Ximo García Domínguez

DOCTORAL THESIS

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DOCTORAL THESIS
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DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

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Ximo García Domínguez

Supervisors

Prof. Francisco Marco Jiménez
Prof. César David Vera Donoso

Valencia, December 2020

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El secreto de la felicidad no está en hacer siempre lo que se quiere,
sino en querer siempre lo que se hace

León Tolstói

Si nada nos salva de la muerte,
al menos que el amor nos salve de la vida

Pablo Neruda

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Valencia, December 2020

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DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

A thesis submitted to the University of Valencia in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Certify that:

The Doctoral Thesis "DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY" has been carried out entirely by Ximo García Domínguez under my direction.

This document has been completed and meets all the requirements for its presentation and public defence.

Valencia, December 27, 2020.

Sig.

Prof. Francisco Marco Jiménez

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ACKNOWLEDGEMENTS

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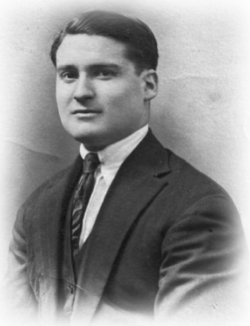

A la memoria de mi bisabuelo,
Manuel Albert Alfonso, por su devoción a la medicina.

"*Tal vez la gratitud no sea la virtud más importante,*
pero sí es la madre de todas las demás".
Marco Tulio Cicerón

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Esta parte, desde mi punto de vista, podría ser considerada la más importante de una tesis, y también la más difícil de escribir, puesto que gracias a lo que implica el contenido de estas líneas seguramente haya sido capaz de escribir el resto del documento. El doctorado es un camino que saca, pero al mismo tiempo exige, lo mejor de ti, tanto a nivel profesional como mayoritariamente personal. Por ello, el apoyo y el cariño de todas las personas que han compartido conmigo este tiempo han contribuido, de una manera u otra, a que este documento esté hoy en tus manos. Y es que cuando alguien empieza una tesis doctoral no solo está asumiendo que su vida cambie radicalmente, sino también la de todos aquellos familiares, amigos y compañeros que le acompañan durante el proceso. Por ello, estas líneas van dedicadas a todas esas personas.

Tal vez sea obvio empezar por quienes me dieron la oportunidad de embarcarme en esta aventura: mis directores, **Paco** y **César**. Sin vuestro tiempo, dedicación y paciencia esto no hubiese sido posible. Tengo la suerte de poder decir que siempre me he sentido valorado, respetado y comprendido, y eso te hace sentir como en casa. Hemos hecho tantas cosas a lo largo de estos años que sería imposible describir en unas pocas líneas cuánto he aprendido con vosotros, tanto en el ámbito de la docencia como en el de la investigación. Gracias por confiar en mí. **Paco**, todavía recuerdo cuando aceptaste ser el director de mi trabajo final de grado en 2014, en aquella práctica de cultivos, donde empezó este proyecto de tesis. En aquel momento no tenía ni idea de cuánto te tendría que agradecer para siempre. Desde entonces hemos trabajado juntos en muchísimos proyectos, sin importar el día, la hora, el lugar o el motivo, y siempre me has hecho sentir a la altura. Gracias, no solo por contar conmigo para todo lo que ha estado en tu mano, sino también por dejarme aportar todo lo que ha estado en la mía. Gracias por tu forma de ser tan cercana, y por hacerlo todo tan fácil, incluso dejando que se me olvide que tú eres el jefe. A día de hoy siento que no he perdido el tiempo, y tú eres el principal culpable. **César**, a día de hoy tengo mucho más que agradecerte de lo que se puede apreciar en ésta tesis, donde tu contribución ha sido esencial para llevar las investigaciones por el buen camino. Más allá, tu figura ha sido para mí un ejemplo de cómo poder conciliar la práctica clínica con una gran actividad investigadora. Créeme que tus estímulos han sido claves para que me decidiese a estudiar el grado de Medicina. Gracias por compartir conmigo tu experiencia, tanto en el laboratorio como en el hospital y su quirófano.

Finalmente, aunque no figure como director de ésta tesis, habría que considerarlo como tal: **José**, creo que es un sentimiento común para todos los que te conocemos el sentir admiración por todo lo que representas. Ha sido una suerte poder aprender contigo, tanto de tus destrezas profesionales como personales, así como de tu visión amplia y crítica de las cosas. Gracias por ser un buen referente y mirar siempre por mí. Estoy convencido de que, sin ti, la tesis no estaría aquí.

Han sido varios los años de compartir el laboratorio con ciertas personas que han conseguido que aquel lugar sea mucho más que un espacio de trabajo. Por ello, me gustaría darle las gracias al resto del equipo: **David, Carmen, Amparo** y, de una forma especial, **Luis**. Tampoco puedo olvidarme de **Carlos**, por ayudarme en toda la gestión de los animales de la granja, pese a no haberle puesto las cosas fáciles… La ayuda de todos vosotros ha sido fundamental para que todos los experimentos fuesen por el buen camino. Al final, el trabajo en equipo es la clave del éxito y, con un equipo así, poco queda que desear. Gracias a todos por haber estado dispuestos a echarme una mano cuando ha hecho falta, y por darme un buen consejo siempre que lo he necesitado. Entre todos habéis conseguido que estos años haya sido fácil saltar de la cama con alegría cada mañana, y que el tiempo haya pasado tan rápido. Siento de corazón que en el 5º hemos formado un gran equipo y una gran familia.

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tesis te cambia la vida, y gracias a ti no me cabe la menor duda. He aprendido muchísimo de ti y creo que eres un ejemplo a seguir en todos los sentidos.

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Y si he dejado a mi familia para el final es simplemente porque es lo más difícil, ya que por mucho que escriba jamás podré reflejar cuánto les tengo que agradecer, no solo en estos últimos años, sino en toda mi vida. A mis abuelos, **Juan** y **Pepica**, **Ramón** y **Carmen**, antes que nada, me gustaría pedirles perdón por no haberles dedicado todo el tiempo que se merecían durante estos años. Gracias por haberme enseñado a valorar las cosas, a ver la vida con perspectiva y a creer en mí. Dicen que uno recoge lo que siembra, y yo no puedo quereros más: gracias por quererme tanto.

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Finalmente, quisiera atribuirles el mérito de esta tesis a mi madre, **Mari Luz**, y a mi padre, **Ximo**, ya que a ellos se lo debo todo en esta vida. Gracias por enseñarme siempre el camino correcto y haberme apoyado siempre en todas mis decisiones y sus consecuencias, menos en una. Todavía recuerdo cuando me fui a València a estudiar Biotecnología y pensé en abandonar a las pocas semanas. Jamás estaré lo suficientemente agradecido de que tú, mamá, no me lo consintieses, y de que tú, papá, la apoyases, ya que no fue nada fácil. Gracias por trabajar tanto y haberos privado de ciertas cosas para que a mí no me haya faltado de nada: siempre habéis considerado mis estudios y mi felicidad como una prioridad. A lo largo de mi vida habéis dejado que yo decida el camino, y vosotros os habéis encargado de allanarlo, fuese cual fuese y costase lo que costase. Si algún día soy padre espero poder darles a mis hijos todo lo que vosotros habéis dado por mí, porque más es imposible. Nadie os avisó de dónde os metíais cuando me tuvisteis, y, pese a lo difícil que os lo he puesto, habéis sido los padres entregados, comprensivos y cariñosos que un hijo necesita. Con vosotros a mi lado no me da miedo nada. Por ello, aunque no siempre os lo demuestro como os merecéis, os quiero y os necesito para seguir alcanzando mis metas. En definitiva, gracias por ser perfectos.

Me gustaría terminar este apartado dedicándole unas palabras a una criatura que llegó a mi vida cuando yo empezaba la tesis, **Cora**. Gracias por ser la única que no se ha movido de mi lado durante días y noches de trabajo. Gracias por saber cuándo debes "molestar" y obligarme a desconectar y descansar. Es increíble cómo alguien tan diferente a ti es capaz de abrirse un hueco en la familia y hacerte sentir tan completo.

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¡Gracias a todos por formar parte de esto!

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ABSTRACT

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Today, it is well recognized that allotransplantation of mature organs are unable to be the solution for all patients suffering from an end-stage organ disease, due to the organ shortage. According to the World Health Organization, transplantation meets only 10% of the global need. Therefore, alternative solutions to organ transplantation are in need. Regenerative medicine is looking for different technologies to generate organs on demand, either through *in vitro* or *in vivo* strategies. Particularly, embryonic kidneys transplantation has long been seen as a possible solution to the end-stage renal disease, whit particular advantages over other options. Metanephroi, extracted at the appropriate time, are able to continue its growth after its transplantation without triggering an immunological response, giving rise to host-vascularized renal structures with excretory and endocrine functionality. The aim of this thesis was to use the rabbit as animal model to further in the establishment of strategies that turns the metanephroi transplantation in an ever closer clinical reality.

In particular, **Chapter I** is aimed to evaluate the feasibility of a laparoscopic approach for the metanephroi transplantation, as a minimally invasive alternative to the open surgeries currently performed. Because of their intermediate size between rodents and larger farm animals, the rabbit model was well-suited for laparoscopic approach modelling. The laparoscopic transplantation technique described in this chapter was validated while the ideal time point to transplant rabbit metanephroi without immunosuppression treatment was determined for the first time. Metanephroi from 15-day-old (E15) and 16-day-old (E16) rabbit embryos grew with an average efficiency near 50%, exhibiting normal glomeruli, proximal and distal tubules and collecting ducts without relevant immunological changes in the hosts. With slight adaptations, this laparoscopic technique could be used for impulse both the preclinical and subsequent clinical trials, guaranteeing the control and safety of the procedure.

Looking for strategies that allow transplanted metanephroi to generate larger renal structures with life-sustaining function, the sildenafil citrate growth-promoting effects were evaluated in **Chapter II**. Here, we demonstrate that metanephroi neovascularization and growth, as well as glomerular filtration in nascent kidneys, can be enhanced using low doses (10 μM) of sildenafil citrate during metanephroi transplantation. Sildenafil citrate can exert its growth-promoting actions through the angiogenesis enhancement, haemodynamic improvement and renoprotective effects. Sildenafil citrate treatment can contribute to the development of a standardized procedure that enables the formation of life-sustaining kidneys, which are essential to guarantee the feasibility of metanephroi transplantation for clinical application.

Finally, **Chapters III, IV,** and **V** were designed to demonstrate if the metanephroi banking is a feasible option through vitrification approaches. Using the Cryotop® as loading device, rabbit metanephroi (E15 and E16) were vitrified using two vitrification solutions (VM3 and M22), characterized by its low toxicity and well-suitability for renal tissue preservation. After long-term cryostorage, metanephroi were warmed and laparoscopically allotransplanted into non-immunosuppressed hosts. Altogether, results showed that transplantation efficiency was similar regardless of the metanephroi origin, either fresh (49.2 ± 8.2 %), VM3-vitrified (37.5 ± 8.0 %) or M22-vitrified (33.5 ± 7.6 %), without the metanephroi age exerting a significant effect (E15 and E16). In all the cases, metanephroi generated host-vascularized renal structures without triggering an immunological response and showed mature glomeruli whose filtration capability was proven by the hydronephrotic state. Therefore, we demonstrated that metanephroi cryopreservation preserves its viability and developmental potential. If the organ supply and demand could be balanced using embryonic kidneys, this cryopreservation approach can dissociate the time of the organ retrieval and its transplantation, allowing their distribution to patients in need to any desired location and guaranteeing an adequate inventory control and quality assurance.

Collectively, these findings should encourage the design of proper studies to support the clinical translation of the metanephroi transplantation to treat patients with end-stage renal disease. Considering the new methods described throughout this thesis, future preclinical studies become more feasible, which makes the clinical translation of the technique one step closer.

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RESUMEN

RESUMEN

Hoy en día, es bien sabido que, debido a la escasez de órganos, el alotrasplante no puede ser la solución para todos los pacientes que padecen una enfermedad orgánica en etapa terminal. Según la Organización Mundial de la Salud, el trasplante de órganos cubre apenas el 10% de la necesidad mundial. Por lo tanto, se necesitan soluciones alternativas. La medicina regenerativa busca diferentes tecnologías para generar órganos a demanda, ya sea mediante estrategias *in vitro* o *in vivo*. En particular, el trasplante de riñones embrionario ha sido considerado durante mucho tiempo como una posible solución a la enfermedad renal en etapa terminal, con ventajas particulares sobre otras opciones. Los metanefros, extraídos y trasplantados en el momento oportuno, son capaces de continuar su crecimiento sin desencadenar una respuesta inmunológica, dando lugar a estructuras renales vascularizadas por el huésped y con funcionalidad tanto excretora como endocrina. El objetivo de esta tesis fue utilizar el conejo como modelo animal para avanzar en el establecimiento de estrategias que conviertan el trasplante de metanefros en una realidad clínica cada vez más cercana.

En particular, el objetivo del **Capítulo I** fue evaluar la viabilidad de un abordaje laparoscópico para el trasplante de metanefros, como una alternativa mínimamente invasiva a las cirugías abiertas que se realizan actualmente. Debido a su tamaño intermedio entre los roedores y los animales de granja más grandes, el conejo constituye un modelo adecuado para modelizar el abordaje laparoscópico. La técnica de trasplante laparoscópico descrita en este capítulo se validó al tiempo que se determinó por primera vez el momento ideal para trasplantar metanefros de conejo sin tratamiento inmunosupresor. Los metanefros de fetos de conejo de 15 (E15) y 16 días (E16) crecieron con una eficiencia media cercana al 50%, exhibiendo glomérulos, túbulos proximales y distales, y conductos colectores normales sin cambios inmunológicos relevantes en los hospedadores. Con ligeras adaptaciones, esta técnica laparoscópica podría utilizarse para impulsar tanto los ensayos preclínicos como los clínicos, garantizando el control y la seguridad del procedimiento.

Buscando estrategias que permitan que los metanefros trasplantados generen estructuras renales más grandes con una funcionalidad compatible con la vida, en el **Capítulo II** se evaluaron los efectos promotores del crecimiento del citrato de sildenafilo. Satisfactoriamente, la adición de pequeñas dosis de citrato de sildenafilo (10 µM) durante el trasplante de los metanefros, permitió incrementar tanto la neo-vascularización como el crecimiento de las estructuras renales resultantes, así como su filtración glomerular. El citrato de sildenafilo podría ejercer sus acciones promotoras del crecimiento a través de la potenciación de la angiogénesis, la mejora hemodinámica y los efectos renoprotectores. El tratamiento con citrato de sildenafilo puede contribuir

al desarrollo de un procedimiento estandarizado que permita la formación de riñones de soporte vital, esenciales para garantizar la viabilidad del trasplante de metanefros para aplicación clínica.

Por último, los **Capítulos III, IV** y **V** se diseñaron para evaluar si era posible la creación de un banco de metanefros mediante técnicas de vitrificación. Utilizando el cryotop® como dispositivo de carga, se vitrificaron metanefros de conejo (E15 y E16) utilizando dos soluciones de vitrificación (VM3 y M22), caracterizadas por su baja toxicidad y su idoneidad para la preservación del tejido renal. Después de un almacenamiento criogénico a largo plazo, los metanefros se desvitrificaron y se trasplantaron por vía laparoscópica en huéspedes no inmunodeprimidos. En conjunto, los resultados mostraron que la eficiencia del trasplante fue similar independientemente del origen de los metanefro, ya fuese fresco (49,2 ± 8,2%), vitrificado con VM3 (37,5 ± 8,0%) o vitrificado con M22 (33,5 ± 7,6%). La edad de los metanefros (E15 y E16) no tuvo un efecto significativo. En todos los casos, los metanefros generaron estructuras renales vascularizadas por el huésped sin desencadenar una respuesta inmunológica, y presentaron glomérulos maduros cuya capacidad de filtración fue probada por el estado hidronefrótico. Por lo tanto, quedó demostrado que la criopreservación de metanefros mediante vitrificación conserva su viabilidad y capacidad de desarrollo. Si se pudiera equilibrar la oferta y la demanda de órganos utilizando riñones embrionarios, este enfoque de criopreservación podría disociar el momento de la recuperación del órgano y su trasplante, permitiendo su distribución a los pacientes que lo necesiten en cualquier lugar, y garantizando un adecuado control de inventario y calidad.

En conjunto, estos hallazgos deberían alentar el diseño de estudios adecuados para respaldar la translación clínica del trasplante de metanefros como terapia de la enfermedad renal en etapa terminal. Con las nuevas herramientas descritas a lo largo de esta tesis en nuestras manos, los futuros estudios preclínicos se vuelven más factibles, lo que hace que la aplicación clínica de la ésta técnica esté un paso más cerca.

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RESUM

RESUM

Hui en dia, és ben sabut que, a causa de l'escassetat d'òrgans, l'alotrasplantament no pot ser la solució per a tots els pacients que pateixen una malaltia orgànica en etapa terminal. Segons l'Organització Mundial de la Salut, el trasplantament d'òrgans cobreix a penes el 10% de la necessitat mundial. Per tant, es necessiten solucions alternatives. La medicina regenerativa busca diferents tecnologies per a generar òrgans a demanda, ja siga per mitjà d'estratègies *in vitro* o *in vivo*. En particular, el trasplantament de renyons embrionari ha sigut considerat durant molt de temps com una possible solució a la malaltia renal en etapa terminal, amb avantatges particulars sobre altres opcions. Els metanefros, extrets i trasplantats en el moment oportú, són capaços de continuar el seu creixement sense desencadenar una resposta immunològica, donant lloc a estructures renals vascularizadas per l'hoste i amb funcionalitat tant excretora com endocrina. L'objectiu d'esta tesi va ser utilitzar el conill com a model animal per a avançar en l'establiment d'estratègies que convertisquen el trasplantament de metanefros en una realitat clínica cada vegada més pròxima.

En particular, l'objectiu del **Capítol I** va ser avaluar la viabilitat d'un abordatge laparoscòpic per al trasplantament de metanefros, com una alternativa mínimament invasiva a les cirurgies obertes que es realitzen actualment. A causa de la seua dimensió intermèdia entre els rosegadors i els animals de granja més grans, el conill constitueix un model adequat per a modelitzar l'abordatge laparoscòpic. La tècnica de trasplantament laparoscòpic descrita en este capítol es va validar alhora que es va determinar per primera vegada el moment ideal per a trasplantar metanefros de conill sense tractament immunosupressor. Els metanefros de fetus de conill de 15 (E15) i 16 dies (E16) van créixer amb una eficiència mitjana pròxima al 50%, exhibint glomèruls, túbulos proximals i distals, i conductes col·lectors normals sense canvis immunològics rellevants en els hostes. Amb lleugeres adaptacions, esta tècnica laparoscòpica podria utilitzar-se per a impulsar tant els assajos preclínics com els clínics, garantint el control i la seguretat del procediment.

