenta una carga a rotura de aproximadamente 1300 N. Se observa claramente que la disminución del grosor del tejido conlleva una disminución de la carga a rotura del mismo. La misma tendencia se observa en la Figura 2, en la que se analiza la carga máxima de MatrACELL y GraftJacket de grueso 1,5 o 2mm
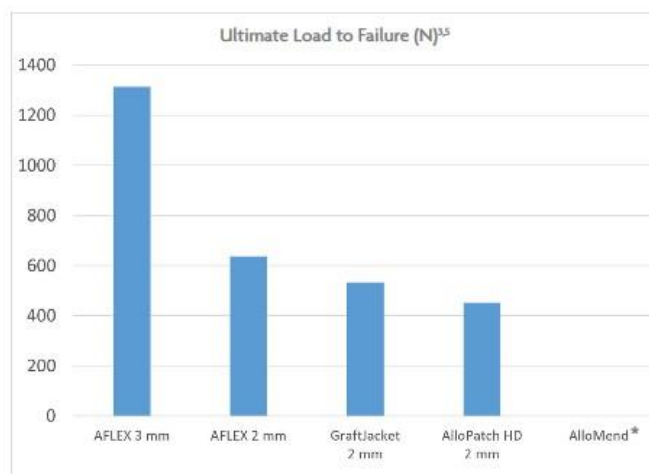
Figura 1: Carga máxima a rotura de 3 productos comerciales (ArthoFLEX®, GraftJacket y AlloPatch), según el grosor del tejido.
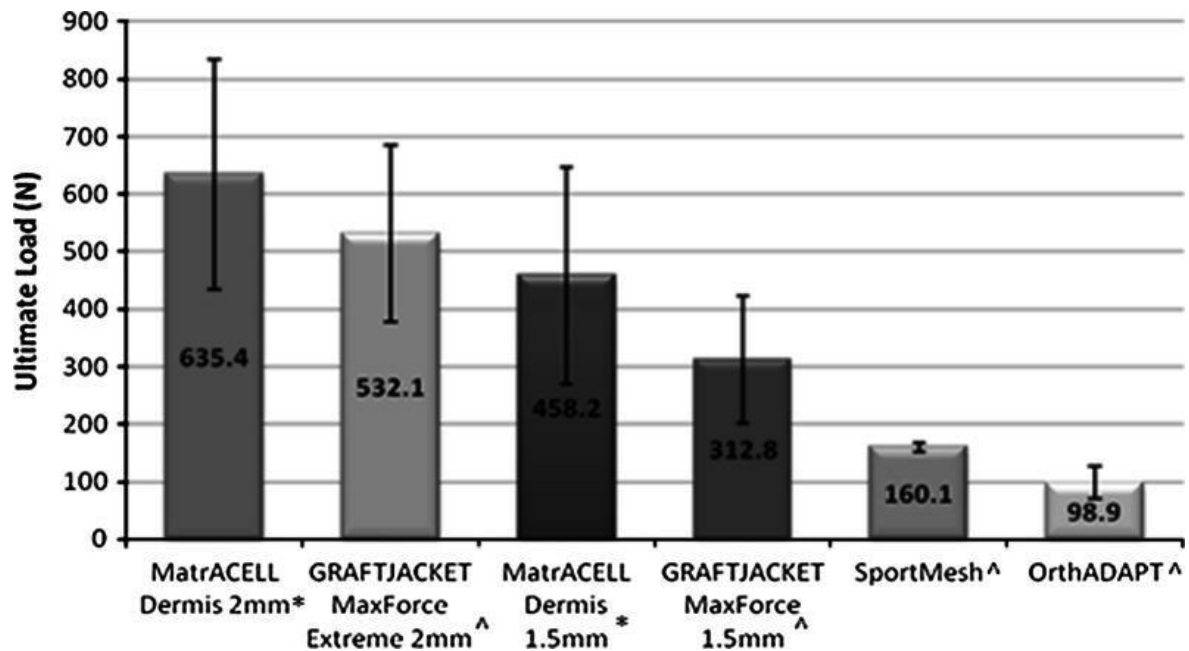
Figura 2: Carga máxima a rotura de diferentes productos comerciales (MatrACELL, GraftJacket, SportMesh y OrthADAPT), según el grosor del tejido(Moore 2011).

Otra propiedad que se puede analizar a partir de un ensayo de tensión uniaxial, es el esfuerzo de tracción. Esta propiedad se calcula dividiendo la carga máxima entre el área de la sección inicial de la probeta en mm^2 (Figura 3).
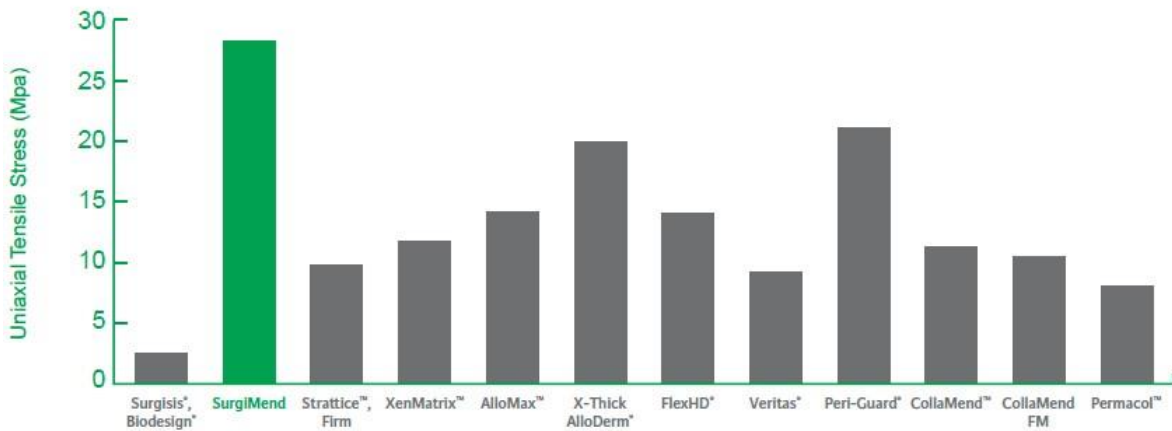
Figura 3: Esfuerzo de tracción a rotura de diferentes productos comerciales.

2. Ensayo de retención de sutura

El ensayo de retención de sutura mide la carga necesaria para extraer una sutura anclada al tejido. Los resultados bibliográficos de diferentes matrices comerciales se pueden observar en las figuras que se presentan a continuación (Figura 4, Figura 5 y Figura 6). En todos los gráficos se observa claramente que la retención de rotura es directamente proporcional al grosor del tejido suturado.
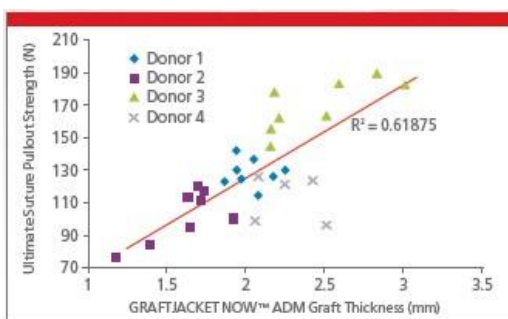
Figura 4: Ensayo de retención de sutura de GraftJacket ADM según el grosor del tejido.
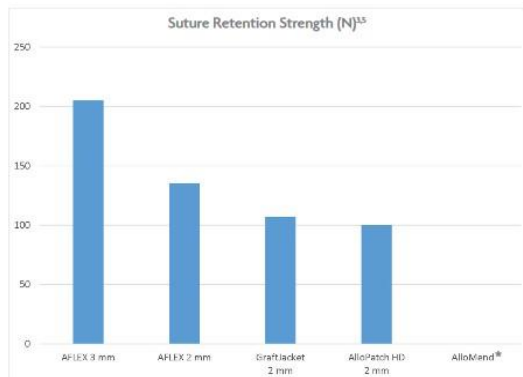
Figura 5: Ensayo de retención de sutura de 3 productos comerciales (ArthoFLEX®, GraftJacket y AlloPatch), según el grosor del tejido.
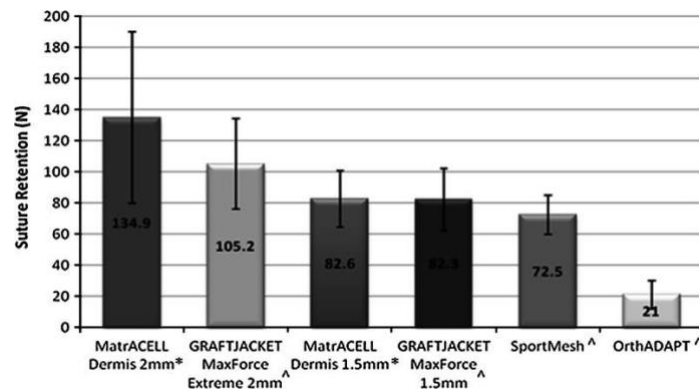
Figura 6: Ensayo de retención de sutura de diferentes productos comerciales (MatrACELL, GraftJacket, SportMesh y OrthADAPT), según el grosor del tejido(Moore 2011).

Moore M a (2011) Decellularization of Human Dermis Using MATRACELL® Technology: Process, Preclinical Studies amd Medical Applications.

ANEXO II: Protocolo de obtención y procesamiento de la MDh

Se presenta el artículo: Pérez ML, Castells-Sala C, López-Chicón P, Nieto-Nicolau N, Aiti A, Fariñas O, Casaroli-Marano RP, Porta O, Vilarrodona A. Fast protocol for the processing of split-thickness skin into decellularized human dermal matrix. Tissue Cell. 2021 Oct;72:101572. doi: 10.1016/j.tice.2021.101572. Epub 2021 Jun 4. PMID: 34119882.

Tissue and Cell

Fast protocol for the processing of split-thickness skin into decellularized human dermal matrix

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ABSTRACT

Background: Dermal scaffolds for tissue regeneration are nowadays an effective alternative in not only wound healing surgeries but also breast reconstruction, abdominal wall reconstruction and tendon reinforcement. The present study describes the development of a decellularization protocol applied to human split-thickness skin from cadaveric donors to obtain dermal matrix using an easy and quick procedure.

Methods: Complete split-thickness donor was decellularized through the combination of hypertonic and enzymatic methods. To evaluate the absence of epidermis and dermal cells, and ensure the integrity of the extracellular matrix (ECM) structure, histological analysis was performed. Residual genetic content and ECM biomolecules (collagen, elastin, and glycosaminoglycan) were quantified and tensile strength was tested to measure the effect of the decellularization technique on the mechanical properties of the tissue.