Buscant estratègies que permeten que els metanefros trasplantats generen estructures renals més grans amb una funcionalitat compatible amb la vida, en el **Capítol II** es van avaluar els efectes promotors del creixement del citrat de sildenafil. Satisfactòriament, l'addició de xicotetes dosis d'aquesta substancia (10 µM) durant el trasplantament dels metanefros, va permetre incrementar tant la neo-vascularització com el creixement de les estructures renals resultants, així com la seua filtració glomerular. El citrat de sildenafil podria exercir les seues accions promotores del creixement a través de la potenciació de l'angiogènesis, la millora hemodinàmica i els efectes renoprotectores. El tractament amb citrat de sildenafil pot contribuir al desenvolupament d'un procediment

estandarditzat que per a la formació de renyons de suport vital, essencials per a garantir la viabilitat del trasplantament de metanefros per a l'aplicació clínica.

Finalment, els **Capítols III, IV** i **V** es van dissenyar per a avaluar si era possible la creació d'un banc de metanefros per mitjà de tècniques de vitrificació. Utilitzant el cryotop® com a dispositiu de càrrega, es van vitrificar metanefros de conill (E15 i E16) utilitzant dos solucions de vitrificació (VM3 i M22), caracteritzades per la seua baixa toxicitat i la seua idoneïtat per a la preservació del teixit renal. Després d'un emmagatzemament criogènic a llarg termini, els metanefros es van desvitrificar i es van trasplantar per via laparoscòpica en hostes sense tractament immunosupressor. En conjunt, els resultats van mostrar que l'eficiència del trasplantament va ser semblant independentment de l'origen dels metanefros, ja foren frescs (49,2 ± 8,2%) , vitrificats amb VM3 (37,5 ± 8,0%) o vitrificats amb M22 (33,5 ± 7,6%). L'edat dels metanefros (E15 i E16) no va tindre un efecte significatiu. En tots els casos, els metanefros van generar estructures renals vascularitzades per l'hoste sense desencadenar una resposta immunològica, i van presentar glomèruls madurs amb capacitat de filtració, tal i com va mostrat l'estat hidronefròtic. Per tant, va quedar demostrat que la criopreservació de metanefros per mitjà de la vitrificació conserva la seua viabilitat i capacitat de desenvolupament.

Si es poguera equilibrar l'oferta i la demanda d'òrgans utilitzant renyons embrionaris, este enfocament de criopreservación podria dissociar el moment de la recuperació de l'òrgan i el seu trasplantament, permetent la seua distribució als pacients que ho necessiten en qualsevol lloc, i garantint un adequat control d'inventari i qualitat. En conjunt, estes troballes haurien d'encoratjar el disseny d'estudis adequats per a afavorir la translació clínica del trasplantament de metanefros com a teràpia de la malaltia renal en etapa terminal. Amb les noves ferramentes descrites al llarg d'esta tesi en les nostres mans, els futurs estudis preclínics es tornen més factibles, la qual cosa fa que l'aplicació clínica d'aquesta tècnica estiga un pas més prop

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ABBREVIATIONS

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#

3D Three-dimensional

^{99}Tc-DMSA ^{99}technetium-2,3-dimercaptosuccinic acid

A

ASC Adult Stem Cells

B

BM Base Medium

BRECS Bioartificial Renal Epithelial Cell System

BS Bowman's space

BSA Bovine Serum Albumin

C

CD Cluster of Differentiation

CKD Chronic Kidney Disease

D

DMSO Dimethyl Sulphoxide

DPBS Dulbecco's Phosphate-Buffered Saline

E

E13 Day 13 of embryonic development

E14 Day 14 of embryonic development

E15 Day 15 of embryonic development

E16 Day 16 of embryonic development

ECM Extra-Cellular Matrix

EEC Environment European Commission

EG Ethylene Glycol

eGFR Estimated Glomerular Filtration Rate

ESC Embryonic Stem Cells

ESRD End-Stage Renal Disease

F

FBS Foetal Bovine Serum

G

G Gauge

G Glomerulus;

GODT Global Observation on Donation and Transplantation

H

H&E Haematoxylin-Eosin Staining

HD Haemodialysis

I

IAK Implantable Artificial Kidneys

IGF-1 Insulin-Like Growth Factor I

iPCS Induced Pluripotent Stem Cells

L

LN Liquid Nitrogen

M

MP 3-Methoxy-1,2-Propanediol

N

NHP Non-Human Primate

NMF N-Methylformamide

NO Nitric Oxide

O

OPTN Organ Procurement and Transplant Network

P

PAK Portable Artificial Kidneys

PBS Phosphate-Buffered Saline

PCS Pluripotent Stem Cells

PD Peritoneal Dialysis

PDE-5 Phosphodiesterase 5 Enzyme

PERV Porcine Endogenous Retrovirus

PVP Polyvinylpyrrolidone

Q

qRT-PCR Quantitative Real-Time Polymerase Chain Reaction

R

RAD Renal Assist Device

RC Renal Corpuscle

RM Regenerative medicine

RRT Renal Replacement Therapies

S

SC Sildenafil Citrate

U

US United States

V

VEGF Vascular Endothelial Growth Factor

W

WAK Wearable Artificial Kidneys

WHO World Health Organization

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1. GENERAL INTRODUCTION

1.1. CURRENT SITUATION IN THE FIELD OF ORGAN TRANSPLANTATION

Nowadays, many patients are suffering degenerative processes or injuries which end in specific irreversible organ failure. In many instances, therapeutic options are limited to supportive measures and preventing further damage [1]. But although substantial progress has been made in the minimisation of irreversible tissue loss in the acute phase of many disease processes, the restoration of lost tissue and organ function after critical damage has occurred has been less successful. Then, transplantation represents the ideal method of restoring full physiological organ function [2]. Paradoxically, the effectiveness of this treatment has used up many organs for transplant, being their availability the main limitation of the technique [2,3].

Based on the Organ Procurement and Transplant Network (OPTN) database, more than 54,000 patients were added into the waiting list for solid organ transplantation (kidney, pancreas, liver, heart, lung and intestine) in 2009, but approximately 28,500 transplants were performed. Ten years later, in 2019, 66,000 patients were added into the same waiting list, being approximately 39,700 the number of transplants performed by this year [4]. Therefore, while waiting lists grows rapidly each year, few patients receive a matched organ (Figure 1), so many of them have to wait long periods of time and dye before receiving the desired organ [5,6].

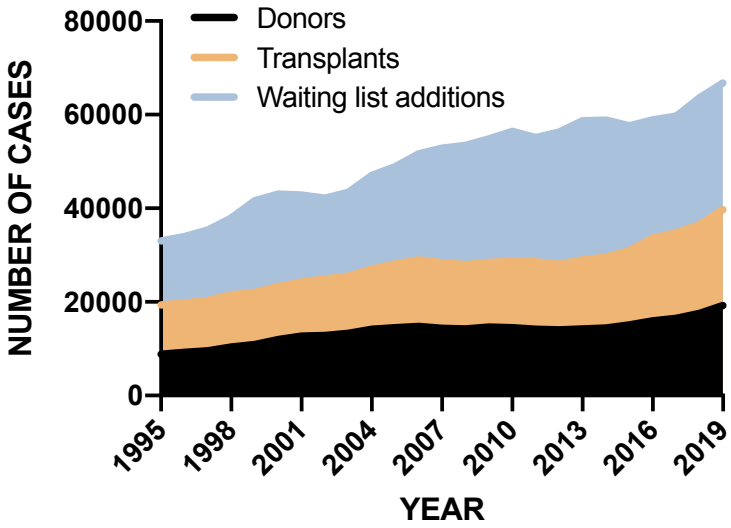

Figure 1. Current status in the field of transplantation in the United States [4].

At present, the current waiting list for organ transplantation in United States (US) rise to 108,762 candidates, of which 16,394 have been waiting for an organ for ≥ 5 years [4]. Worryingly, it is well recognized that official organ waiting lists represents just a fraction of a much bigger problem related to the organ shortage, as nearly 730.000 annual US deaths attributable to end-stage organ disease could be avoided by an organ transplant [7,8]. In this sense, it has been suggested that without organ shortage for

transplantation, >30% of all deaths in the United States could theoretically be prevented [9,10]. Even so, this situation is more complicated in other countries (Figure 2). While US contains roughly 4% of the world's population and perform 25% of its organ transplants, the continent of Africa contains roughly 16% of the world's population but perform fewer than 0.5% of its organ transplantation [11]. Worldwide, the World Health Organization (WHO) estimates that organ transplants currently meet less than 10% of global needs [12].

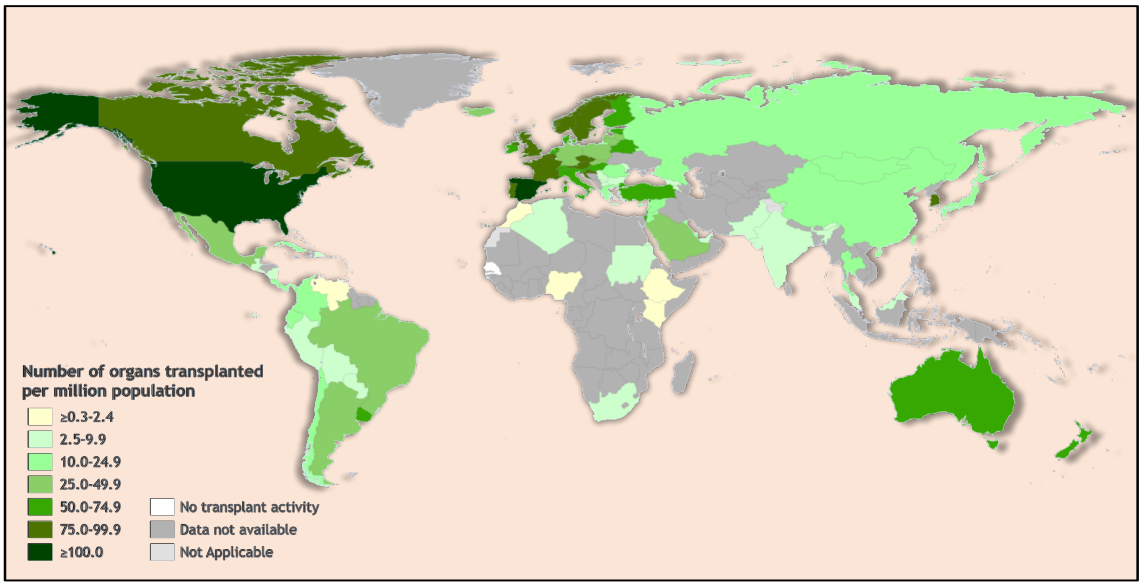

Figure 2. Global unmet need for transplantation are extended worldwide. Based on the Global Observation on Donation and Transplantation (GODT) database [12].

In this context, the global shortage of organs for transplantation has long been recognized as a major public health challenge, whose drastic situation is commonly attributable to the rare condition that must exist for organs to be suitable for recovery and transplantation. This is because, organ donation/transplantation process is necessarily complex and must include some critical steps [13]:

i. **Donor identification:** All potential donors should be identified to facilitate donor screening and donor management.
ii. **Donor screening:** Some serological and other screening methods should be used to minimise the risk of transmission of infectious or malignant diseases from donors to the recipients.
iii. **Donor management:** Organs procured must be in good condition prior to retrieval, so the donor physiological state should be managed prior to and during organ retrieval. Poor donor management can make organs unusable.
iv. **Consent/authorization:** Despite countries have different legal requirements, appropriate consent or authorisation has to be obtained before organs can be removed.

v. **Organ retrieval:** The surgical method for removing organs from the donor's body and how those organs are subsequently handled and preserved prior to and during transportation are critical to the successful outcome of the donation/transplant. If organs are damaged during removal and/or transportation, some can be repaired, but others will have to be discarded.
vi. **Organ allocation:** The successful long-term outcome of the transplant is highly dependent on appropriate matching between donor and recipient, so it is necessary a well organised system for allocating and transporting donated organs to the most appropriate recipient. Sometimes, optimum allocation will require exchanges between transplant organisations and countries.

Unfortunately, it must be taken into account that, even after having obtained an organ, half of these organs fail within 10 years of being transplanted despite the great advances in immunosuppression [11]. Therefore, transplant recipients must adhere to lifelong immunosuppressant drug regimens to delay rejection, whose side effects put patients at increased risk for life-threatening infections, cancer and other diseases [11,14,15]. Therefore, although organ transplantation represents today the ideal method to recover the full function lost in end-stage organ disease, its clinical application is limited by the organ availability, the risks of allograft loss rejection and immunosuppressive therapy toxicity. The above considerations should place technologies and research that can substantially increase the availability of transplantable organs and its long-term survival at the top of our scientific priority list.

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1. GENERAL INTRODUCTION

1.2. IMPLICATIONS OF KIDNEY SHORTAGE FOR THE UROLOGY PATIENTS

On a world scale the total number of individuals with chronic kidney disease (CKD) exceeds 840 million, placing the kidney diseases as one of the most common diseases worldwide [16]. When CKD progresses to the end-stage renal disease (ESRD), renal replacement therapies (RRT), such as dialysis (haemodialysis or peritoneal dialysis) or kidney transplantation, are required [17]. The first successful kidney transplantation was performed in a dog by Emerich Ullmann, in 1902 in Vienna (Austria) [18]. More than half a century later, the Nobel Prize winner Joseph Murray was the first to report a successful kidney transplant between identical twins, in 1954 in Boston (US) [18,19]. Since then, kidney transplantation represents the best therapeutic option for patients with CKD with progression to ESRD, which incur a fatal irreversible decline of kidney function [20]. Compared with other RRT, kidney transplantation restore the full physiological renal functions [2], also providing best outcomes of survival [21–24], quality of life [25–28], and cost-effectiveness [29–33].

However, the success of this option is limited by the scarcity of available kidneys for transplant [12,34]. Worldwide, it has been estimated that 2.6 million people received RRT in 2010, but only 73,179 kidney transplantations (44% from living donors) were performed according to WHO [35], and at least 2.2 million people might have died prematurely because RRT could not be accessed [36]. Over the course of nearly a decade, the kidney transplantation activity has risen to 94,997 transplants worldwide (35% from living donors) [37]. However, and worryingly, the worldwide use of RRT is projected to more than 5.4 million people by 2030, driven by the increasing ESRD prevalence due to population ageing and an increasing prevalence of diabetes and hypertension [36,38]. For this reason, it is expected that kidney diseases become the 5th leading cause of life-years lost in 2040 [39]. This organ shortage and the continued increasing list is haunting the nephrology community even in the transplants leading countries. Nowadays, Spain is the leading country in organ donation for 28 consecutive years [40], with a rate of donation > 40 per million population and > 55 kidney transplantations per million population (Figure 3). However, only 3,423 kidney transplantation were performed in 2019, instead of the 7,356 that were necessary according to the waiting list [41]. In US, of the total patients in the waiting list for an organ transplantation, 92,906 candidates (>80%) need a kidney donor. According to the statistics, in 2018, 40% of patients listed for kidney transplant were still waiting since 2015 and 34,591 patients were removed from the list due to death or decline in medical condition [4,42].

Close to 10% of the population are diagnosed with CKD around the world [43,44]. However, given the current circumstances and the long waiting list for kidney transplantation, patients with advanced disease are habitually obliged to resort to RRT alternative to transplant, mainly haemodialysis (HD) and peritoneal dialysis (PD). Unfortunately, prolonged dialysis increases the risk of develop cardiovascular disease

and therefore mortality is 20 times higher in these patients than in the general population [45–47]. Thus, the overall survival of patients on chronic dialysis is 78% at 1 year, 57% at 3 years, and only 42% at 5 years [48]. Moreover, both HD and PD dialysis not only fail to meet the full physiological endocrine, metabolic, and immunomodulatory functions of the native kidney [2,49], but also entails a prohibitive annual costs [33,50–52]: €1436 million in Spain, £1.4 billion in UK, and $120 billion in US. One patient with CKD can costs upwards of €80,000 for dialysis alone, equating to €14 billion per year to the EU health system [53]. In this sense, the worldwide cost of treating end-stage renal disease totals over $1 trillion in the course of a decade [54]. In contrast, the estimated cost for a kidney transplant is one eighth of the estimated cost for a dialysis treatment per life-year saved [31]. In this context, an in an attempt to increase the number of transplantable organs that are available, the selection criteria have been expanded to include marginal kidneys from suboptimal donors [55]. Nonetheless, given the serious donor shortage, kidney transplantation only can be considered as the utopic solution to control this public problem.

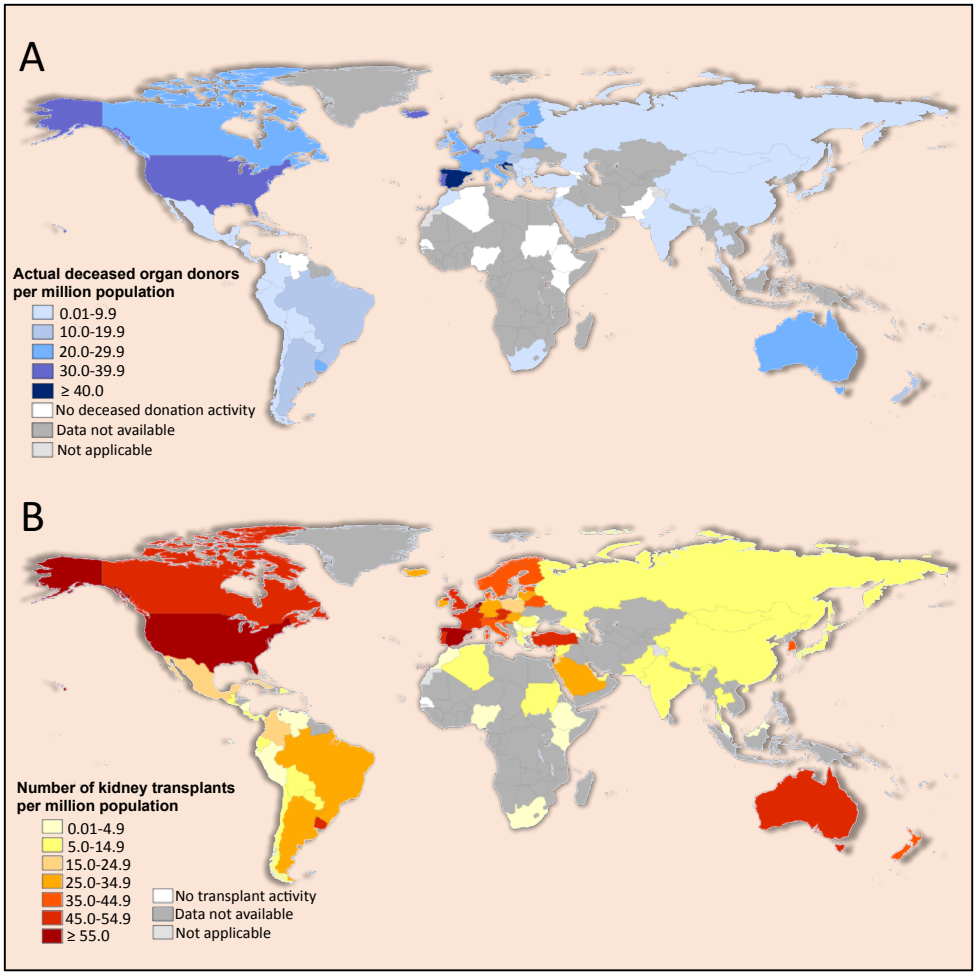

Figure 3. Rates of deceased organ donors (A) and kidney transplantation (B) per million population. Data from the Global Observatory on Donation and Transplantation [12].

Of note, although results with organ transplantation have progressively improved over time, thanks to the advance in surgical techniques and availability of new immunosuppressive drugs, the survival of kidney graft is still limited [11,56–60]. In the firsts 5 years after transplantation, around 20% of recipients will experience an episode of acute rejection, and approximately 40% of recipients will die or lose graft function within 10 years after transplantation [56]. Therefore, even in the extraordinary event of getting a transplantable kidney, in many cases this will not be a definitive solution for the patient. In fact, in 2018, 11.8% US patients in the kidney transplant waiting list were waiting for a retransplant [42]. To reduce this risk of allograft rejection, the use of immunosuppressive drugs is unavoidable in a renal transplant recipient, but it incur some severe associated complications [61–66]. About 70% of all renal transplant recipients experience at least one infection episode by 3 years, being infections the leading cause of hospitalization in this patients and the cause for 18% of all deaths with functioning grafts [64]. On the other hand, immunosuppression following kidney transplantation is known to increase the risk of post-transplant lymphoproliferative disorder greater than 10 times compared to the general population, being an aggressive malignancy that lead to 5-year survival rates of less than 40% [65]. In light of the above reported considerations, kidney transplantation is not a viable long-term solution for the dialysis treatments. So there is a need for a next-generation therapy that can be employed as a substitute for dialysis or transplantation and offer a therapeutic option for patients with ESRD.

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1. GENERAL INTRODUCTION

1.3. CURRENT BIOENGINEERING AND REGENERATIVE STRATEGIES FOR THE GENERATION OF FUNCTIONAL RENAL GRAFTS ON DEMAND

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Regenerative medicine (RM) could offer some of the next-generation therapies for ESRD. In the last twenty years, much effort has been put toward manufacturing bioengineered organs that would be able to generate new functional grafts for transplantation, providing the ultimate treatment for various end-stage organ diseases. From cell-based microsystems to macroscopic devices, the bioengineered kidney scenario is increasingly expanding and sustains high expectations in the RM field [67–70]. However, the kidney offers a major challenge to scientists in this field than other simpler organs, due to structural and functional reasons. Mammalian cells, as part of multicellular organisms, function in tissue units that contain several types of cells, which together form an organ. In this sense, the development of an organism involves cell differentiation, morphogenesis and appropriate patterning to form the architectural context of tissues and organs [71]. Particularly, kidney exhibits a remarkable architectural complexity coupled with the presence of at least 26 different specialized cells [72]. This critical organization is crucial for the recapitulation of the complex renal functions, including glomerular filtration, and reabsorption of fluid and noble solutes [67,69]. However, despite of these technical challenges, the idea of generating functional kidney grafts *in vitro* or *in vivo* on demand cannot be overlooked, as it could extend the option of restoring renal function to more patients. The idea is that the long waiting list for a kidney transplant in patients with ESRD will be eliminated, providing a definitive solution to these patients. Therefore, in the field of renal bioengineering and RM, several strategies are being explored [67,68].

1.3.1. Functional organoids from stem cells

The creation of a whole kidney remains today a technical challenge, but the current technologies led to the development of nephron parts that could be used in the future as the elementary units of bigger structures [67]. Kidney organoids are self-organizing and three-dimensional aggregations that respond to environmental cues and derive from pluripotent stem cells (PSC), which generally include both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). PSC have the potential to differentiate into any cell type and self-assemble into heterogeneous organoids, having been used for the first time in 2014 to generate kidney organoids [67]. Since then, a vast amount of publications has been reported using this strategy, which are comprehensively reviewed recently [73,74]. Kidney organoids are composed of structures resembling the nephron architecture with glomeruli and proximal/distal tubules, but their organization does not mimic one of mammalian kidneys, which still hamper the use of these organoids as transplantable grafts. The majority of developed glomeruli lack a vascular network and, although these organoids can become vascularized after its transplantation under the renal capsule of an immuno-deficient mouse, the neovessels are much smaller than native ones. In addition, kidney organoids exhibit a more

immature degree than adult kidneys, so the size increase and maturation of the renal organoids would therefore be essential to obtain transplantable and functional grafts without a compromised blood filtration [73–76]. In particular, on study reports the generation of higher-order kidney organoids (i.e. branching ureter, differentiated renal components and nephron progenitor niches…) in a murine model [77], but unfortunately this protocol did not recapitulate the same results in human cells. On the other hand, adult stem cells (ASC) also receive great interest because it avoids the potential tumorigenicity of PSC, being clinically safe and isolatable from adult kidneys [68,69]. Using ASC, the reconstitution of a 3D kidney-like structure *in vitro* has been described [78]. However, it had no functional vasculature and, therefore, non-vascularized kidney structures did not produce urine. These results suggest that tissue-specific stem cells may only have the ability to reconstitute the minimum unit of its organ origin by differentiating into specialized cells in the correct niche. Therefore, although it is difficult to recapitulate the renal complexity using techniques based on individual cells, these organoids may be a promising cellular source for kidney repair and regeneration. Furthermore, these cells could be used in the different gene-editing platforms, such as CRISPR/Cas9, to generate kidney disease animal models that could be useful to elucidate novel mechanisms to treat ESRD [68,69].

1.3.2. Blastocyst complementation

If PSC are injected into a blastocyst (embryonic stage 5-days after fertilization), these cells become synchronized with the inner cell mass, generating a chimeric body. Thus, if normal PSC are injected into a blastocyst that lacks the potential to form a particular cell lineage, these cells lines in the resultant organisms will exclusively derive from the injected PSC. It is because normal-PSC assume the role that deficient cells cannot accomplish due to the lack of any functional gene [79]. This phenomenon is known as blastocyst complementation. Since its first use in 1993 [80], this technology has been applied to reconstruct several different tissues and organs, including heart [81], pancreas [82], liver [83], lungs [84] and kidney [85]. It has been reported that rat iPSC injected into a pancreatogenesis-disabled mouse blastocyst produced normal chimeric mice, with almost entirely rat pancreas that produced insulin, and whose pancreas islets improved hyperglycaemia when transplanted into a diabetic rodent model [82]. These studies indicated that PSC could occupy and develop in a vacant developmental niche, a fact that could be used to *in vivo* generation of organs derived from donor PSC using a xenogeneic environment.

In the case of the kidney, deficient mice blastocyst in spalt-like transcription factor 1 (Sall1; a transcription factor essential in renal organogenesis) injected with mouse iPSC resulted in mice with kidneys originated from the injected iPSC [85]. But with the

exception of structures that do not depend on Sall1 expression to develop, such as collecting ducts and microvascular endothelial cells [85]. Similar results were recently observed by Goto et al. [86], who generated PSC-derived mouse kidneys in Sall1-targeted anephric rats. While all the metanephric mesenchyme cells were of mouse PSC origin in Sall1-deficient rats, the ureteric bud-derived collecting tubes and blood vessels were composed of a mixture of donor and host cell types. Authors recognize that, for transplantation therapy, further elimination of host blastocysts derived cells would be essential to reduce the use of immunosuppressive treatment. Therefore, in order to generate an entire organ from PSC-derived cells, all renal lineages must be absent from the blastocyst. The use of genetically modified animals could provide the appropriate developmental niche to this end [86]. Then, although blastocyst complementation remains one of the most promising strategies for obtaining a whole functional kidney, this technique is still in its early stage.

1.3.3. Decellularization/recellularization technology

Decellularization is a process by which biological scaffolds can be obtained from native organs/tissues. To this end, cellular components are removed by the perfusion of detergents, enzymes or other cell-lysing solutions through the organ vasculature. However, the extra-cellular matrix (ECM), composed by collagen, fibronectin, laminin, glycosaminoglycans and growth factors, and its three-dimensional architecture and biochemical composition is not affected [87–91]. This acellular scaffolds can be subsequently recellularized through different cell seeding strategies. Being known that the ECM influences the behaviour and phenotype of the resident cells, decellularized biological scaffolds can induce cell migration, proliferation, and differentiation, also influencing its three-dimensional spatial arrangement [87–91]. This strategy can be used to obtain decellularized cadaveric scaffolds that can provide a niche for stem cells differentiation into an appropriate cell type, which contributes to whole organ generation. Supporting this idea, decellularization before recellularization has been employed to develop a functional heart [88], liver [92] and lungs [93].

Several attempts were made to regenerate a kidney following this methodology. In 2013, Song et al. reported a successful whole kidney regeneration [56]. They used cadaveric kidneys from rats, pigs and humans to produce acellular renal scaffolds, which were repopulated by perfusion of endothelial and epithelial cells, leading to the formation of viable renal tissues. The newly regenerated artificial kidney after cell infusion produced rudimentary urine both *in vitro* and following transplantation. Promising results has been obtained by other groups, but an insufficient degree of recellularization has been the main limiting factor [94,95]. This is mostly due to the complexity of the renal structure that hinder the establishment of a satisfying

recellularization protocol that repopulate the whole kidney. In addition, both the parenchyma and the vasculature need to be entirely regenerated to regenerate the kidney function and, ideally, with patient-derived cells that would not trigger immune rejection [67]. Optimized bioreactor was designed to infuse cells at high pressure, allowing to recellularize about 50% of the renal volume [96]. Engineering other simpler urinary tissues, such as the ureter, through decellularization/recellularization has exhibited more success [97]. However, this technology is far to be translated into clinical settings due to uncertainty of complete decellularization methods, scarce recellularization and the potential host immunological response to the new bioengineered construct [89,96,98].

1.3.4. Bioprinting in 3D

Three-dimensional (3D) bioprinting is based on depositing living cells together with supporting biomaterials into precise positions to build biological structures or organs [99–101]. The idea is to generate a layer-by-layer deposition of cells and supporting components that forms complex 3D functional living tissues. For this purpose, biomaterials that support the growth of living cells, together with high spatial resolution devices that dispense the appropriate component into 3D complex geometries, are required. While still in its early stages, bioprinting strategies have demonstrated their potential use in RM, generating a variety of transplantable tissues, including skin, cartilage, and bone [99–101]. However, several technical challenges can emerge when these methodologies are applied to generate more complex tissue, like the kidney: (i) the technical difficulty to reproduce the complex renal architecture; (ii) the diversity of cell types required to mimic a kidney environment; (iii) the choice of proper biomaterials that allows the preservation of renal structure and functionality [102]. Therefore, currently, 3D bioprinting is principally employed for the generation of portions of the nephron. In 2016, one group create a 3D-bioprinted renal tissue, consisting of a 3D proximal tubule model able to replicate human kidney physiology with functional properties [103]. In 2019, the same group created a vascularized proximal tubule model with active reabsorption of solutes through tubular-vascular exchange [104]. With the current level of technology, the fully recapitulation of the intricate kidney architecture and its complex composition would be a technically difficult task, if not impossible [105]. Therefore, despite its enormous potential, there is still a long way to go in the field of 3D bioprinting to achieve a functional transplantable kidney tissue.

1.3.5. Renal device

Currently, renal substitution therapy with haemodialysis or haemofiltration has been the only successful long-term ex vivo organ substitution therapy to date [106]. Although this treatment has proven life sustaining for patients suffering from a renal failure, it falls short of fully replacing kidney functions, as evidenced by the continuing high rates of morbidity and mortality in treated patients. Between RM and RRT, the tissue engineering of a bioartificial kidney, as a Renal Assist Device (RAD), represents a novel possible solution to create a structure to replace a kidney function [106–108]. This device consists of a bioengineered structure that contains a hybrid "living membrane" with functional proximal tubule epithelial cells, supported by an artificial functionalized hollow fibre membrane. Renal cells grow in monolayers until confluence and perform different reabsorption and secretory functions, constituting a device with absorptive, metabolic, and endocrine functions. Although this functions are less efficient than those in native proximal tubules, RAD technology was able to improve acute haemodialysis performance in uremic dogs [106–108] and has been the subject of some clinical trials [109,110]. Up to now, the RAD is the only bioengineered kidney device that has been successfully tested in humans. However, despite this success, its manufacturing times and costs, its delicate storage requirements and distribution, and the required cell sourcing, has been proved to be important limitations to an extensive use of RAD device.

In an attempt to overcome these limiting factors, a Bioartificial Renal Epithelial Cell System (BRECS) was developed by the same group [111–113]. The BRECS is a perfusion cell-attached bioreactor designed to be fully cryopreservable, based on niobium-coated carbon disks seeded with human renal tubular epithelial cells into a polycarbonate-stainless steel hardware. The BRECS device has been demonstrated its efficacy in a porcine septic shock model and in an anephric sheep model, but this technology is yet to be applied in clinical trials [111–113]. In the meantime, this pioneering work has paved the way to multitude of promising studies and prototypes of the so-called Portable Artificial Kidney (PAK), Wearable Artificial Kidney (WAK) and Implantable Artificial Kidney (IAK), reviewed elsewhere [114,115]. Overall, this bioengineered artificial kidney devices could represent a feasible alternative to renal replacement therapy and transplantation, despite its current limitations mostly represented by high costs of production and storage, the durability of the implanted device, and the ability to reabsorb important solutes and water from the filtrate.

1.3.6. Organ xenotransplantation

1.3.6.1. Of adult organs

Organ transplantation between organisms from different species, or organ xenotransplantation, offers an attractive possibility to overcome the shortage of kidneys from deceased and living donors. Xenotransplantation offers several advantages over allotransplantation, such as unlimited supply of organs, elective organ availability and the avoidance of the detrimental effects of brain death on the allogeneic organs [48]. Clinical xenotransplantation has a long history going back to blood transfusions across species in the 17th century [116]. The first attempt to graft kidneys from a non-human primate (NHP) into human recipients goes back to 1963, when Reemtsma et al. transplanted chimpanzee kidneys into 13 patients with ESRD [117]. Unfortunately, two months after surgery, all hosts died from rejection or infection, with the exception of one patient who survived for 9 months. There are several disadvantages with the use of NHPs as organ donors, such as its limited availability, poor breeding potential, high maintenance costs, slow growth, high risk of xenozoonosis and mixed public opinion [118]. Nowadays, with the advent of genetic engineering and cloning technologies, pigs are considered the preferred sources of organs for xenotransplantation, being possible to delete pig antigens or to insert a human transgene that provides protection from human complement and/or coagulation activity [118]. These important breakdowns, in combination with specific immunosuppression regimens, has allowed to extend the survival time of life-sustaining pig-to-NHP renal xenografts up to more than a year [119]. With the utilization of CRISPR/Cas9 technology, scientist have the capability to rapidly genetically engineer porcine donors, suggesting that life-sustaining kidney xenotransplantation is much closer to clinical reality than previously thought. However, there are certain hurdles that still need to be overcome before xenotransplantation can be considered a routine therapeutic option, which are reviewed elsewhere [120–127]:

- **Appropriate immunosuppressive regimens must be founded**. These regimens are more intensive than those used in allotransplantation, increasing the risk of recipient death. Currently, as many as 40% of kidney allografts are lost because of recipient death with a functioning graft. In addition, preclinical xenotransplant models have utilized immunosuppressive drugs that are not yet approved for clinical use.

- **Zoonotic transmission of infectious diseases must be avoided**, both to the recipient and to human populations more broadly. Some concerns have been ameliorated by the use of pathogen-free facilities and the development of extensive testing. However, particular concerns involve porcine endogenous retroviruses (PERVs), which are integrated into the pig genome and are therefore

present within all transplanted tissues. However, PERVs can be eliminated through gene editing techniques, or controlled by available antiretrovirals, bringing clinical trials a step closer.

- **Ethical concerns must be addressed**. Crossing the species barrier has always been seen as potentially morally problematic. Xenotransplantation implies the creation of chimeras (i.e. organism made up of cells of different embryonic origins), which can cause some ethical problems related to conflicts of interests or values. Cultural and religious concerns, together with the need to respect both animal rights and human dignity, force that ethics of chimeric research must be examined on an individual basis and approved by an oversight committee. No blanket statements can be made.

- **Clinical trials of xenotransplantation must be legally acceptable**, besides medically and ethically justified. For now, xenotransplantation is mainly conceived and regulated as a pharmaceutical/medical product. Therefore, regulatory frameworks should contain specific conditions about the safety of the donor source, xenograft, and "manufacturing" process (i.e. genetically modified or wild-type), also indicating safety and efficacy of the xenotransplantation procedure. In addition, these guidelines should ensure that risk management protocols are in place to identify, contain, and combat any outbreak of infection in a timely manner. Coordinated international action to harmonize global practices in this field may be necessary due to the fragile balance between individual and collective rights and the tensions of globalization.

1.3.6.2. Of embryonic tissues

This technique constitutes the cornerstone of this thesis, so it will be treated in more detail, explaining its advantages over the above described techniques and the points in which we will try to take a step forward during this project.

Mammalian embryonic organs or primordia, once morphologically defined (Figure 4), can maintain its growth capability and undergo differentiation after its transplantation into the appropriate niche. Therefore, embryonic organ transplantation results in differentiated and functional structures, able to replace the lost function of diseased organs. Promising results has been obtained in this field using neuronal tissue [128], stomachs [129], intestines [129], tracheas [129], lungs [129,130], livers [130,131], spleens [132] and hearts [133]. But, at present, the main progress in this area revolve around the pancreas [134,135] and kidney [136].