Results: Biomolecules quantification, residual genetic content (below 50 ng/mg dry tissue) and histological structure assessment showed the efficacy of the decellularization process and the preservation of the ECM. The biomechanical tests confirmed the preservation of native properties in the acellular tissue.

Conclusions: The acellular dermal matrix obtained from whole split-thickness skin donor with the newly developed decellualrization protocol, maintains the desired biomechanical and structural properties and represents a viable treatment option for patients.

1. Introduction

The clinical use of skin grafts is the current gold standard for the treatment of major burn patients, due to the lack of skin donor site available for autografting. First experiences with the cryopreservation of tumours and biopsies demonstrated that the tissues maintain their viability after very low temperatures have been applied. Skin autografts, in particular, preserve the capacity to take and produce hair after being frozen (Taylor,

1949). Previous studies described procedures that allow
the use of post mortem homograft as biological dressing in burns (Brown et al., 1953; Jackson, 1954). The development of long-term preservation techniques enabled skin banks to ensure the quality, safety and availability of tissue to meet the needs of a burn unit. In this sense, the aim of the skin grafts is to cover the wound to avoid fluid and protein loss, preventing microbial infection and acting as

production of new natural dermis, help re-epithelization due to the presence of the basement membrane, and the formation of new vessels. The effect of ECM scaffolds in tissue regeneration is related to their 3D structure and the bioactive components (Eweida and Marei, 2015).
The source of the scaffold as well as the decellularization technique and the final preservation method directly affect the final ECM composition and properties (Gilbert et al., 2006).

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pain relief. When the affected area is more than 30 % of the total body surface area, homografting results to be a life-saving treatment (Cleland et al., 2014). However, skin grafting works as temporary coverage since it will be
rejected some days after the implant due to an immunological process in the recipient.
More recently, skin allografts evolved into skin bio-substitutes with the aim of being permanent grafts for repairing or restoring lost or damaged soft tissue, and seeking to increase the survival rate of the patients, and the long-term functioning of the healed wounds. For this purpose, grafts must lack their original cellular content in order to avoid inducing immunological response in the recipient. The process leading to acellular tissues with an intact ECM is known as decellularization. Many skin substitutes emerged in the market with promising properties from different origins: synthetic, biological, xenogeneic, and composites of all of them with or without *in vitro* cultured cells or even crosslinked with molecules that modify its biomechanical properties (Haddad et al., 2017; Sheikholeslam et al., 2018). The most widely used biological substitutes are cadaveric skin allografts, porcine skin xenografts and amnion (Halim et al., 2010). Acellular Dermal Matrices (ADMs) constituted by a dermal ECM, stimulate the

The removal of the antigenic components in the tissues is achieved through different decellularization approaches, such as physical methods (freezing, heating, stirring, sonication, and homogenization), chemical methods (detergents, acids, bases, chelating agents and either hypertonic or hypotonic solutions) and biological methods (enzymes) (Crapo et al., 2011). The effectiveness of the decellularization methods depends on different factors such as the amount of cells colonizing the tissue, the density of the ECM structure, the fat content and the thickness (Kawecki et al., 2018; Ratcliffe and Niklason, 2002). Submitting the tissue to hypo or hypertonic solutions cause cellular lysis, which encompasses the first step to decellularization. The next step is the removal of the cell content from the tissue by surfactants, like detergents, that disorganize the lipid bilayer of the cell membranes and wash out cellular remnants. In the case of ADM, in order to maintain the structure and the native properties of the tissue, low concentration of surfactants should be used (White et al., 2017). Enzymatic treatments are often applied to remove DNA remnants in the tissue. It was initially thought that an enzymatic treatment could damage the tissue if it acted upon structural proteins or removed the basement membrane, but in the correct concentration, an enzymatic treatment helps

to reduce DNA content, to obtain tissues with no genetic material while maintaining the structural proteins of the ECM intact. Moreover, the quantification of residual DNA has become a threshold (Crapo et al., 2011) for defining an acellular tissue.

ADM are currently being used for different clinical indications, generally for soft tissue regeneration or reinforcement. Therefore, depending on the clinical application, graft characteristics may vary. For instance, the use of ADM grafts in the reconstruction of abdominal wall and tendon augmentation, must ensure the preservation of its mechanical properties to withstand daily functional activity (Barber et al., 2012; Cole et al., 2018; Deeken and Lake, 2017; Mirzayan et al., 2020); whereas for wound closure, the ideal graft must ensure structural support through collagen contribution to help regenerate the tissue loss (Balaji et al., 2016; Kawecki et al., 2018).