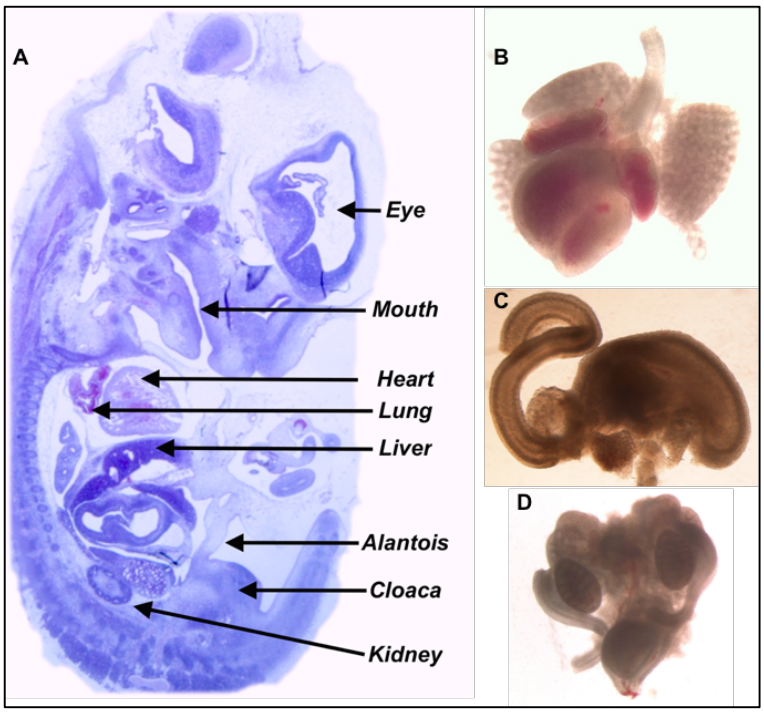

Figure 4. Histology of 15-day-old rabbit embryo and retrieved organs [137]. (A) Embryo. (B) Lung and heart. (C) Stomach and intestines. (D) Gonads and cloaca.

If primordia are retrieved at the proper time of the embryonic development, embryonic organ transplantation offers theoretical advantages versus other bioengineering strategies or fully differentiated (adult stage) organ transplantation. A minimum degree of differentiation in the primordia ensures that embryonic organs has committed to tissue-specific genetic developmental program, in which cells are "knowing" its destination cell type and how should be assembled [138]. Therefore, exogenous steer differentiation or assembly are not required, and the risk of teratoma formation by more undifferentiated cell-states is avoided [138]. On the other hand, and in opposition to terminally differentiated adult organs, embryonic cells possess an enhanced growth potential and a relative attenuated immunogenicity [139]. The immunological advantages could be due to a failure of direct antigen presentation resulting from the lack of donor antigen presenting cells in embryonic primordium [140]. Furthermore, major histocompatibility complex class I and II proteins, as well as complement genes associated with innate immunity, had restricted expression in embryonic tissues compared to older organs, skewing the immunological response [141–143]. Interestingly, embryonic organs obtained at sufficiently early stage are avascular, but they are able to attract its own vasculature from the host, being therefore less susceptible to humoral rejection than developed organs with donor blood vessels transplanted across a discordant genetic barrier [134,144–146]. For the same reason, renal primordia do not require immediate vascular anastomosis upon transplantation, as is the case in a vascularized mature organ.

This means that equilibrium between undifferentiated and full differentiated stages is required (optimal time window). This optimal point depends on the embryonic tissue and the gestational duration of the specie (Figure 5), coinciding with the gestational age in which organ primordia have the maximal capacity of grow and differentiate with minimal risk of teratoma formation and immunorejection [130,139,147].

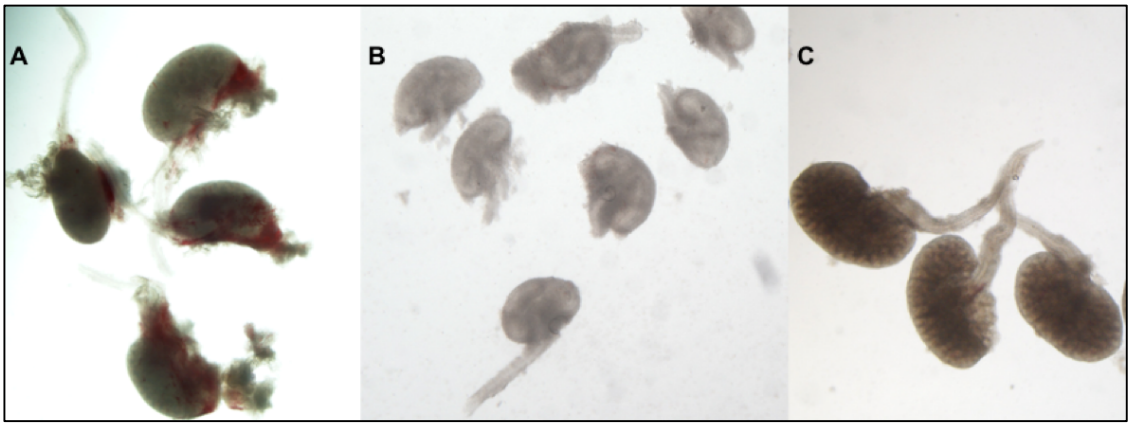

Figure 5. Embryonic kidneys (metanephroi) from different species [137]. (A) Porcine 28-day-old metanephroi. (B) Rabbit 15-day-old metanephroi. (C) Mouse 13.5-day-old metanephroi.

These intrinsic properties of embryonic organs have led to encouraging results across allogeneic barriers into immune-competent hosts, but also across concordant or highly disparate xenogeneic barriers [138]. Of note, pancreatic primordia undergo growth, differentiation, and function after transplantation into non-immunosuppresed hosts, normalizing the glucose levels in diabetic rodents [135,138,148]. In the same way, if embryonic pancreas was xenotransplanted, glucose intolerance can be corrected in diabetic rats [149–151] and ameliorated in rhesus macaques [152,153] by porcine developing beta cells without immunosuppression treatment. But focussing on the renal primordia, data from different animal models has demonstrated that embryonic kidney (metanephroi) allotransplanted into non-immunosuppressed hosts results in fully differentiated and vascularized renal structures with excretory (urine production) and endocrine functions (renin and erythropoietin activity) [138,147,154–158]. Following this strategy for de novo renal organogenesis, survival of rats with all native renal mass removed can be increased by prior metanephroi development and ureteroureterostomy [159], being survival time proportional to the mass of functional renal tissue developed [146]. Moreover, developed metanephroi demonstrated a renin activity that increase the arterial blood pressure in a rat model of acute hypotension [156], and inhibit the progression of vascular calcification in rats with renal failure [160].

Successfully, metanephroi xenotransplantation experiences also result in fruitful outcomes. Metanephroi from rats become enlarged and vascularized, forming mature tubules and glomeruli after being transplanted in mice that received tolerance-inducing agents [161]. Development also occurs after transplantation of pig metanephroi into mice [147] and rats [162] receiving costimulatory blockade. Specifically, Dekel et al. xenotransplanted metanephroi of both human and pig origins into immunodeficient mice, which generate functional nephrons as evidenced by the dilute urine they produce [143]. In addition, these authors demonstrated *in vivo* the decreased immunogenicity of the embryonic grafts compared with adult kidney transplants, showing that embryonic primordia require lower immunosuppressive regimens to survive [143]. Interestingly, it is known that xenotransplanted metanephroi can provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue [163]. Taking advantage of an organogenic niche, it has been shown that if human mesenchymal stem cells are injected into the site where the metanephroi will develop, these cells can be integrated into the embryo development programme and become part of the newly generated structures [164]. Then, if metanephroi are recovered and transplanted, the new developed kidney contains human nephrons and the vasculature from the host, being able to produce urine and secrete human erythropoietin that improve anaemia in hosts [164,165]. With a view to the future clinical application, the xeno-tissue component could be eliminated using metanephroi from transgenic donor that carries a regulated suicide gene, leaving autologous tissue and alleviating immunosuppression effects and ethical concerns [163–165].

However, before the clinical applications of the metanephroi transplantation to resolve ESRD, there are some factors that should be addressed in order to facilitate the clinical translation of the technique. Of the most important is the transplant surgery and graft site. To date, metanephroi have been transplanted into different sites such as the omentum, kidney capsule, anterior eye chamber and lymph node [136,140,154,155,166,167]. However, the influence of the insertion site is not indifferent. For example, hydrostatic pressure stimulus from the paraaortic area increases renin production of developing metanephroi, but there were no site-specific differences in erythropoietin production [157]. Classically, omental transplantation was deemed to be particular favourable due to some reasons [136,138,157]:

- The vascular precursors present in renal primordia provide one source for the microvasculature if metanephroi are transplanted in the anterior eye chamber or beneath the renal capsule. But, if primordia are transplanted into the omentum, these precursors may regress and be replaced by precursors originating from hosts. In order to minimize the donor antigen recognitions by immune circulating cells of the receptor, blood supply of developed kidneys should be through host vessels.

- This site is not confined by a tight space, allowing undisturbed expansion of a growing organ primordium. Hence, morphogenesis is not physically constrained.
- Transplantation to omentum facilitates the transplantation technique.

Intriguingly, to our best knowledge, all the metanphroi transplant surgery has been performed through open surgery, probably due to the small size of the animal model used (currently murine models) or by the lack of an effective process properly described [137,168]. However, a practical and safe laparoscopic procedure should be described to guide the metanephroi transplantation technique to the clinical setting due two reasons: the facilitation of the preclinical trials in larger mammals and the possession of a minimally invasive technique for transplantation in humans [168]. Inevitably, going forward clinical scenario, it is necessary to validate the procedure in models that reliably predict human performance and whose physiological, anatomical, and immunological characteristics closely resemble the human features [169]. However, without the laparoscopic approach, the major difficulties of the large animal operations could complicate the preclinical phases of this line. Once arrived at the clinical phase, laparoscopy-assisted surgery has advantages compared with open surgery, such as significantly less pain, incision-related complications and better quality of life during the early postoperative period [170]. In favour, it has been known that although laparoscopic procedures could enhance the duration of intervention, and the ischemia time accordingly, similar grafts outcomes and survival rate was obtained compared to open surgery [171]. So laparoscopic approach becomes an important point to consider in order to hopefully guide the metanephroi transplantation towards its clinical alternative to the shortage of allogenic organs.

Another issue that deserves our attention is that once metanphroi has developed, its renal mass and clearances are still too low to sustain life in long-term [140,155,159,172,173], and there is no increase even after a long time after transplantation [173]. Of the most plausible explanations is the hydronephrotic state to which nascent kidney arrive, due to its functionality and the lack of a urine excretion channel, which turn it dysplastic and non-viable and stops its growth. However, if metanephroi were transplanted beside cloacas (Figure 6), and cloacal-developed bladder was connected to the host ureters, the new kidney can excrete urine thought the recipient ureter, avoiding hydronephrosis. This strategy allows a sustainable growth and maturation of the nascent kidneys, and prolonged the lifespan of anephric rats in the short-term [158]. However, allowing the new kidney to grow larger and sustain life in the long-term is a remaining obstacle to guarantee the feasibility of this strategy for clinical application.

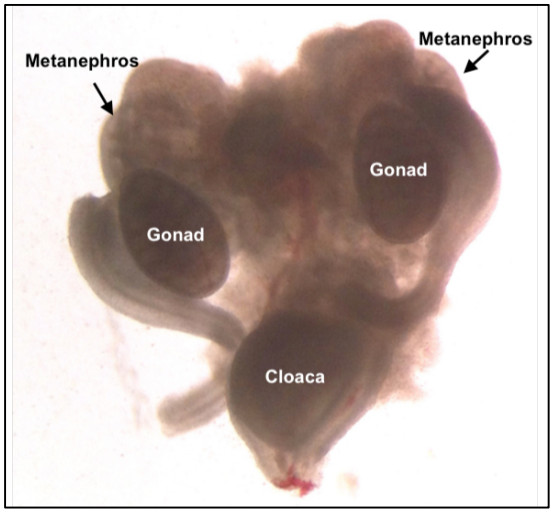

Figure 6. Detail of a rabbit embryonic urogenital structure [137].

Interestingly, both weight and clearances of the nascent kidneys can be enhanced by compensatory renal growth following reduction of host native renal mass, which stimulus is accompanied by changes in the expressions of several growth factors [155]. Besides, organ culture experiments have demonstrated *in vitro* that some growth factors are essential for kidney development, and blocking them inhibits kidney growth [172]. In this sense, being well described the renal growth-promoting actions of the insulin-like growth factor 1 (IGF-1), it has been shown that clearances can be increased by the pharmacological administration of IGF-1 to hosts [173]. By the other hand, clearances and urine volumes were increased significantly in developing metanephroi pre-treated (prior implantation) with vascular endothelial growth factor (VEGF), and increased further in post-treated (during ureterouretostomy) nascent kidneys [172]. Subsequently, using combinations of growth factors to pre-treat and post-treat metanephroi, Hammerman's group have achieved rates of clearance in transplanted metanephroi almost 300 times those measured without any treatment [155], approximately 6% of the clearance achieved by a normal kidney [144,172]. In these experiences, metanephroi secrete a concentrated urine and, in some animals, clearances achievable by a single transplanted metanephros approaches the level that supports life in patients on dialysis (10% of baseline) [144,159,174]. Therefore, it is of especial importance to investigate whether growth factors could be used to enhance the growth and function of developing metanephroi. In this sense, sildenafil citrate (SC) is a well-known drug used for treatment of pulmonary hypertension and male erectile dysfunction (Viagra™) due to its vasodilatory effect. SC mediates its effect via inhibition of phosphodiesterase 5 enzyme (PDE-5), which up-regulates cGMP and nitric oxide (NO), leading to improved renal haemodynamics and renoprotective effects through anti-inflammatory, anti-oxidant, and anti-apoptotic mechanisms [175,176]. Besides, SC triggers angiogenic gene/protein expression up-regulation, increasing angiopoietin-1 and VEGF systems, which are essential for renal vascularization [172,177]. By this

mechanisms, SC cause neovascularization, but also increase blood flow via NO vasodilatory effects, and reduces thrombus formation by affecting platelet functions [177,178]. These SC properties could exert a positive effect on the avascular metanephroi after its transplantation, whose survival may depend on the vascularized host bed, oxygenation of local tissue, and the patient's current systemic status [178]. Therefore, positive effects are expected from SC to increase graft survival and function rates after metanephroi transplantation.

Hence, the most immediate objectives of the thesis are to develop a laparoscopic procedure for the transfer of metanephros and to verify whether the use of SC leads to positive effects on the transplant performance and on the new kidney function. The rabbit (*Oryctolagus cuniculus*) is the chosen model along this thesis for several reasons. In contrast to smaller animal models, a healthy rabbit (3-3.5 kg) provides an experimental subject with a good capacity for the laparoscopic approach [137,168]. Although it is crucial to validate any procedure before its clinical application in higher species (more similar anatomically and physiologically to the human), rabbits are widely bred, docile, have short life cycles and are very economical compared to larger animals, providing an excellent animal model for the first experimental steps [168]. Besides the rabbit model has been proposed as a better model for several human diseases than rodents, as rabbit genes are apparently more similar to those of the human than are rodent genes [179,180].

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1. GENERAL INTRODUCTION

1.4. ORGAN CRYOPRESERVATION HOLDS THE PROMISE TO TRANSFORM MEDICINE

1.4.1. Advances in organ preservation

The primary graft function and its long-term outcomes are dependent on ischaemia–reperfusion injury, defined as the paradoxical exacerbation of cellular dysfunction and death, following restoration of blood flow to previously ischaemic-damaged tissues (comprehensively reviewed in [181,182]). There is evidence that different organs have different resistance thresholds to the ischaemic insult, occurring during the preservation period after retrieval and before implantation, which could be affected by the preservation modality [183]. The three main organ preservation techniques include static cold storage, continuous hypothermic perfusion and cryopreservation [183,184]. To date, static cold storage remains the most common preservation modality due to its simplicity and lower cost, despite its higher risk of subsequent transplant damage that allows a storage time limited to 4-12 h [183,184]. Although continuous hypothermic perfusion can extend preservation time up to a few days (no more than 5-7 days), this methodology causes metabolic changes that can impairs the organ function and involves a constant risk of microbiological contamination [10,184]. Besides, even if it were technically feasible to maintain manufactured tissues and organs indefinitely in culture through perfusion, the cost would become prohibitive over few months [10]. Therefore, although organs used for clinical transplantation are currently preserved at above-zero temperatures, cryopreservation is believed to be an ideal and inexpensive approach for the long-term preservation [10].

Cryopreservation under extreme low temperature allows to achieve the “cryogenic suspension of life”, allowing the metabolic arrest and preventing ischemic damage until organs are warmed when necessary. Therefore, the aim of cryobiology is to shift the pendulum from cell death to immortality at low temperatures, which can be accomplished by slow-freezing and vitrification methods depending on the concentration of cryoprotectants and de cooling-warming rates used [137,185,186]. It is now accepted that uncontrolled ice formation during slow-freezing is the single most critical factor inducing mechanical damage in structured tissues and organs, which severely restricts the survival rates [187]. In recent years, this major problem has been effectively circumvented in some tissues by using ice-free cryopreservation techniques based upon vitrification [188]. Vitrification, is a method developed by Rall and Fahy that bursts in the 80’ with the promise to the long-term cryopreservation [189]. Vitrification refers to the transformation of an aqueous phase into the glassy state (Figure 7),due to a highly increase of the viscosity without ice crystals formation at low temperatures [189,190].

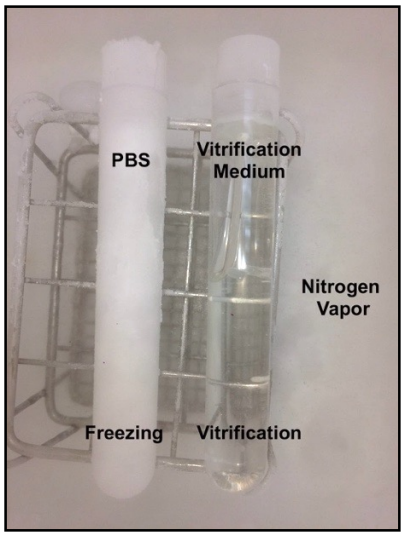

Figure 7. Macroscopic view of frozen vs vitrified state.

However, on the basis of the current knowledge, vitrification success is directly proportional to the cooling and warming rates and viscosity, but inversely proportional to the sample volume, being this last the major problem regarding whole vital organ vitrification [184,187,191]. Large volumes incur worse heat transfer, hindering thus the high cooling-warming rates required, which maximum is achieved and currently limited to the usage of liquid nitrogen for cooling and a warm bath for warming [191]. Therefore, vitrification requires higher concentrations of cryoprotectants to increase viscosity, introducing greater risks of both osmotic damage and cryoprotectant toxicity [188]. Although the combination of different cryoprotectant is often used to increase viscosity and reduce the level of toxicity of each individual component, to date small ovaries, blood vessels, heart valves, corneas, cartilage, and similar structures that can be cooled and rewarmed rapidly, are the only macroscopic structures that overcome vitrification procedures [10,192]. Large-sized organs, due to their small ratios of superficial area to volume, require further efforts in two directions [184]: (i) looking for low-toxic and highly-penetrating cryoprotectants to control its concentration and exposition time to achieve its uniform distribution; (ii) developing better techniques to more precisely control the internal and external organ temperatures. Until this technology is available, cryobiology experts gathered at the first Organ Banking Summit, convened in 2015 in Palo Alto (California, US), discussed the major remaining scientific challenges that need to be overcome in order to bank organs [9]: (i) controlling ice crystal formation, (ii) reducing cryoprotectant and osmotic toxicity, (iii) preventing thermal and mechanical stress, (iv) control of heat and mass transfer profiles, (v) minimizing ischemic injury, (vi) limiting chilling injury, and (vii) developing protocols for optimal rewarming, repair, and functional assessment of organs. In the meantime, some experimental successes in recent years suggest that organ banking is possible, including the successful re-implantation of rodent hearts[193], livers [194] and gonads [195], porcine livers [194], and sheep ovaries [194] after low-temperature storage. Fahy et al.

reported successful rabbit kidneys storage at -45 ^{o}C, which support life after transplantation [196], as well as successful re-implantation of a functioning rabbit kidney after long-term storage below -120 ^{o}C in the glass state [192]. Besides, Chinese labs have recently made multiple advances related to the successful cryopreservation and reattachment of functioning rat hind limbs [197] and human digits [198]. However, it remains the case that no vital mammalian organ has ever been cryopreserved to a temperature low enough for long-term storage and subsequently thawed, transplanted, and found to support life for a long time [9,10,192].

1.4.2. Implications of preservation constrains for organ transplantation

As reviewed in the previous introductory sections, having enough organs and tissues to meet public health needs has been the subject of extensive efforts in science, medicine, and public policy; ranging from increasing organ donation/utilization to developing bioengineered or laboratory-grown organs. But until we are able to preserve this biological structures during procurement or manufacturing, storage and transport, these efforts will not reach their lifesaving potential [10,11]. Therefore, developing an organ and tissue supply (either from living/deceased donor or derived from regenerative medicine strategies) that can meet the healthcare demands of the XXI century must meet two challenging requirements: first, having enough of these lifesaving resources; and second, having the means to store and transport them for a variety of logistical needs. In this sense, the promise of organ and tissue preservation to transform medicine has been recently reviewed [9–11,187]. Without proper preservation procedures, the future of these treatments would still be compromised by the ability to physically distribute the organs to patients in need and produce these products in a way that allows adequate inventory control and quality assurance.

In the case of organs derived from deceased or living donors, its problematic shortage are fuelled by severe logistical constrains related to organ preservation limits, being today measured in hours, so that transplantation is required almost immediately after the organ is recovered [11]. This fact limits the lifesaving potential of transplantation in many ways. Since the assignment must be done in short time periods, donor and recipient must be stay over relatively short distances, so that organs often resulted not well-matched with recipients from the immunological point of view. Therefore, patients could have an increased risk of organ rejection, requiring intensive immunosuppression regimens [70] or a new organ in the case of final rejection [57]. Besides, limited matching distances leave unequal access to transplant, affecting patients with fewer resources, who cannot relocate to join more favourable waiting lists [199]. But in the worst case, this phenomenon contributes to thousands of organs being discarded, which have a profound impact on waiting lists. In United States, 12% recovered organs become not

transplanted annually, which could avoid 52% removals of patients from waiting list due to death or illness, or treat 17% new additions to the waiting list (Table 1 [4]).

***Table 1**. Organ procurement and transplantation data in the United States (2019).*

	Organs recovered	Organs transplanted	Organs discarded	Waiting list patients removed for death or illness	Waiting list additions
Kidney	29070	24610	4460	8284	43224
Liver	9675	8897	874	2421	13448
Pancreas	1374	1028	346	64	480
Heart	3635	3604	31	527	4717
Lung	5252	4914	338	314	3248
Intestine	86	81	5	19	103
Total	49092	43134	6054	11629	65220

Based on Organ Procurement and Transplantation Network (OPTN) data [4].

Worldwide, although each cadaveric donor can provide up to eight lifesaving vital organs, on average only 3-4 per donor are transplanted, being nearly 20% of kidneys currently discarded mainly because they exceed their maximum preservation times [9]. Trying to avoid organ loss, almost $1 million could be needed each time to fly organs from donors to their recipients, being lengthy operations performed day or night without time to adapt procedures to individual circumstances [9]. A successful long-term organ preservation would revolutionize the world for organ transplantation, allowing more organs to be available, improving transplant outcomes and safe, mitigating risks, and decreasing costs. Several key opportunities to accelerate progress toward this goal were identified at the first Organ Banking Summit, which should be encouraged by the potential public health impact of organ banking [9]:

- **Increasing pool of donor organs**: improving donor utilization and reducing organ discard, decreasing costs and logistical burdens of transplantation, allowing time for new matching approaches to guarantee donor-recipient compatibility, supplying successive organs in case of organ rejection.
- **Enhancing transplant viability and function**: assessing organ function and allowing organ repair before transplantation, enabling time to immune tolerance induction in recipients, screening of transmissible diseases from donors, preventing ischemic injury during organ transport.
- **Expanding transplantation access**: allowing a flexible scheduling of transplant surgeries both in time and geographically, extending live kidney donation chains, providing enough organs in emergency care (situations of natural disasters or terrorist attacks).

Nonetheless, as reviewed above, donor sources are unlikely to be able to provide all of the transplants required. Fortunately, the gap between organ supply and demand can

be eliminated in principle by the field of regenerative medicine over the next few years, providing organ replacements created in the laboratory. However, once these new lifesaving organs arrive, its therapeutic promise depends on the ability to physically distribute these laboratory-grown organs to patients in need and to produce these regenerative products in a way that allows for adequate inventory control and quality assurance. For this purpose, cryopreservation may be indispensable. Regrettably, cryopreservation of complex systems remains also the missing link in the regenerative medicine supply chain, whose consequences has been discussed by members of the Organ Banking Summit on several occasions [9–11]:

- This scenario limits the economic viability, as grafts cannot be mass-produced nor generated long in advance of need for cases of acute injury or illness.
- With adequate cryopreservation protocols, it must be possible to ship an inventory of the regenerative medicine products to any desired location without product deterioration, storing them at destination until use.
- Besides, as any medical product, long-term storage is required to allows quality analysis and quarantine of the produced organs, but also to protect them from contamination and genetic drift.

Therefore, as tissue engineering and regenerative medicine matures, complex tissue preservation will increasingly become a bottleneck limiting the entire field, being necessary cryopreservation protocols compatible with both living (i.e. cells) and non-living components (i.e. extra cellular matrix or non-organic hardware of the bioartifical organs) of the grafts or bioartificial organs [111]. As proof, tissue storage has already become a major barrier for tissue engineering companies working to manufacture skin graft substitutes, blood vessels, and other engineered products [9]. If measures are not taken, tissue storage constraints are likely to plague regenerative medicine just as the organ shortage limits transplantation today [9–11,187].

However, if the graft supply and demand could be balanced by embryonic organ transplantation in a most favourable future situation, most of drawback intrinsic to the mature organs cryopreservation would be directly surpassed because of the small size of the primordia. In 1986, Groscurth et al. reported that different human embryonic organs are able to be long-term cryopreserved by slow-freezing, maintaining its morphology, viability and differentiation capacity after its xenogeneic transplantation into immune-deficient mice [200]. As case report, of the two transplanted metanephroi, one become developed and showed mature glomeruli [200]. More recently, Bottomley et al. reported that no differences in tissue viability were observed *in vitro* between fresh, slow-frozen and vitrified metanephroi [201]. These authors ratify that the small metanephroi size (<1mm in rats), allows a rapid perfusion and equilibration of the cryoprotectants within the tissue, making vitrification feasible. However, although

cryoinjuries were dominant in the frozen group, cryoprotectant-related toxic effects were observed in the vitrified one [201]. However, nowadays new and less toxic vitrification solutions has been designed for diverse biological systems [196,202]. Therefore, our last purpose in this thesis was to use two vitrification mediums, characterized by its low toxicity (VM3 [196,202]) and its optimized composition to cryopreserve the renal tissue (M22 [192,196]), in order to demonstrate *in vivo* if developing metanephroi retain its developmental and functional properties. If metanephroi banking could be carried out successfully, the organization of its transplantation could fuel that its therapeutic potential turns in a reality for patients in need.

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2. OBJECTIVES

OBJECTIVES

The general aim of this thesis was to establish strategies that turns the metanephroi transplantation in an ever closer clinical reality.

This aim was itemised into the following specific objectives:

→ Development of a minimally invasive laparoscopic procedure well-suited for the metanephroi transplantation into the omentum, as an alternative to the current techniques involving opening surgeries.

→ Determine if the addition of sildenafil citrate during the metanephroi transplantation can exert any growth-promoting action, enhancing both the graft size and function, towards the generation of renal structures with life-sustaining potential.

→ Evaluate the feasibility of the long-term metanephroi banking by using ice-free cryopreservation techniques based upon vitrification, which could dissociate the time between the organ retrieval and its transplantation, allowing time to allocate the graft where necessary and guarantee an adequate inventory control and safety assurance.

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3. CHAPTER I

LAPAROSCOPIC TRANSPLANTATION OF METANEPHROI: A FIRST STEP TO KIDNEY XENOTRANSPLANTATION

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3.1. ABSTRACT

Embryonic kidney xenotransplantation could represent a new solution to the scarcity of kidneys for transplantation. The aim of this study was to determine the feasibility of laparoscopic metanephroi allotransplantation in rabbits. To this end, microscopic dissection was conducted to obtain metanephroi from 14- day-old (E14), 15-day-old (E15) and 16-day-old (E16) rabbit embryos. Using single-port abdominal laparoscopy, a spinal needle was inserted percutaneously, through which the metanephros were deposited (using an epidural catheter) close to a patent blood vessel in the retroperitoneal fat. Seventy metanephros (24 E14, 20 E15 and 26 E16) were transplanted to 18 rabbits. Three weeks later, the animals were examined through open surgery. We compared the embryonic maturity, the morphometric variables of the metanephros and the development rate of the transplanted metanephros. The results showed that the lower time limit for the metanephroi extraction was E14. Three weeks after transplantation, only 3/24 E14 metanephroi grew at minimal expression (12.5%). In contrast, 10/20 (50%) E15 and 12/26 (46.1%) E16 metanephroi grew. These metanephroi had differentiated sufficiently towards normal glomeruli, proximal and distal tubules and collecting ducts. We detected no relevant immunological changes in the peripheral blood. In conclusion, here we have described for the first time in the literature the laparoscopic metanephroi allotransplantation from embryos as a feasible and minimally invasive technique. The recipients did not require immunosuppression.

3.2. INTRODUCTION

Chronic kidney disease (CKD) is a public health problem of great magnitude. Approximately 9-10% of the population has an estimated glomerular filtration rate of (eGFR) <90ml/min/1.73m2, and 0.3% of that population suffer from that disease in stages 4-5 that are advanced and symptomatic (eGFR < 30 ml/min/1.73 m2) [203]. Allogeneic renal transplantation is the most effective therapeutic method so far; however, it is limited by the shortage of available organs. One solution might be the transplantation of vascularized renal xenografts, but for many reasons, those types of organs are not available for humans as of now [67,120]. In the field of medicine, embryonic precursor transplantation has been seen as a promising option. There are several advantages with the use of metanephros rather than mature kidneys [155]. The transplanted metanephros obtain their vascularization mainly from the receptor [204]. Therefore, both hyperacute and acute rejection are attenuated, possibly allowing for transplantation and breaking the species barrier [143,147]. So far, metanephros transplants were performed using open surgery from mouse to rat, from pig to rat, etc. It has not been described the use of a minimally invasive technique such as laparoscopy for metanephros transplant, nor the use of rabbit as an animal model in this field. The

goal of this study was to develop morphologically functional kidneys from transplanted metanephros through laparoscopy in the retroperitoneal fat in immunocompetent recipients.

3.3. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

3.3.1. Animals and ethical statements

New Zealand females (5 months old) were used as embryo donors and metanephroi recipients. These animals came from the experimental farm of the Universidad Politécnica de Valencia, an accredited animal care facility (code: ES462500001091). All animals were kept in an alternating cycle of 16 h of light and 8 h of dark, with free access to food and water, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively.

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the "Universitat Politècnica de València" ethical committee prior to initiation of the study (code: 2015/VSC/PEA/00170). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. An authorisation certificate issued by the Valencian governmental administration to experiment on animals is held by XGD (code: 2815), FMJ (code: 2273) and JSV (code: 0690).

3.3.2. Metanephroi recovery and characterization

The rabbit belongs to the few species where ovulation is induced at the time of mating, resulting in a defined chronology of gestation, which allows us to know the embryonic age precisely [179]. In order to characterize the metanephroi, donor rabbits were artificially inseminated to generate embryos of 13 (E13), 14 (E14), 15 (E15) and 16 (E16) days. Rabbits were anesthetized with intramuscular xylazine (5 mg/kg) and euthanized with intravenous thiopental (20 mg/kg). Embryos were removed from the uterus and weighed individually for characterization. Some of them were fixed in Bouin solution, embedded in paraffin and stained with hematoxylin and eosin for histological evaluation. The remaining metanephroi in the embryos were dissected under a stereomicroscope and transferred to a petri dish with Dulbecco's phosphate buffered

saline solution. Each metanephros was photographed for morphometric characterization (area, perimeter, length and width) using the free available software Image J. (http://rsb.info.nih.gov/ij/).

3.3.3. Metanephroi laparoscopic transplantation

Metanephroi obtained from E14 (*n*=24), E15 (*n*=20) and E16 (*n*=26) embryos were transplanted in order to assess its developmental ability. The maximum time between recovery and transplantation was 45 min. Female hosts were sedated by an intramuscular injection of xylazine (5mg/kg) and morphine chloride (3 mg/kg). As surgical preparation, anaesthesia was performed by intravenous injection of ketamine hydrochloride (35mg/kg) in the marginal ear vein. After shaving the abdomen of the animals, they were placed on an operating table in the Trendelenburg position. The procedure is performed with a single port for endoscopic camera into the abdominal cavity. An epidural needle was inserted into the inguinal region under vision (Figure 8 A-B). A 17 gauge (G) epidural needle was used for a E14 and E15 metanephroi (Figure 8 C-E). For E16 metanephroi a Cystofix® needle was used (Figure 8C-E).

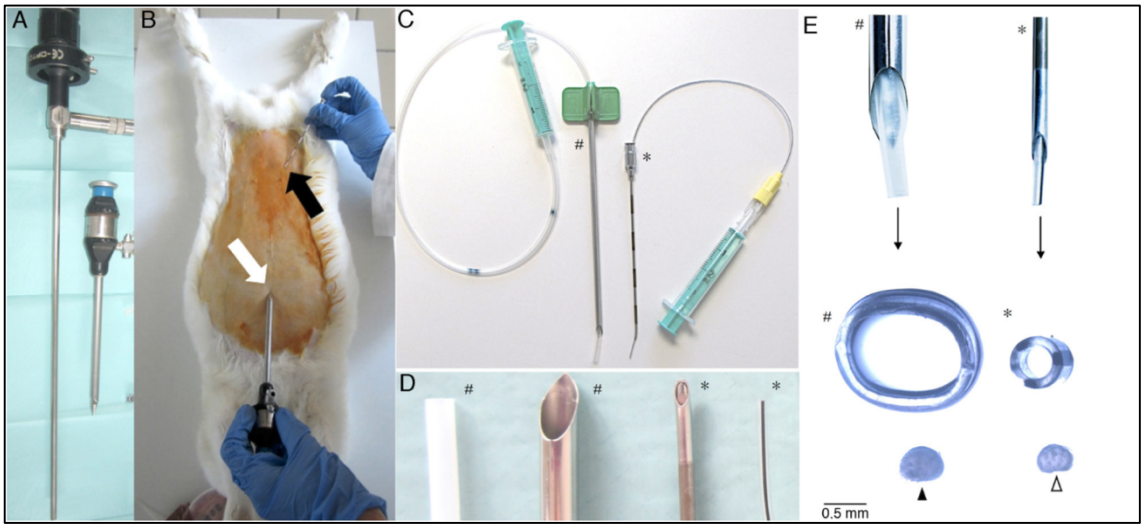

Figure 8. Equipment for laparoscopic transplantation of metanephroi. (A) Endoscope, camera and laparoscopic trocar. (B) Animal in the operating table: insertion position of the endoscope trocar (white arrow) and the epidural needle (black arrow). (C) Transfer systems of laparoscopy for 15-day-old (*) metanephroi and for 16-day-old (#) metanephroi. (D) Epidural catheter Cystofix® detail and needle used for 16-day-old (#) metanephroi and detail of epidural catheter and needle used for 15-day-old (*) metanephroi. (E) Details of internal diameter of Cystofix® (#) catheter and epidural (*) catheter calibers. 16-day-old metanephros (black arrowhead) and 15-day-old metanephros (white arrowhead).

A striking blood vessel in the retroperitoneal fat was identified and a niche (hole) was performed (Figure 9). Then, metanephroi was aspirated into the corresponding catheter, which was introduced through the corresponding needle to release the metanephroi in the performed niches (Figure 9). Four metanephroi (one in each niche) were transplanted in each host without immunosuppression. After surgery, analgesia was administered every 12 h for 3 days, as previously described [168].