In this context, it is of general interest for tissue banks to define easy and quick protocols in order to avoid repetitive manipulations, therefore diminishing the possibility of contamination. Although, it is a challenge to translate the research scale to large-scale tissue banking routine, in this paper, we present a new approach for a decellularization protocol to obtain an ADM from complete amount of skin from human cadaveric donors, in a short time. This optimized protocol is tested to ensure cell removal while maintaining the biological, biochemical, and biomechanical characteristics of the skin ECM. The hADM may be used as a skin substitute or for different applications such as wound-healing (Helliwell et al., 2016), immediate breast reconstruction (Antony et al., 2010; Kim et al., 2012; Ortiz, 2017) and burns medicine (Wainwright et al., 1996). **2. Materials and methods**

2.1. Skin procurement

Approximately 1500 cm^2 of skin per donor, were procured from five human cadaveric donors after obtaining informed consent for research purposes. Ethical Committee's approval was issued by CEIm Hospital Valle Hebron, ´ Barcelona; PR (BST) 314/2019. Donor screening included, but may not be limited to, the review of complete social and

medical history, physical examination of the donor, complete serological and microbiological testing during retrieval, histopathological analysis, as well as any other information pertaining to risk factors for relevant communicable diseases. Skin fragments were retrieved from the back and the lower limbs of the donor with a dermatome (ACCULAN® 3Ti Dermatome GA 670, Aesculap Inc). The skin was placed into a sterile container with RPMI media and antibiotic cocktail (penicillin 1000U/ mL, streptomycin 500 µg/mL and vancomycin 500 µg/mL) until it was processed in a clean room environment.

2.2. Decellularization protocols

The skin decellularization protocol was defined to remove the cellular content while maintaining the structure and mechanical properties of the tissue. The fast protocol consisted of the following steps: (i) decontamination, (ii) de-epithelization, (iii) DNA content removal, and (iv) cell debris elimination. Firstly, skin fragments were screened in order to select regular fragments while discarding those with hair, tears and nevus. Selected fragments (an average number of 15 fragments of about 20 × 4 cm^2 per donor with thickness between 0.8 and 1.4 mm) were sized and transferred into a sterile container with the antibiotic cocktail solution for decontamination. Thereafter, the skin was incubated in 0.5 L of 1 M NaCl (S5150, Sigma Aldrich) which leds to cellular lysis by osmosis and facilitates the de-epithelization, performed manually with forceps. Afterwards, an incubation in 0.2 L of 0.2 mg/mL DNAse (6922859, Roche) was performed, which led to the lysis of amino acid sequences resulting in the elimination of genetic material. In a last step, an incubation in 0.5 L 0.5 % SDS (sodium dodecyl sulphate; 05030- 1L-F, Sigma Aldrich) was performed to wash out cellular debris. All the incubations were performed under gently stirring. Rinsing steps with sterile water after each incubation and a final washing with 0.9 % NaCl solution were performed. Before packaging, 10 × 4 cm^2 hADM samples were measured with manual micrometre in order to confirm the final thickness. The dermis obtained was preserved in a glycerol solution. The complete process lasts three

days, including two days of the decellularization protocol (Fig. 1).

2.3. Microbiological assessment

Tissue samples were taken in each step of the decellularization protocol and included in thioglycolate broth media (28410, Biomerieux) for aerobic/anaerobic growth.

2.4. Analysis of the extracellular matrix

2.4.1. Qualitative assay: (hADM) histology and structure

The structure of the extracellular matrix was assessed by means of a histology approach. Biopsies from each donor were procured prior and after decellularization and prepared in 4%

2.4.2. Quantitative assays: (hADM) ECM proteins analysis

After freeze-drying, tissue ECM proteins were quantified using commercially available kits according to manufacturer's instructions: Soluble Collagen Assay Sircol™ (Bicolor life science assays, S1000) for total collagen (acid-soluble and pepsin-soluble) testing; Fastin Elastin Assay (Bicolor life science assays, F2000) for elastin (α-elastin after acid- treatment) testing; Glycosaminoglycan Assay Blyscan (Bicolor life science assays, B1000) for total glycosaminoglycan (GAGs; sulphated GAG content after papain extraction). All are dye-binding methods and absorbance was read with an Epoch microplate spectrophotometer (Biotech) at 570 nm (collagen), 513 nm (elastin), and 656 nm (GAGs). The results were presented as μg specific protein/mg dry weight tissue.

8.2 2.5. DNA quantification

A commercially available kit (QIAamp DNA Mini Kit; Qiagen, 51304) was used to extract the DNA from the tissue before and after decellularization. Tissue biopsies were freeze-dried and 10 mg of

Fig. 1. Schematic diagram of split-thickness decellularization protocol.

Day 1. Processsing
- Fragment screening and sizing.
- Decontamination incubation

Day 2. Decellularization (I)
- Rinsing with water and incubation in 0.5L of 1M NaCl

Day 3. Decellularization (II)
- De-epithelization and rinsing steps
- Enzimatic treatement: 0.2L of 0.2mg/mL DNAse.
- Rinsing steps and 0.5L 0.5% SDS.
- Rinsing steps and packaging in glycerol solution.

paraformaldehyde fixing solution at 4 °C, overnight. Tissues were subsequently washed with PBS and preserved with 30 % ethanol solution until a paraffin embedding was achieved. Serial sections (3 μm intervals) were stained in accordance with haematoxylin-eosin (HE) and Masson trichrome (MT) protocols. Images were taken from each slide using the bright-field microscope Axio Scope A1 (Zeiss) with the AxioCam MRc5 camera.

each sample were used for DNA quantification using affinity columns. Levels of extracted DNA were visualized with PicoGreen (Thermo Fisher, p11496) and measured on Triad Multi-Mode Microplate Reader (Dynex Technologies). The amount of DNA remaining in decellularized tissue was compared with native samples and the percentage of DNA remaining was calculated. The

results were presented as ng DNA/mg dry weight tissue.