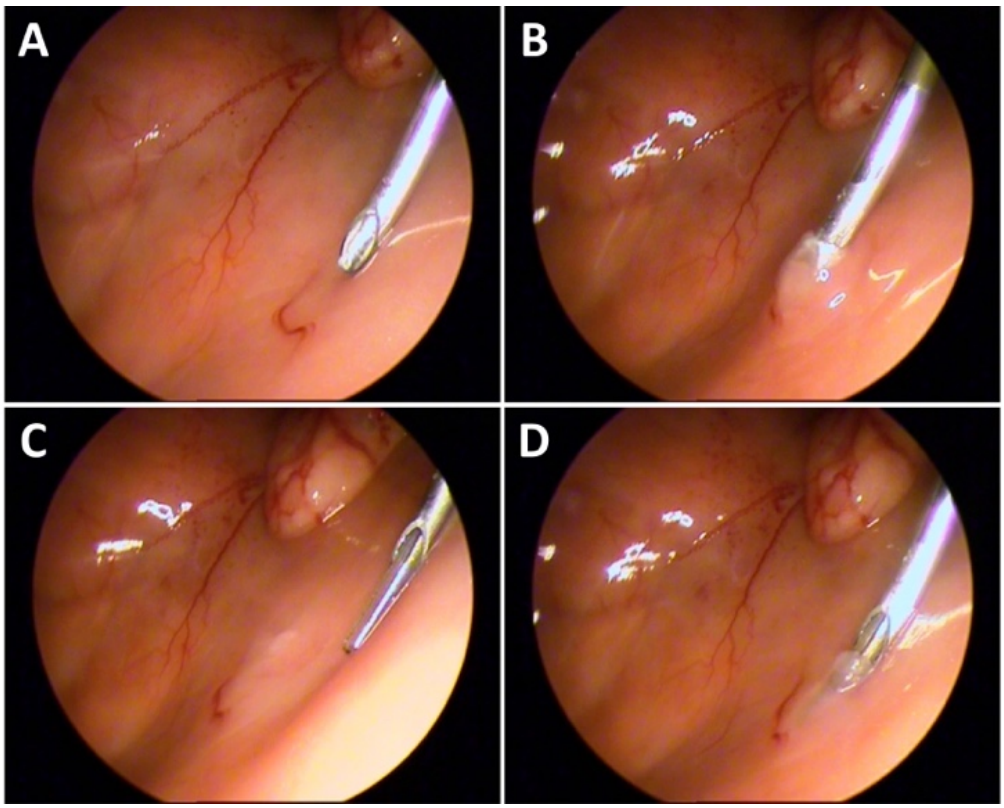

Figure 9. Procedure for laparoscopic transplantation of metanephros [137]. (A) Identification of the renal vessels in the retroperitoneal fat. (B) Making a niche in the retroperitoneal fat, where metanephros will be transferred. (C) Inserting the catheter through the needle with the metanephros. (D) Catheter insertion in the niche and metanephros release.

3.3.4. Metanephroi development and histology

Twenty-one days post-transplantation, the hosts were euthanized with intravenous thiopental (20 mg/kg), performing an open laparotomy for identification and recovery of the new kidney. The presence of urinary ectasia was observed. Subsequently, the new kidneys were removed to determine the presence of glomeruli. Hence, the new kidneys were fixed in a formaldehyde solution and embedded in paraffin wax for histological analysis using a haematoxylin-eosin staining. The functionality of the new kidneys was considered in accordance of the presence of mature glomeruli.

3.3.5. Determination of peripheral white blood cells

By flow cytometry we determined whether metanephroi transplants (E14 and E15) generated an immunological response in rabbit hosts. To do so, blood samples were obtained before transplantation (day 0) and before euthanasia (day 21). The percentage of lymphocytes and the number of leukocytes were determined before performing the flow cytometry. All samples were processed in duplicate. The total lymphocyte count was calculated as the product of leukocyte counts and percentages of lymphocytes, the counts and lymphocyte subset percentages. This methodology has been conducted as other authors have described [205–207].

3.3.6. Statistical analysis

The maturity degree of embryos (E13, E14, E15, and E16) was assessed by comparing its weights using a general linear model. Similarly, morphometric analysis of E14, E15, and E16metanephros (area, perimeter, length, and width) was performed using a general linear model. The recovery rate (recovered kidneys/transplanted metanephroi) depending on the embryonic age (E14, E15 and E16) was analysed using a Chi-square test. Finally, the peripheral blood parameters were analysed using a general linear model, including the embryonic age (E14 and E15), the extraction day (0 and 21), and their interactions as fixed effects. All the analyses were carried out using the SPSS 21.0 statistical package.

3.4. RESULTS

This initial experiment allowed us to characterize the embryos, identifying the position and the morphometric characteristics of the rabbit metanephroi (Figure 10). Depending on the embryonic age, embryos ranged in weight from 0.15 ± 0.014 to 0.68 ± 0.007 g (Table 2). In the E13 embryos, metanephroi are not present (Figure 10A and 10E). In the E14 embryos, the transitions mesenchyme-epithelium required for the formation of nephrons are just beginning (Figure 10B, 10F and 10I). In the E15 and E16 embryos it is possible to identify immature glomeruli (Figure 10C and 10G for E15; Figures 10D and 10H for E16) in the constituted metanephroi, consisting of undifferentiated metanephric mesenchyme with rudimentary epithelial structures (Figures 10G and 10J for E15; Figures 10H and 10K for E16). However, differences in the size and maturity are evident between both ages of embryos (Table 2; Figures 10I, 10J and 10K for E14, E15 and E16, respectively).

Table 2. *Embryo and metanephroi characterization.*

Embryonic age	Embryo		Metanephroi				
	n	Weight (g)	N	Area (mm^2)	Perimeter (mm)	Length (mm)	Width (mm)
E13	6	0.15 ±0.014^{d}	-	-	-	-	-
E14	12	0.28 ±0.009^{c}	18	4.47 ±0.176^{c}	10.02 ±0.195^{c}	0.92 ±0.047^{c}	2.22 ±0.100^{b}
E15	9	0.49 ±0.011^{b}	14	8.47 ±0.199^{b}	11.51 ±0.221^{b}	3.87 ±0.053^{b}	3.28 ±0.088^{a}
E16	20	0.68 ±0.007^{a}	20	12.86 ±0.342^{a}	13.77 ±0.215^{a}	4.82 ±0.090^{a}	3.25 ±0.074^{a}

n: number of embryos; *N*: number of metanephros. a, b, c, d: data in the same column with different superindex are significantly different ($p < 0.05$).

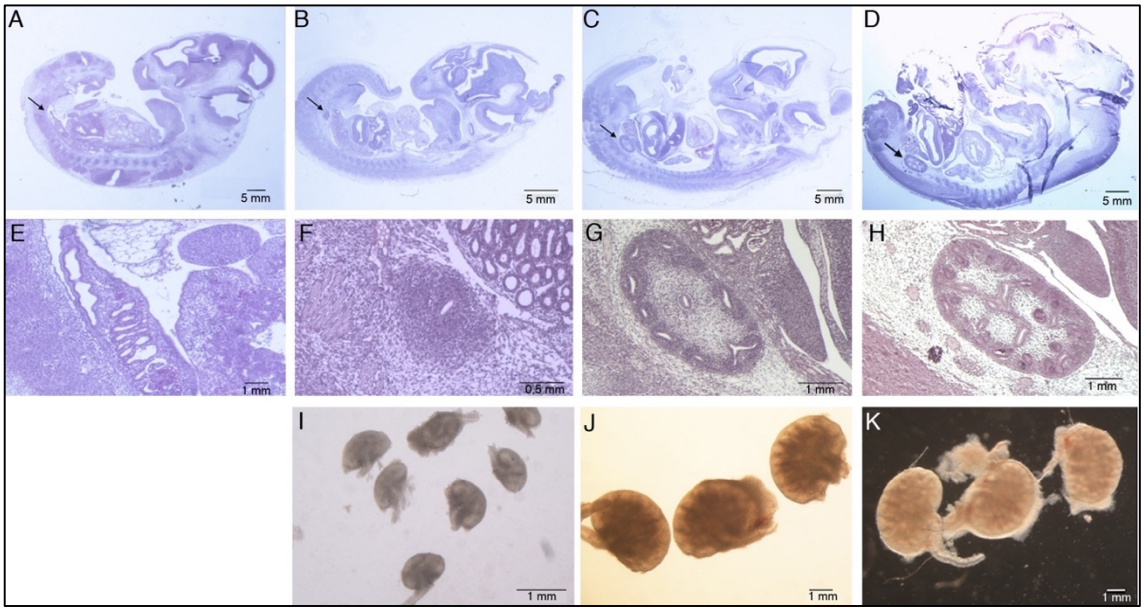

Figure 10. Histology of rabbit embryos and metanephros recovered depending on age. (A) 13-day-old embryo. The arrow indicates the position of the metanephros. (B) 14-day-old embryo. The arrow indicates the position of the metanephros. (C) 15-day-old embryo. The arrow indicates the position of the metanephros. (D) 16-day-old embryo. The arrow indicates the position of the metanephros. (E) Absence of metanephros. (F, I) Detail of a 14-day-old metanephros. (G) Detail of a 15-day-old metanephros. (H) Detail of a 16-day-old metanephros. (I) Detail of a dissected metanephros from 14-day-old embryo. (J) Detail of a dissected metanephros from 15-day-old embryo. (K) Detail of a dissected metanephros from 16-day-old embryo.

The metanephroi from E14, E15 and E16 embryos were transplanted to evaluate the influence of the embryonic stage in the growth and the differentiation potential. After twenty-one days, more than 35% (25/70) of all grafts survived. All new kidneys increased its size with no evidence of malignancy (Figure 11 A-C). Nonetheless, the results were different depending on the embryonic age. Specifically, 12.5% (3/24) E14, 50.0% (10/20) and 46.1% (12/26) E16 metanephroi were recovered, being lower the recovery rate for E14 metanephroi than for its E15 and E16 counterparts ($p<0.05$). All the new kidneys developed from E15 and E16 metanephroi exhibited significant growth and mature glomeruli at 3 weeks after transplantation (Figures 11E and 11H for E15; Figures 11F and 11I for E16). In contrast, the transplanted E14 metanephroi became into structures without glomeruli (Figure 11D and 11G).

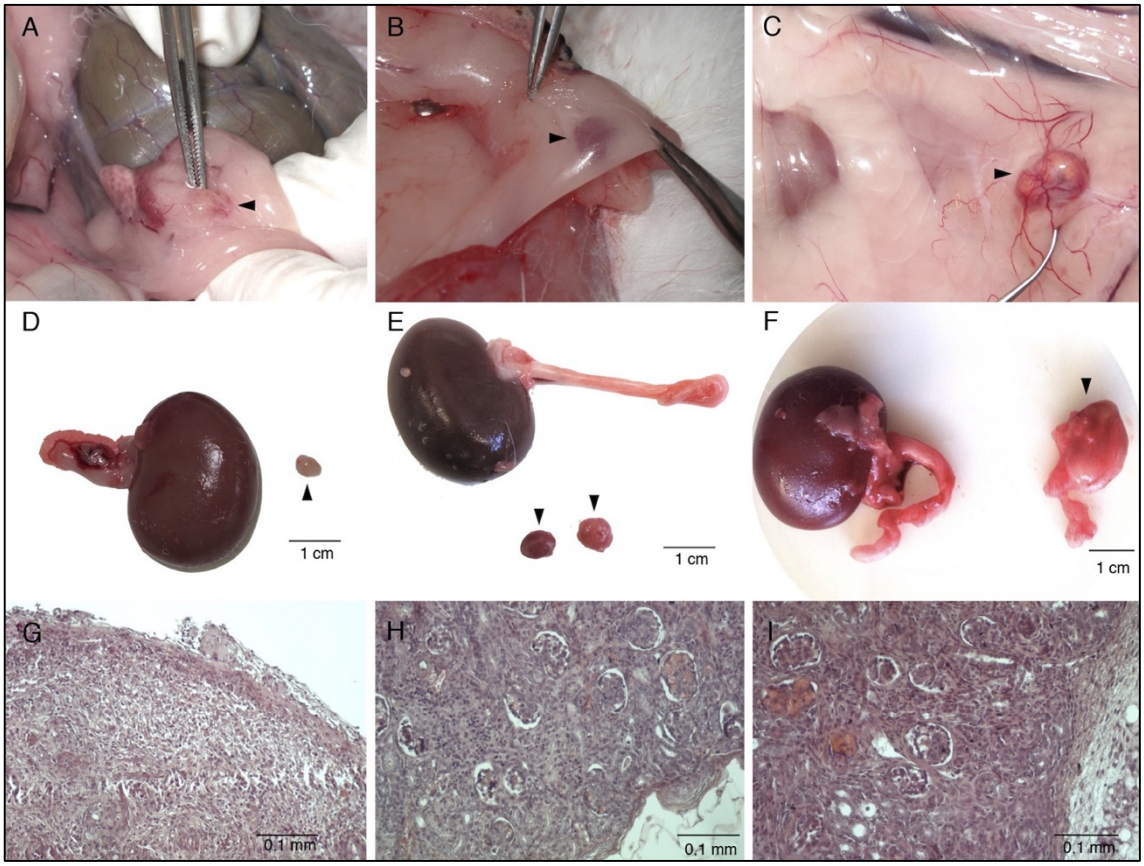

Figure 11. Developed metanephroi twenty-one days after the transplantation. (A) Development of 14-day-old metanephros (black arrow). (B) Development of 15-day-old metanephros (black arrow). (C) Development of 16-day-old metanephros (black arrow). (D) The own kidney host and new evolved kidney from 14-day-old transplanted metanephros. (E) The own kidney host and the new evolved kidney from 15-day-old transplanted metanephros. (F) The own kidney host and evolved neo- kidney from 16-day-old transplanted metanephros. (G) Histological section of the new evolved kidney from 14-day-old transplanted metanephros. (H) Histological section of the new evolved kidney from 15-day-old transplanted metanephros, observing a complete differentiation of mature glomeruli. (I) Histological section of the new evolved kidney from 16-day-old transplanted metanephros, observing a complete differentiation of mature glomeruli. Hematoxylin-eosin, ×400.

Rabbit hosts showed no change in lymphocyte populations (total number of lymphocytes, B lymphocytes, T CD5+ lymphocytes, CD4+ CD8+ and CD25+). Consequently, lymphocyte populations were similar before and 21-days after transplantation (Figure 12).

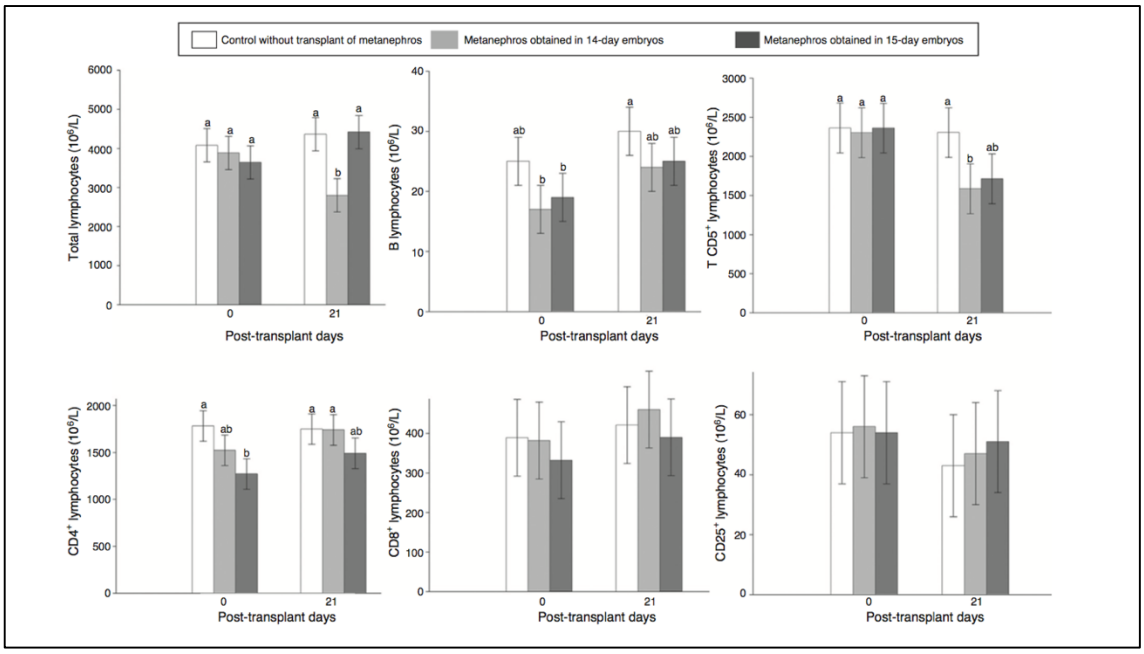

Figure 12. Effect of 14-day-old and 15-day-old metanephroi transplantation on the peripheral white blood cells in non-immunosuppressed recipients. (A) total lymphocytes. (B) B lymphocytes. (C) T CD5+ lymphocytes. (D) CD4+ lymphocytes. (E) CD8+ lymphocytes. (F) CD25+ lymphocytes. [a,b]Significant differences ($p<0.05$).

3.5. DISCUSSION

Although renal transplantation saves thousands of lives, transforming the quality of life of the transplanted patients, many patients die or should continue to receive replacement therapy for chronic renal failure because the supply of organs for transplantation does not cover the current needs [43,44]. Worldwide, it has been estimated that 2.6 million people received renal replacement therapies (RRT) in 2010, but only 73,179 kidney transplantations (44% from living donors) were performed according to WHO [35], and at least 2.2 million people might have died prematurely because RRT could not be accessed [36]. Therefore, it is important to implement new and affordable treatment options. Our group, in line with the Hammerman's work [138,155], has explored the transplant of kidney precursors as a possible alternative to adult grafts. But, as a team of urologists, we have taken a translational standpoint due to the existence of organ shortage. The first evidences relating to the origin of the vascular supply revealed that vascularization is derived from the host [208]. When the

rat metanephroi is transplanted into the peritoneal cavity of the mouse, most glomerular capillary loops originate from the host [145,155].

The transplanted metanephroi causes an immunological silence or a very attenuated immune response from the host [209–211]. That is probably due to the reduced expression of tissular antigens in embryonic kidneys compared to the developed ones (e.g. the major histocompatibility complex I and II) and the relative absence of dendritic cells in the transplanted metanephroi, contrary to what happens in mature kidneys [140]. Moreover, transplanted metanephroi obtain their vascularization from the host-receptor, which elicit a silent immune response or attenuated [138,212]. These intrinsic properties of embryonic organs have led to encouraging results across allogeneic barriers into immune-competent hosts, but also across concordant or highly disparate xenogeneic barriers with few immunosuppression [138,143]. Therefore, the use of metanephroi can be an effective alternative to the use of human renal allografts [172]. Several authors have shown that the life of anephric animals was significantly prolonged with the metanephroi transplantation and the subsequent anastomosis to the host's urinary tract [146,159]. Moreover, the increase of the renal mass through additional metanephroi in the same host increases even more the chances of survival [146]. On the other hand, it has been reported that the transplanted metanephroi significantly raises the plasma renin levels and maintains the average blood pressure in an anephric animal model with induced hypotension [156]. Other studies show that the xenotransplanted embryonic kidney provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue [163]. With a view to the future clinical application, the xeno-tissue component could be eliminated using metanephroi from transgenic donor that carries a regulated suicide gene, leaving autologous tissue and alleviating immunosuppression effects and ethical concerns [163–165].

Today, the life-sustaining ability of the metanephroi-developed renal structures are still too low to allow us to consider the translation of this technique to the clinical practice [140,155,159,172,173]. However, we must not forget that human renal allotransplantation required more than half a century to reach its full clinical application. As in that situation, we have to see now, hopefully, this line of research, which can provide an unlimited supply of kidneys. To date, transplantation surgery has been performed through open laparotomy mainly in rodent models experiences, probably due to the lack of an easy procedure to develop preclinical trials in higher species. In order to overcome this limitation, here we describe a feasible and minimally invasive laparoscopic approach to transplant metanephroi into the rabbit host omentum with high efficiency. This approach should encourage the design of proper preclinical studies that propel the metanpehroi transplantation to its full therapeutic potential.

3.6. CONCLUSION

As far as we know, we have described for the first time in the literature the allogeneic transplantation of E15 and E16 rabbit metanephroi through a minimally invasive laparoscopic approach. Our results show that after the metanphroi transplantation into non- immunosuppressed hosts, they grow and develop mature glomeruli without triggering any immune response.

ACKNOWLEDGMENTS

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VNIVERSITAT DE VALÈNCIA

DOCTORAL THESIS
Doctoral Programme in Medicine

DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

4. CHAPTER II

SILDENAFIL CITRATE ENHANCES RENAL ORGANOGENESIS FOLLOWING METANEPHROI ALLOTRANSPLANTATION INTO NON-IMMUNOSUPPRESSED HOSTS

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4.1. ABSTRACT

Generation of a functional kidney graft on demand would extend the option of kidney transplantation to more patients. In this sense, embryonic kidneys transplanted into adult non-immunosuppressed hosts are able to trigger the formation of a vascular system from the host, avoiding immune response and exhibiting functional renal properties. However, it is not easy to obtain a large organ that allows the connection surgery, precisely because, during its growth, its functionality makes it hydronephrotic and not viable. Sildenafil citrate (SC) is widely known as a potent inductor of the formation of new blood vessels (angiogenesis), offering renoprotective properties due to its anti-inflamatory, antifibrotic and antiapoptotic effects. Based on, the main objective of this study was to evaluate if the SC addition during metanephroi transplantation favours its integration into the host through its properly vascular connection, improving the development of the new organ. Then, 15 metanephroi were transplanted through laparoscopic procedure embedded in phosphate-buffered saline (untreated SC group), while 34 were equally transplanted with 10 µM (low dose) and 30 µM (high dose) SC solution. Twenty-one days after transplantation, 10/15, 9/17 and 8/17 new metanephroi-developed kidneys were recovered from 0, 10 and 30 µM of SC groups, respectively. Recovery rate was similar between all experimental groups, being observed satisfactory growth of new kidneys with histologically mature glomeruli and a comparable endocrine functionality with the host kidneys. Metanephroi treatment with low SC dose treatment allows to recover renal structures with higher weights than untreated ones. The histomorphometry analysis revealed that developed kidneys from SC groups exhibit dilated renal corpuscle, consistent with the hydronephrotic state. Histological mature glomeruli and hydronephrosis state prove the new kidneys' excretory function, while renin and erythropoietin gene expression reveals its endocrine functionality. In conclusion, we have described a procedure, based on the use of SC, that improved the vascular connexion and growth of the transplanted metanephroi. This study gives hope to a pathway that can lead to an unlimited supply of kidney transplants, which could put an end to the current long waiting lists.

4.2. INTRODUCTION

Currently, renal diseases affect epidemic numbers of people worldwide, and have continued to escalate in their prevalence globally in recent years [213]. Kidney organs are responsible for vital functions, including the excretion of metabolic wastes and toxins, the regulation of body fluids, and the endocrine control of the blood pressure and erythrocyte maturation. Hence, when renal degenerative processes end in an organ failure, organ transplantation becomes the ideal method to restoring full physiological organ function [2]. However, insufficient donor availability difficult this way, and

patients with end-stage renal disease (ESRD) are habitually forced to receive dialysis as a palliative treatment. In Spain, the world's leading country in organ donation for 28 consecutive years [40], only 3,423 kidney transplantation were performed in 2019, instead of the 7,356 that were necessary according to the waiting list [41]. In 2018, 40% of US patients listed for kidney transplant were still waiting since 2015, and 34,591 patients were removed from the list due to death or decline in medical condition [4,42]. Therefore, required organs do not arrive in time for all the patients, and 5%-10% of patients die on the waiting list every year [214]. In this precarious situation, emerging technologies in the field of regenerative medicine seek to address the limitations of current treatment strategies exploring new frontiers. The common idea is to generate kidney grafts on demand able to function as native kidneys, based on strategies ranging from stem cells therapy, blastocyst complementation, decellularization-recellularization, or 3D bioprinting [67,69]. But, the kidney is one of the most difficult organs for de novo formation due to its complex architecture and composition, containing numerous highly specialized and differentiated cell types [69,72]. Therefore, to date, cell therapies with individual cells are far from achieve fully functional transplantable renal graft. As a promising solution for these limitations, xenotransplantation of embryonic kidneys showed that if this intact renal primordia was transplanted into adult non-immunosuppressed hosts, it can mature as if they had not been extracted from the embryo, without triggering an immune response in the hosts [139,143]. These embryonic kidneys or metanephroi are able to attract the formation of a vascular system from the host, undergoing maturation and exhibiting excretory and endocrine functional properties [139,143,145,147,155–158,160,163]. Glomerular filtration in developing metanephroi was demonstrated firstly by Woolf et al. [154] and Abrahamson et al. [166] in the 90'. Today, metanephroi transplantation remains a promise to treat renal injury, as metanephroi has been successfully transplanted across concordant or highly disparate xenogeneic barriers [143,147,161,162]. Specifically, Dekel et al. transplanted human and porcine metanephroi into mice, obtaining kidney structures that produce urine [143]. These findings suggest that, if embryonic organs are retrieved from pathogen-free animals [67,215], this source could provide an unlimited and elective supply of organs for clinical transplantation.

But to date, the renal mass and clearances exhibited by the metanephroi-developed renal structures are still too low to sustain life in the long-term [140,155,159,172,173]. Due to the lack of a urine excretion channel, developing metanephroi become hydronephrotic and stops its growth. But, if nascent kidneys are connected to the host ureter via direct ureterouretostomy [159] or other novel strategies [158], they become enlarged and can extend the life of anephric hosts in the short-term [158,159]. However, allowing the new kidney to grow larger and sustain life in the long-term is a remaining obstacle to guarantee the feasibility of this strategy for clinical application. Interestingly, it has been demonstrated that both weight and clearances of the nascent kidneys can

be enhanced by compensatory renal growth following reduction of host native renal mass, which stimulus is accompanied by changes in the expressions of several growth factors [155]. Therefore, it is of especial importance to investigate whether growth factors could be used to enhance the growth and function of developing metanephroi. Using combinations of growth factors to pre-treat (prior transplantation) and post-treat metanephroi (during ureterouretostomy), Hammerman's group have achieved rates of clearance in transplanted metanephroi almost 300 times those measured without any treatment [155]. These values were approximately 6% of the clearance achieved by a normal kidney [144,172], which approximates a level of renal function that would be expected to preserve life [144].

Sildenafil citrate (SC) is a well-known drug used for treatment of pulmonary hypertension and male erectile dysfunction (Viagra™) due to its vasodilatory effect. Via inhibition of phosphodiesterase 5 enzyme (PDE-5), SC up-regulates cGMP, nitric oxide, and angiogenic systems, causing angiogenesis and renoprotective effects through anti-inflammatory, anti-oxidant, and anti-apoptotic mechanisms [175–178,216]. SC has demonstrated beneficial properties as a preconditioning or protective drug during kidney transplantation [217,218]. Besides, SC enhances the cartilage graft viability and survival, which is highly dependent on the vascularized host bed, oxygenation of local tissue, and the patient's current systemic status [178]. To some extent, the survival and development of the avascular metanephroi could depend on the same variables, being crucial its connection to the host vascular system. The aim of this work was to determine if the addition of SC during metanephroi transplantation enhances its integration into the host, improving its development.

4.3. MATHERIAL AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

4.3.1. Animals and ethical statements

New Zealand females (5 months old) were used as embryo donors and metanephroi recipients. These animals came from the experimental farm of the Universidad Politécnica de Valencia, an accredited animal care facility (code: ES462500001091). All animals were kept in an alternating cycle of 16 h of light and 8 h of dark, with free access to food and water, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively.

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the "Universitat Politècnica de València" ethical committee prior to initiation of the study (code: 2015/VSC/PEA/00170). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. An authorisation certificate issued by the Valencian governmental administration to experiment on animals is held by XGD (code: 2815), FMJ (code: 2273) and JSV (code: 0690).

4.3.2. Experimental design

Metanephroi were recovered from 15 days old embryos. Then metanephroi were placed in small drops containing one of the following SC concentrations: 0 µM (untreated group), 15 µM (low dose) and 30 µM (high dose). After that, metanephroi were transplanted by laparoscopy with 5 µL of each SC solution into non-immunosuppressed hosts. Total white blood cells and lymphocytes were estimated to identify any immunological response. After 21 days, the transplantation efficiency (recovery rate: developed kidneys recovered/metanephroi transplanted), degree of neovascularization, nascent kidneys growth (weight), and its endocrine function (mRNA) were assessed to elucidate the effect of the SC addition during metanephroi transplantation. Kidneys from 5 weeks old rabbit kits (coeval with metanephroi age) were used as a control for growth study, meanwhile host kidneys (which are under the same physiological environment that nascent kidneys) were used as control for endocrine analysis. The experimental design was summarized in Figure 13.

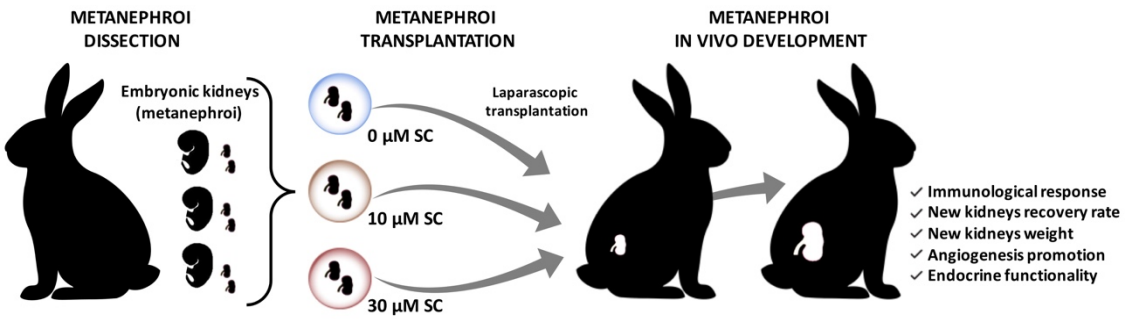

Figure 13. Experimental design. Metanephroi were recovered, placed in raising concentrations of sildenafil citrate (SC), and laparoscopically transplanted into non-immunosuppressed hosts. The immunological response, recovery rate (developed kidneys recovered/metanephroi transplanted), angiogenesis, and the new kidneys weight and endocrine function were assessed.

4.3.3. Metanephroi recovery and transplantation

Metanephroi were carefully dissected from 15-day-old (E15) rabbit embryos under a dissecting microscope. One embryo was fixed directly for histological examination. Just before its allotransplantation, metanephroi were deposited in a 5 µL droplets of phosphate-buffered saline (PBS) solution containing SC concentrations of 0 µM (untreated group), 10 µM or 30 µM. All manipulations were performed at room temperature (25 ± 1 °C). The allotransplantation was performed using a minimally invasive laparoscopic technique, as described previously [219], within 45 min after metanephroi were retrieved. Briefly, animals were placed on an operating table in a vertical position (head down at 45-degree angle). Only one endoscope trocar was inserted into the abdominal cavity. Then, an epidural needle was inserted into the inguinal region. After a renal vessel was identified in the retroperitoneal fat, a hole (as a pouch) was performed adjacent to the vessel. Then, each metanephros was aspirated with 5µL of each SC solutions (0, 10 or 30 µM) in an epidural catheter (Vygon corporate, Paterna, Valencia, Spain), introduced into the inguinal region through an epidural needle, and deposited (transplanted) into the pouch previously created. Between 2 to 4 metanephroi were transplanted in each host (one metanephros per hole). No immunosuppression was given to recipients. Metanephroi transplantation was assessed in three sessions. Anaesthesia, analgesia and the postoperative care were performed as we previously described for laparoscopic procedures [168].

4.3.4. Determination of peripheral white blood cells

Before the transplantation (day 0), one blood sample of each recipient (n=7) was collected from the central ear artery and dispensed into an EDTA-coated tube (Deltalab S.L., Barcelona, Spain). Then, basal levels of total white blood cells and lymphocytes were estimated at most 10 minutes after blood collection using an automated veterinary haematology analyser (MS 4e automated cell counter, MeletSchloesing Laboratories, France) and according to the manufacturer's instructions. After metanephroi transplantation, two blood extractions were analysed weekly along the experiment to detect significant variations of the total white blood cells and lymphocytes populations.

4.3.5. Metanephroi development and histomorphometry of the renal corpuscle

All animals were euthanized 3 weeks after transplantation, retrieving all the new kidneys developed to annotate the recovery rate (recovered kidneys/transplanted metanephroi). Then, renal structures were weighted, fixed in formaldehyde solution

and embedded in paraffin wax for histological analysis. Samples for histology were cut into 5-µm sections and stained with haematoxylin and eosin. The stained sections were observed with light microscopy for histological and histomorphometric examination. To measure histomorphometric parameters, a minimum of 25 renal corpuscles and glomeruli were evaluated (area and perimeter) for each experimental group. Photomicrographs were taken at a total magnification of x400. Measurements were determined using ImageJ software (public domain http://rsb.info.nih.gov/ij/). In addition, the glomerular tuft cellularity was estimated by counting the total number of nuclei of each glomerulus. Kidneys originating from a 5-week-old rabbit (coeval with the metanephroi age) were used as controls.

4.3.6. Renin and erythropoietin mRNA gene expression

After the euthanasia was performed, developed metanephroi samples were obtained by retrieving biopsies randomly from different sites. Immediately, samples were washed with phosphate buffered saline (PBS) to remove blood remnants, and stored in RNA-later (Ambion Inc., Huntingdon, UK) at -20 ºC until the analysis. Five samples were analysed in each experimental group (control, 0 µM, 10 µM and 30 µM of SC). Hosts kidneys under the same physiological environment that nascent kidneys were used as controls. RNA was extracted with Dynabeads kit (Invitrogen Life Technology) according to the manufacturer's instructions and treated with DNase I to eliminate genomic DNA contamination. Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen). Quantitative real-time PCR (qRT-PCR) reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every qRT-PCR was performed from 5 µL of diluted 1:40 cDNA template, 250 nM of forward and reverse primers (Table 3) and 10 µL of PowerSYBR Green PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 µL.

Table 3. *Information about primers used for qRT-PCR.*

Gene Symbol	Forward Primer	Reverse Primer
REN	GGGACTCCTGCTGGTACTCT	CTGAGGGCATTTTCTTGAGG
EPO	ACGTGGACAAGGCTGTCAGT	TGGAGTAGATGCGGAAAAGC
GAPDH:	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT

REN: Renin; EPO: Erythropoietin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (60s). After qRT-PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were

confirmed by SYBR Green-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. A ΔΔCt method adjusted for PCR efficiency was used [220], employing the geometric average of GAPDH as housekeeping normalization factor [221]. Relative expression of cDNA pool from various samples was used as the calibrator.

4.3.7. Statistical analysis

Differences in the recovery rate between groups were assessed using a probit link model with binomial error distribution, according to a mixed model including the experimental group as a fixed effect. Variations in the pheripheral blood values were evaluated using a general linear model including the day post-transplant as a fixed factor. The new kidney weight, hitomorphometric measures (area and perimeter) and the glomerular tuft cellularity were compared using a general linear model including the experimental group as a fixed effect and replicate as a random factor. The replicate was non-significant and was removed from the model. Data of relative mRNA abundance were normalized and analysed using a general linear model as previously. All statistical analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, USA). Significance was attributed to analyses where p is less than 0.05. Data were reported as least-square means ± standard error of the mean.

4.4. RESULTS

Three rabbit females were used as embryo donors, generating a total of 28 embryos. Metanephroi were carefully micro-dissected and transplanted into 7 adult non-immunosuppressed hosts. A total of 49 whole metanephroi were allotransplanted: 15 embedded in a 0 µM SC solution (untreated group), 17 embedded in a 10 µM SC solution (low dose), and 17 embedded in a 30 µM SC solution (high dose). No immunological response was observed after metanephroi transplantation, as total white blood cells and total lymphocytes values remains similar to the basal levels (day 0) along the experiment (Figure 14). Twenty-one days after transplantation, we observe that transplanted metanephroi grew and promoted angiogenesis (Figure 15). Metanephroi, treated with SC exhibited deeper vascular integration with the host than untreated ones, as more pronounced vascular network was developed both in the low and high dose group (Figure 15). However, a similar recovery rate was observed for all the groups: 10/15 (67%), 9/17 (53%) and 8/17 (47%) for metanephroi transplanted embedded in 0 µM, 10 µM and 30 µM of SC, respectively.

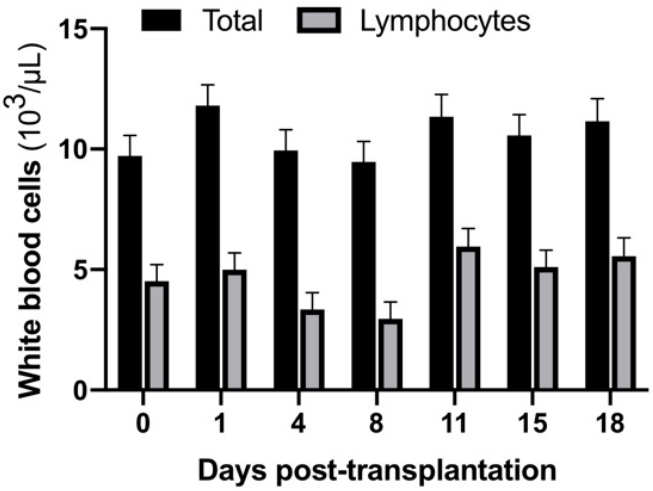

Figure 14. Effect of metanephros transplantation in peripheral blood levels of total white blood cells and total lymphocytes, comparing basal values before transplantation (day 0) and that post-transplantation along the experiment (day 1, 4, 8, 11, 15, 18).