2.6. Biomechanical testing by uniaxial mechanical assay

Skin samples (native and decellularized) were tested using an uniaxial tensile test, increasing stress until reaching fracture by means of a universal tensile tests machine (Instron 3366) which measures the resistance of the material to an applied force. Control samples stored at 4 °C were tempered at RT, and then measured within 72 h after recovery. Decellularized samples stored in 50 % glycerol at RT, were washed 5 times with 0.9 % NaCl before measuring.

Bone-shaped samples of 10 × 4 cm were prepared and gripped (3 cm per end) to measure the mechanical properties of 4 cm as it is schematically described in Fig. 5E. The specific thickness of each sample was measured using a micrometre. Samples were pre-conditioned using a velocity of 12 mm/min until reaching a load of 0.5 N, which was defined as an unstretched length (L_o). The samples were stretched 12 mm/min until reaching rupture and the mechanical properties of each sample were obtained from the stress-strain (σ- ε) curve. The following mechanical properties were analysed: maximum load (N), Young's modulus (N/mm), stiffness (N/mm^2) and elongation at maximum load (%).

2.7. Remnant reagents quantification

2.7.1. Remnant SDS analysis

Residual traces of SDS in the tissue after decellularization were analysed by means of a SDS Detection & Estimation Reagent kit (G-Biosciences, cat# 786− 129) following the manufacturer's instructions. Biopsies were freeze-dried and 10 mg of each sample were evaluated. Absorbance at 600 nm was measured using Epoch microplate spectrophotometer (Biotech) at 600 nm. Results were presented as μg SDS/mg dry weight tissue.

2.7.2. Remnant glycerol analysis

Glycerol remaining in the hADM tissue after each of 5 rinsing steps was analysed using commercially available Glycerol assay kit (Sigma Life Science, MAK117-1KT) following the manufacturer's instructions. The absorbance was read at 570 nm using the Epoch microplate spectrophotometer (Biotech). The results were presented as μM glycerol per total protein.

2.8. Statistical analysis

The PRISM software version 5.00 (GraphPad Software, San Diego CA, USA, www.graphpad.com) was used for statistical analysis. All results are presented as the mean ± standard deviation (MD ± SD) obtained from five independent donors per group and three independent values per donor. The non-parametric two-tailed Mann-Whitney test was used and *p* values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Structural evaluation of hADM

No evidence of cellular material remnants were observed after the decellularization protocol (Fig. 2A). The structural architecture was maintained in hADM when compared to the dermis in the native skin (Fig. 2B). Epidermis, basement membrane and dermis were present in the native skin while hADM was constituted only by dermis.

3.2. Microbiological assessment

There was no evidence of bacterial or fungal growth on thioglycolate media after 30-days culture for any of the samples taken at each step of the protocol.

3.3. DNA content

After hADM decellularization DNA quantity was below 4 ng/mg dry tissue (Fig. 3), value which clearly fulfil the requirements proposed by Crapo et al. (Crapo et al., 2011). Therefore, the proposed protocol allows the elimination of 99 % of the genetic material, giving a statistically significant DNA reduction. Moreover, electrophoresis analysis

demonstrated that there were not remnant genetic fragments of more than 200bp (data not shown).

3.4. ECM structural biomolecules content

The decellularization protocol did not affect the quantity of total collagen (Fig. 4A), despite significant reduction of elastin content in hADM compared to native tissue (Fig. 4B). GAGs also decreased in hADM (Fig. 4C), although this difference was not statistically significant.

3.5. Biomechanical properties

Decellularized skin maintained their intrinsic elastic properties compared with native skin. Moreover, the results of the mechanical properties, after its preservation in 50 % glycerol for a minimum of 6 months, demonstrated that the preservation method does not affect the mechanical properties of the hADM (Fig. 5). Comparing hADM with native skin, no significant differences were observed for maximum load (Fig. 5A), young modulus (Fig. 5B) or elongation at maximum load
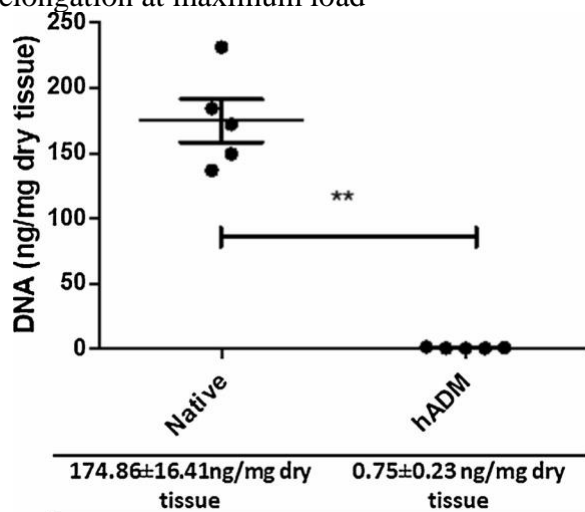

Fig. 3. DNA content in the native and decellularized samples of each donor. DNA content within hADM was compared with native skin. The results are presented as mean ± SD and statistical differences were performed using non- parametric two-tailed Mann-Whitney test (N = 5, in triplicate). Differences are significant with p-value <0.05.

(Fig. 5C), neither after preservation in glycerol for 6 months.

3.6. Remnant reagents quantification

At the end of the protocol the skin was washed with a several immersions with 0,9% NaCl in accordance with our protocol. Remnants of SDS were around 0.2 ± 0.46 % once the skin was rinsed (Fig. 6A) and no remnants of glycerol were observed (Fig. 6B). The elimination of residual glycerol turned out to decrease progressively and significantly after 15 min.

4. Discussion

Nowadays, human or animal grafts and synthetic materials are used as skin bio-substitutes. The historical use of skin allograft as temporary dressing in major burns entails their degeneration as a result of an immune response activation in the recipient (Benichou et al., 2011) directed against epidermis and dermal cells (Dettin et al., 2017; Dragúnovˇ a et al., 2017´). Dermal ECM is a relatively non-immunogenic structure capable of providing a support for cellular repopulation and vascular in-growth (Ferrando et al., 2016; Terzini et al., 2016).
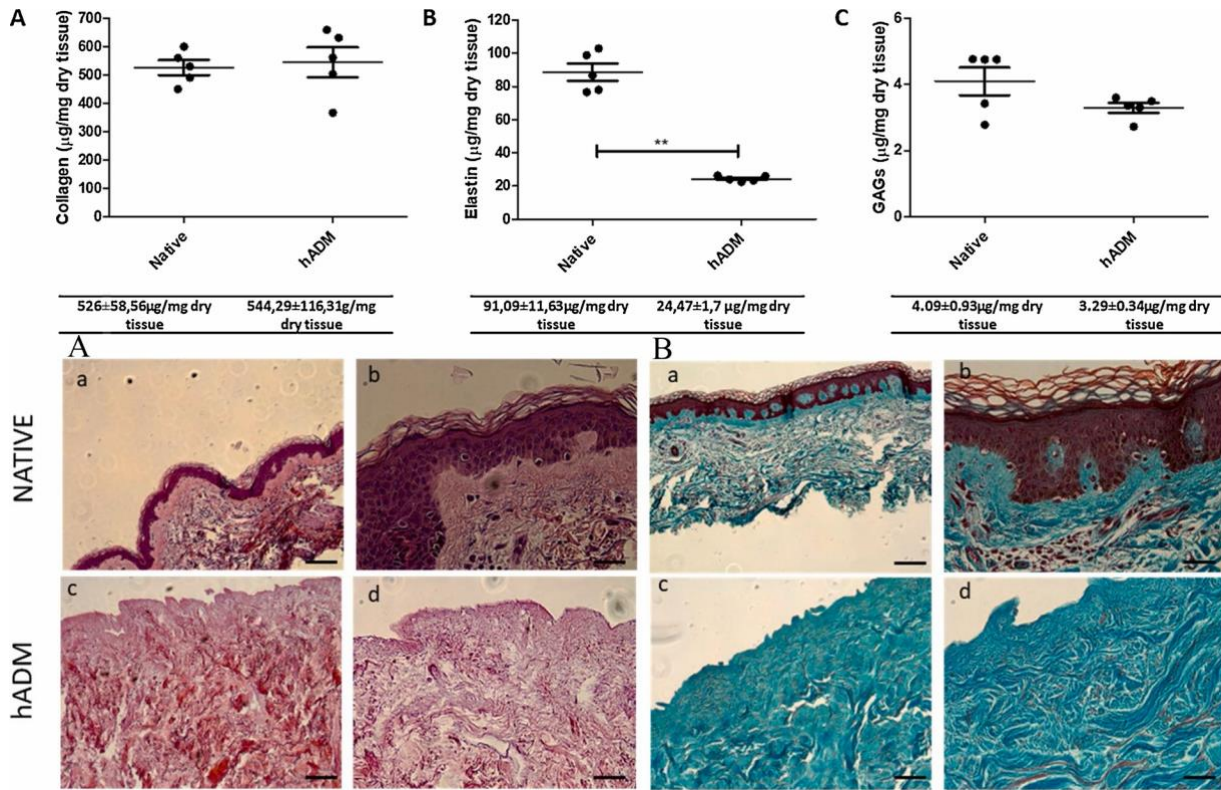
Fig. 2. Histological sections of decellularized and native skin stained. (A) Haematoxylin-Eosin staining. (B) Masson Trichrome staining. Scale bar (a. c) 100 μm and (b, d) 20 μm.
Fig. 4. Structural ECM biomolecules content. (A) Collagen (B) Elastin and (C) GAGs concentration. The results are presented as mean ± standard deviation and statistical differences were performed using non-parametric two-tailed Mann-Whitney test (N = 5, in triplicate).
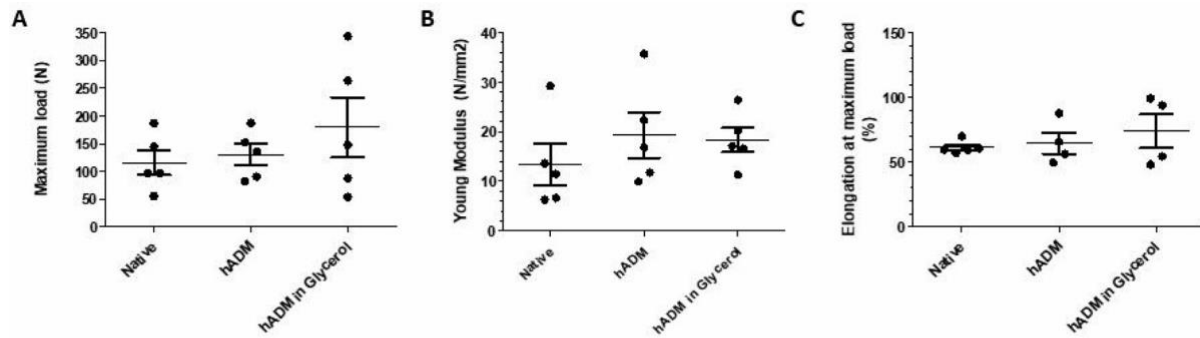
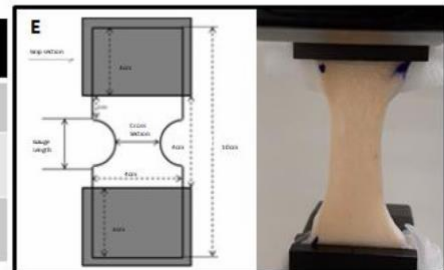| D	Native	hADM	hADM after preservation in 50% Glycerol
Maximum load (N)	117,1±50,29	129,8±43,5	179,65±121,95
Young Modulus (N/mm²)	14,27±8,71	19,26±10,35	18,30±5,54
Elongation at maximum charge (%)	67,5±5,9	64,69±11,92	56,3±19,1

Fig. 5. Mechanical uniaxial assay. The mechanical properties of decellularized skin before and after its preservation in glycerol 50 % were compared with the native tissue. (A) Maximum load (N), (B) Young Modulus (N/mm²), (C) Elongation at maximum load (%), (D) summary table of numerical results and (E) schematic diagram of bone-shaped-samples and picture of skin sample clamped for mechanical assay. The results are presented as mean ± standard deviation and statistical differences were performed using non-parametric two-tailed Mann-Whitney test (N = 5, in triplicate).
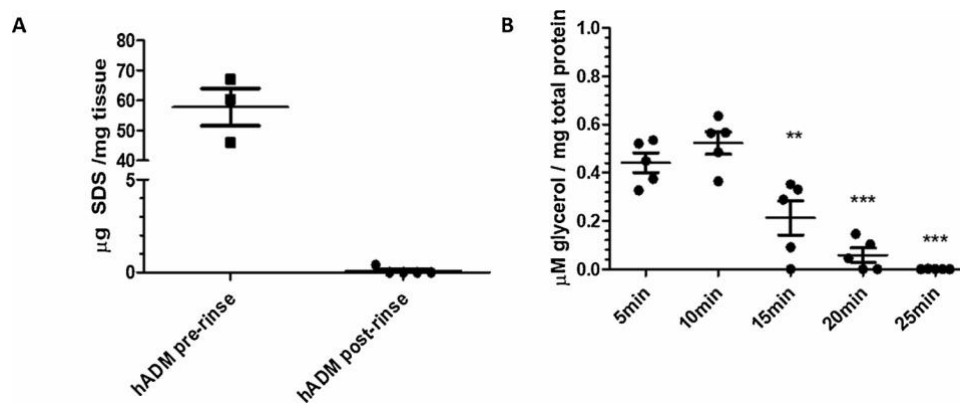
Fig. 6. Remnant reagents (A) SDS elimination was ensured after the decellularization protocol. (N = 5). (B) Glycerol quantification after serial washes of 5 min. Five successive washes were performed (N = 5, n = 3). For both analysis, the results are presented as mean ± standard deviation and statistical differences were performed using non-parametric two-tailed Mann-Whitney test No statistical differences were observed in any case.

Moreover, it has been reported that the use of skin allografts improves wound-healing and regulates cell behaviour promoting cell adhesion, spreading, migration, proliferation and differentiation (Greco et al., 2015; Helliwell et al., 2016; Yu et al., 2016). In this work, we present a novel and rapid protocol to obtain decellularized human skin for clinical application. This improved protocol is able to achieve 99 % of DNA removal, with skin native-like structure and preserve the main ECM biomolecules and biomechanical properties. Thus representing a suitable graft for skin replacement. The proposed protocol reduces the manipulation (time and the number of entries in the clean rooms) while ensuring the decellularization of the complete donor skin. Despite the thickness of the tissue, the histology and DNA quantification demonstrate that the reagents are capable to penetrate to the deepest layers accomplishing effective decellularization.

Decellularized human tissues are promising biological scaffolds to support tissue growth and *in vivo* tissue regeneration for preclinical research and clinical practice (Belviso et al., 2020; Carbonaro et al., 2020; Putame et al., 2020). In the context of clinical practice, tissue decellularization has been the subject of growing interest in the last decades due to its potential, either for using the tissue for its original biological function or for new indications (Yu et al., 2016). Acellular dermal matrices have been tested for their use in different indications such as wound-healing (Helliwell et al., 2016), immediate breast reconstruction (Antony et al., 2010; Bullocks, 2014; Chun et al., 2010; Kim et al., 2012; Ortiz, 2017; Salzberg, 2006), burns medicine (Wainwright et al., 1996), abdominal wall reconstruction (Garvey et al., 2017; Ghetti et al., 2017), maxillofacial surgeries (treatment of gingival recession) (Cairo, 2017), leg ulcers (Cazzell, 2019; Guo et al., 2017), tendon reinforcement and joint repair (Cockcroft and Markelov, 2018) among others. Each clinical application needs special tissue specifications, but in all cases an acellular dermal matrix must fulfil the following characteristics: integrate within the recipient's own tissue and promote regeneration of the autologous tissue (Benichou et al., 2011; Terzini et al., 2016).

Different protocols for skin decellularization have been described and multiple ADM products have been commercialized during the last decades (Gilbert, 2012; Gilbert et al., 2006; Hogg et al., 2013; Shevchenko et al., 2010). All of them are based on the sequential combination of decellularization agents using incubations at different times and temperatures. Some of these protocols can take until four weeks. It is noteworthy that the efficiency of decellularization highly depends on different tissue factors such as type and quantity of cells, ECM density, lipid content and tissue thickness (Crapo et al., 2011). While short protocols have been published and demonstrated to be effective to decellularize dermis in small skin fragments in the context of research, our improved protocol proved to be effective when applied to high amounts of tissue (Belviso et al., 2020). The method proposed in this work is capable of significantly reduce the DNA content below the accepted threshold 50 ng/mg dry tissue (Crapo et al., 2011), while maintaining the architecture of the dermis in terms of histology. Compared with native skin, collagen and GAGs quantity were preserved, while elastin decreased significantly. Elastin reduction after decellularization could be caused by the removal of the epidermis, which contains high amount of elastin, or due to some chemical reagent involved in the process.

The dermis provides a major contribution to the overall mechanical characteristics of the skin due to its main constituents, which allow high levels of deformation and flexibility as the fibrils stretch and re-orientate (Hussain et al., 2013; Nemoto et al., 2012; Silver et al., 2003; Terzini et al., 2016). Collagen is the main structural protein of the skin as well as the primary mechano-structural element and confers tensile strength and proteolytic resistance, being stiff and lacking extensibility (Greco et al., 2015). Elastin fibres dictate the mechanical behaviour of skin at small stresses and strains. Elastin has an intimate relationship with collagen promoting the return of collagen to its wavy posture at rest (Hussain et al., 2013). The combination of these two components provides the skin a non-linear mechanical behaviour and allow high levels of deformation and flexibility as the fibrils stretch and re-orientate (Terzini et al., 2016). Furthermore, it has been described that the contribution of glycosaminoglycans to the elasticity and tensile strength of the skin is minimal (Hussain et al., 2013). The analysis performed in this work shows that our improved protocol is able to maintain of the primary mechanical properties of the tissue which correlates with its native collagen content. The protocol described here results in a safe product without cells and with potential use in burn treatment, wound healing and soft tissue regeneration surgeries. Moreover, our laboratory is currently developing a new decellularization skin protocol capable of obtaining full-thickness hADM grafts with higher mechanical properties, which makes them suitable for clinical indications such as tendon reinforcement or abdominal wall reconstruction.

The preservation medium chosen for hADM, is a glycerol solution that confers antimicrobial properties (Cameron et al., 2000), allows room temperature storage and easy handling. The study of glycerol removal has demonstrated that an acceptable level of preservation reagent elimination is obtained after 5 washes with 0.9 % NaCl. Another important factor using skin substitutes is the biocompatibility of the graft: a detailed animal model study will be presented in a subsequent article by our group.

European tissue establishments perform their activities according to a set of guidelines that demand an ethical approach for tissue procurement, a manufacturing license granted in accordance with the current state-of-the art in GMP; and a procedure ensuring a minimum load of viral, bacterial and fungal and quality control measurements that continuously guarantee tissue quality. A decellularized human skin graft manufactured on this basis may be viewed as being both safe in terms of disease transmission and adverse reactions. Dermal matrix production in GMP facilities with a strict sterility control lead us to state that it can be applied with the confidence that the risk of disease transmission and/or adverse reaction is remarkably low. Moreover, we hypothesize that the use of hADM may improve results in situations where synthetic or

xenogeneic products did not succeed, like septic fields. As opposed of all these benefits, some limitations affect this study. The procurement technique is complex and it was difficult to obtain homogeneous thickness for all fragments. We used to procure split-thickness skin for coverage in major burns, where the homogeneity of the thickness was not standardized. Instead, for hADM the pieces need to be homogeneous, so that the fragments to be decellularized were carefully selected. The use of detergent implies repetitive serial washing at the end of the protocol to assure the biocompatibility of the allograft; although the results show that this rinsing steps are necessary and sufficient for the elimination of the reagents to obtain a decellularization protocol suitable for tissue banking needs. Finally, the elastin content in the hADM diminish when compared to native skin, but this effect does not result in a decrease of mechanical properties, obtaining a hADM suitable for the aims of the study.

5. Conclusion

This work describes a step-by-step easy and quick process from the retrieval of the skin until the preservation of a human acellular dermal matrix. The two days decelluarization procedure, from whole donor skin, delivers a human cell-free dermal matrix that accomplishes the proposed specifications, presenting negligible levels of residual genetic material, maintaining major ECM biomolecules content, and with the hyper elastic biomechanical properties. The developed acellular dermal matrix is therefore considered a safe allograft with a set of mechanical, structural, biochemical and storage properties which suggest its suitability for a broad range of clinical applications that do not requires high mechanical properties.

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