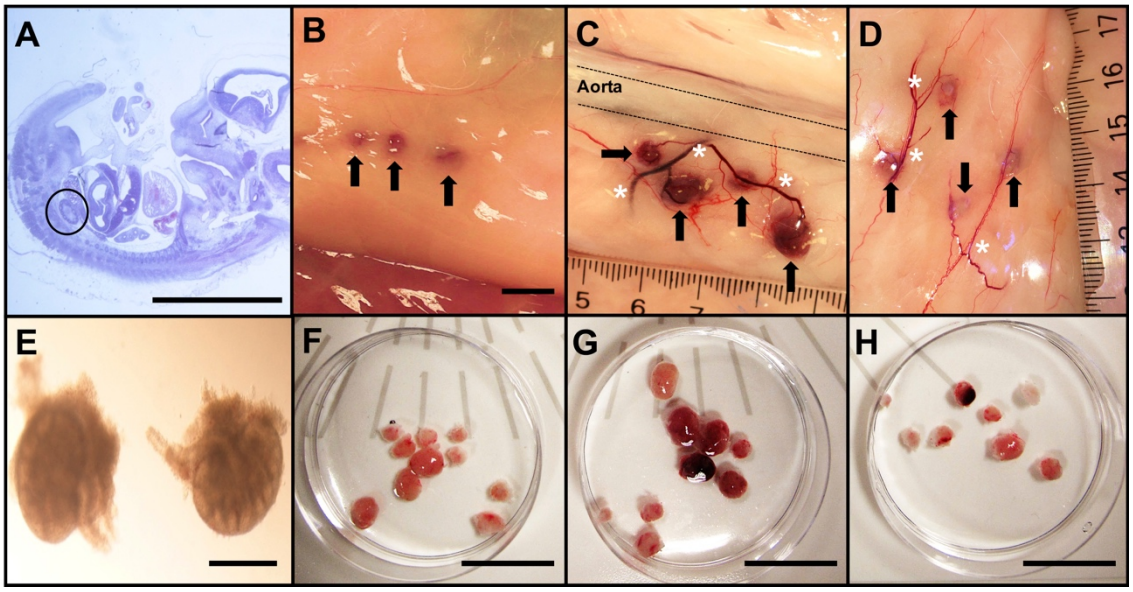

Figure 15. Development of metanephroi after its allotransplantation with or without sildenafil citrate. (A) Histology of 15-days-old rabbit embryo. Circle indicates metanephroi position. Scale bar: 0.4 cm. (B) Nascent kidney from metanephroi without sildenafil citrate (SC) treatment. Scale bar: 1 cm. (C) Developing kidney from metanephroi treated with 10 µM SC solution. Asterisk indicates neoangiogenesis. Arrows indicates the growing metanephroi. Scale in cm. (D) Developing kidney from metanephroi treated with 30 µM SC solution. Asterisk indicates neoangiogenesis. Arrows indicates the growing metanephroi. Scale in cm. (E) Fifteen-days-old metanephroi. Scale bar: 0.5 mm. (F) New kidneys recovered from transplanted metanephroi without SC treatment. Scale bar: 2 cm. (G) New kidneys recovered from metanephroi trasplanted with 10 µM SC solution. Scale bar: 2 cm. (H) New kidneys recovered from metanephroi trasplanted with 30 µM SC solution. Scale bar: 2 cm.

Significant increase in developing kidney weight was observed after low SC dose treatment (0.13±0.021 g), but not for the high dose (0.07±0.025 g) compared with the untreated metanephroi (0.08±0.020 g). All nascent kidneys weight was lower than control samples (0.74±0.028 g), independently of the experimental group ($p < 0.05$). All nascent kidneys become hydronephrotic, demonstrating its excretory function. Concordantly, in all the groups metanephroi underwent differentiation and developed new kidney graft explants with histologically mature glomeruli (Figure 16), whose histomorphometric analysis was shown in Table 4.

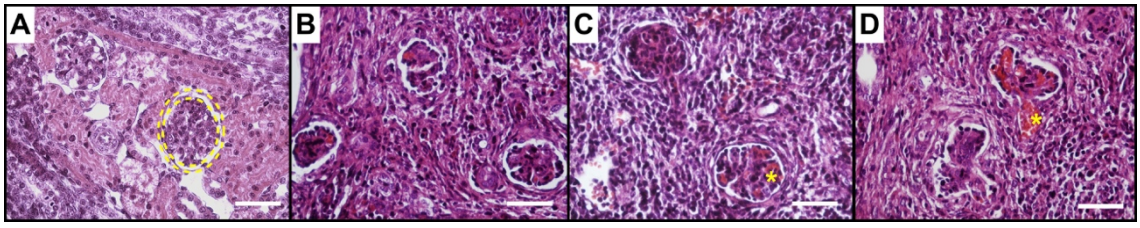

Figure 16. Histology of developing kidneys with or without sildenafil citrate. (A) Micrograph showing glomeruli of the control kidney originating from a 5-week-old rabbit. External circle surrounds the renal corpuscle. Internal circle surrounds the glomerulus. Space between both circles indicates the Bowman's space. (B-D) Micrograph showing glomeruli of new kidney after metanephroi transplantation: (B) without sildenafil citrate (SC) treatment, (C) with a 10 µM SC solution, and (D) with a 30 µM SC solution. Asterisk indicates blood flow into the glomerulus.

Table 4. *Histomorphometric quantification of renal corpuscle of kidneys developed after allotransplantation of metanephroi.*

Group	n	Renal corpuscle		Glomerulus		
		Area (μm^2)	Perimeter (μm)	Area (μm^2)	Perimeter (μm)	Cell number
0 µM SC	10	3034.6 ± 176.44^b	201.1 ± 6.06^b	2132.5 ± 142.56^b	170.9 ± 5.70^b	41.0 ± 2.22^b
10 µM SC	9	3639.7 ± 179.94^a	218.5 ± 6.18^a	2749.5 ± 145.39^a	192.0 ± 5.81^a	49.9 ± 2.26^a
30 µM SC	8	3582.44 ± 187.59^a	218.3 ± 6.45^a	2655.7 ± 151.85^a	190.2 ± 6.06^a	48.3 ± 2.36^a
Control	6	2633.4 ± 92.31^c	184.2 ± 3.17^c	2104.7 ± 74.58^b	165.3 ± 2.98^b	52.7 ± 1.16^a

n: Number of new kidneys or control kidneys. Data are expressed as least-square means ± standard error of the mean. a,b,c: Data in the same column with uncommon letters are different ($p < 0.05$).

Histomorphometric data showed that all the metanephroi-developed kidneys exhibited higher renal corpuscle values (area and perimeter) than control samples, demonstrating the hydronephrotic state of the former and its filtering activity. Moreover, both renal corpuscle and glomerulus measurements were increased in the SC developed metanephroi compared to the untreated ones, indicating that SC increased both

capillary dilatation and glomerular filtration. Tuft cell density in the SC developed metanephroi were also higher than in the untreated ones and similar to the control samples, showing a trophic effect of the SC on the glomeruli development. Finally, renin and erythropoietin gene expression was similar between control samples (host's kidney) and metanephroi-developed kidneys, regardless of the experimental group (Figure 17).

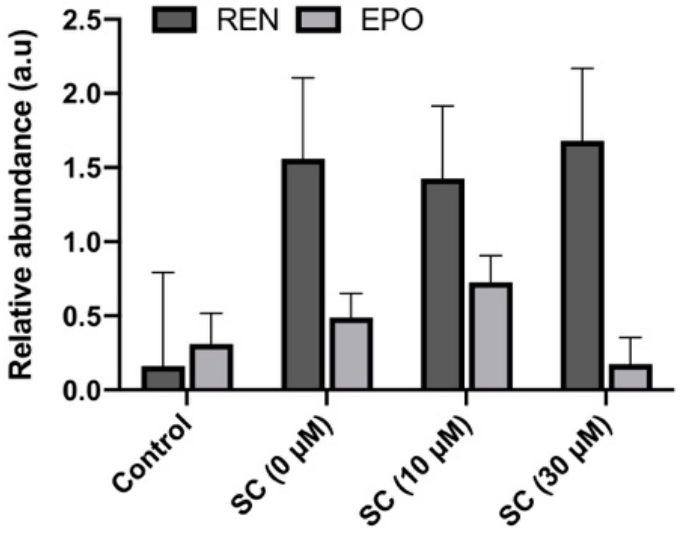

Figure 17. Gene expression of the renin (REN) and erythropoietin (EPO) between control samples (host kidneys) and developing kidneys from metanephroi treated with sildenafil citrate (SC) at 0 µM (untreated group), 10 µM (low dose) and 0 µM (high dose).

4.5. DISCUSSION

To our best knowledge, here we describe for the first time the valuable use of SC during metanephroi transplantation to enhance the angiogenesis and growth of the nascent kidneys. Moreover, our findings revealed that SC also exert a positive effect on the glomerular development and function, without affecting the endocrine function of the developing metanephroi. Besides, no immunological response was observed after metanephroi transplantation, regardless of the treatment received. Altogether, our results represent firm evidence of the SC trophic effects for the metanephroi development. Looking for the strategies that allow to obtain life-sustaining renal structures, the use of SC becomes a potential factor towards the clinical translation of the metanephroi transplantation therapy.

Severely damaged kidneys possess a limited regenerative potential and therapeutic interventions are not sufficient to restore renal function in patients with ESRD, turning transplantation the ideal method to restore full physiological functions [69,222]. Given

the graft shortage, either from living or deceased donors, well recognized for the WHO [12], some regenerative and bioengineering strategies are trying to generate kidney grafts on demand [67–69]. Metanephroi transplantation remains one of the most promising approaches, but obtaining larger and life-sustaining renal structures after its transplantation remains an obstacle for its therapeutic potential [140,155,159,172,173]. In this sense, we evaluate the SC effect during metanephroi transplantation on their *in vivo* development. Being previously proposed as a useful agent in instances where neo-vascularization is desired [223], SC treatments during metanephroi transplantation stimulated angiogenic responses. As a result, SC-treated developing metanephroi exhibited deeper vascular irrigation than untreated ones. Blood impregnation in the nascent kidneys can be observed both macroscopically and microscopically (histology). Previously, Rostaing et al. demonstrated that SC exerts a dilatation of glomerular afferent arterioles, promoting an increase in the filtration process [224]. Likewise, our histomorphometric data showed that SC increases the area and perimeter of the glomerulus, but also of the renal corpuscle. It means that if SC promotes the glomerular filtration in nascent kidneys, they must accumulate more filtrate in the bowman's space due to the lack of a urine-excretion channel (hydronephrosis). Taking into account that SC act as a potent inductor of VEGF release [223,225,226], our results support those of the Hammerman's group, who demonstrated that inulin clearances and urine volumes were increased significantly in VEGF-treated metanephroi [172]. However, although VEGF-treatment did not affect the weights of transplanted metanephroi [172], SC-treatment allows us to obtain larger renal structures. In agreement, it has been proven that low SC doses resulted in a more angiogenic responses than that produced by a saturating VEGF concentration [223]. Moreover, SC-treatments exert renoprotective effects [175–178,216] that could retard hydronephrosis-related damages, allowing metanephroi growth for longer. However, this overgrowth appeared to be dependent on the SC-dose used, occurring at low doses but not at high doses. Maybe, high SC doses could accelerate glomerular filtration, exacerbating a hydronephrotic state that arrests the metanephroi growth earlier. Comparative results were observed by Yardimci et al. [227], which allow us to speculate that metanephroi development could be better at high doses before urine production was started. In this particular case, it seems that low doses (10 µM) of SC are the optimal treatment to promote the vascularization of developing kidneys and to recover larger renal structures with higher degree of glomerular development and function. Moreover, developed metanephroi not only showed glomerular filtration activity, but also endocrine functions, which are consistent with the previous literature [143,155–158,163,172]. The present study showed that renin and erythropoietin gene expression levels were comparable between developed metanephroi and host kidneys, regardless of the experimental group. Interestingly, as reported before, harvesting metanephroi at the optimal age avoids the immunological response from hosts [138,219].

Compared to coeval native organs, developed metanephroi reach a diminished renal mass that could incur potential life-sustaining limitations. However, it has been demonstrated that the survival time of anephric recipients transplanted with metanephroi is proportional to the renal mass developed [146]. This concept is similar to that used in kidney transplantation from paediatric donors, in which both kidneys are transplanted in bloc into adult recipients to guarantee acceptable glomerular filtration rates [228,229]. This strategy should be combined with those designed to avoid hydronephrosis [158], or other developed in our group, such as the minimally invasive laparoscopic transplantation procedure [137,219], effective banking protocols [230–232] and now the use of SC. All these strategies in conjunction could constitute a path increasingly consolidated by which metanephroi xenotransplantation could provide transplantable renal grafts for the treatment of patients with ESRD.

4.6. CONCLUSION

Here, we have described a procedure based on the use of SC during the metanephroi transplantation that improves the neovascularization, growth and glomerular filtration of the nascent kidneys. This treatment enhances the glomerular developmental degree without compromising neither the endocrine activity nor the immunological silence of the new renal structures. This SC-based strategy gives hope to a way that can lead to an unlimited supply of renal grafts and end to the current long waiting lists.

ACKNOWLEDGMENTS

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VNIVERSITAT DE VALÈNCIA

DOCTORAL THESIS
Doctoral Programme in Medicine

DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

5. CHAPTER III

VITRIFICATION OF KIDNEY PRECURSORS AS A NEW SOURCE FOR ORGAN TRANSPLANTATION

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5.1. ABSTRACT

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand, and by the risk of allograft loss rejection and immunosuppressive therapy toxicity. In recent years, xenotransplantation of kidney precursor cells has offered a novel solution for the limited supply of human organs. Specifically, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties, and avoiding humoral rejection from non-immunosuppressed hosts. Even if supply and demand could be balanced using xenotransplants or lab-grown organs from regenerative medicine, the future of these treatments would still be compromised without the ability to physically distribute the organs to patients in need and to produce these products in a way that allows adequate inventory control and quality assurance. Therefore, long-term cryopreservation is required. Kidney precursors originating from 15-day-old (E15) rabbit embryos were vitrified using Cryotop® as a loading device and VM3 as vitrification solution. After 3 months of storage in liquid nitrogen, 18 kidney precursors were transplanted into non-immunosuppressed adult hosts by laparoscopy surgery. Twenty-one days after allotransplantation, 9 new kidneys were recovered. All the new kidneys recovered exhibited significant growth and mature glomeruli. Having achieved these encouraging results, we report, for the first time, that it is possible to create a long-term biobank of kidney precursors, understood as an unlimited source of organs for transplantation, facilitating the inventory control and distribution of organs.

5.2. INTRODUCTION

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand, and by the risks of allograft loss rejection and immunosuppressive therapy toxicity [3]. In recent years, xenotransplantation of kidney precursor cells has provided a novel solution for the limited supply of human donor organs [139,143]. Specifically, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties, and avoiding rejection from non-immunosuppressed hosts [138,139,212]. However, even if supply and demand could be balanced using xenotransplants or lab-grown organs from regenerative medicine, the future of these treatments would still be compromised by the inability to physically distribute the organs to patients in need and produce these products in a way that allows adequate inventory control and quality assurance [10,201]. To this end, organ cryopreservation will be indispensable.

The long-term banking of human organs or their engineered substitutes for subsequent transplantation is a long-sought and important goal [9–11,187,196,233–236]. To date, small ovaries, blood vessels, heart valves, corneas and similar structures are the only macroscopic structures having the capacity to recover, at least in part, after vitrification [192,196]. Kidneys and hearts have been the most widely studied organs, but neither has been reproducibly recovered after cooling to temperatures lower than -45ºC, being mechanical damage from ice of the main causes [192,196]. Fahy et al. [192] reported a case history of one rabbit kidney that survived after vitrification and supported the life of a recipient animal for an indefinite period of time. To our best knowledge, only Bottomley et al. [201] evaluated the cryopreservation of metanephroi immediately after thawing, but only under *in vitro* conditions.

In an effort to advance in organ cryopreservation, this study was conducted to evaluate whether vitrified kidney precursors can develop morphologically normal glomeruli after their warming and allotransplantation in non-immunosuppressed rabbits.

5.3. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

5.3.1. Animals and ethical statements

New Zealand females (5 months old) were used as embryo donors and metanephroi recipients. These animals came from the experimental farm of the Universidad Politécnica de Valencia, an accredited animal care facility (code: ES462500001091). All animals were kept in an alternating cycle of 16 h of light and 8 h of dark, with free access to food and water, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively.

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the “Universitat Politècnica de València” ethical committee prior to initiation of the study (code: 2015/VSC/PEA/00170). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. An authorisation certificate issued by the Valencian governmental administration to experiment on animals is held by XGD (code: 2815), FMJ (code: 2273) and JSV (code: 0690).

5.3.2. Metanephroi recovery

Donor does were artificially inseminated with 0.5 mL of fresh heterospermic pool semen from fertile males at a rate of $40x10^6$ spermatozoa/mL in Tris-citric-glucose extender [237]. Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg buserelin acetate and the females were euthanised at day 15 post-insemination. Recovered 15 day old embryos (E15) were placed in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2% of bovine serum albumin (BSA) at 38.5ºC. Metanephroi were surgically dissected under a dissecting microscope using previously described techniques [219]. Some of the recovered embryos were placed in Bouin's solution to fix. They were then dehydrated through ethanol series, cleaned with xylol and embedded in paraffin. Next, 5-7µm sections were cut for hematoxylin-eosin staining and the slides were studied by light microscopy in order to identify the position and the size of the metanephroi (Figure 18).

5.3.3. Vitrification procedure

Vitrification was performed within 1 h after recovery following the minimum essential volume method, using Cryotop® as device [238] (Kitazato-dibimed, Valencia, Spain) and VM3 as vitrification solution [192,196] (21st Century Medicine, Fontana, CA, EEUU). Cryotop® is a special container, consisting of a fine thin film strip attached to a hard handle. This allows us to minimise the volume of vitrification easily. All manipulations were performed at room temperature (25 ± 1 °C) and all the media were used at room temperature, except for the first warming solution, which was used at 37.5 °C.

Metanephroi were first submerged into 2.5 ml of equilibration solution that containing 1.7% w/v ethylene glycol (EG), 1.3% w/v formamide, 2.2% w/v dimethyl sulphoxide (DMSO), 0.7% w/v PVP K12 (polyvinylpyrrolidone of Mr 5000 Da) and 0.1% w/v final concentrations of commercially available SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium (BM: DPBS + 20% foetal bovine serum, FBS) for 3 min. Then, the metanephroi were submerged into 2.5 ml of solution containing 4.7% w/v EG, 3.6% w/v formamide, 6.2% w/v DMSO, 1.9% w/v PVP K12 and 0.3% w/v final concentrations of ice blockers in BM for 1 min. Finally, the metanephroi were submerged into 2.5 ml of vitrification solution consisting of 16.84% w/v EG, 12.86% w/v formamide, 22.3% w/v DMSO, 7% w/v PVP K12 and 1% w/v final concentrations of ice blockers in BM before being loaded into Cryotop® devices (Figure 18) and directly plunged into liquid nitrogen (LN) within 1 min. For warming, metanephroi were submerged into 2.5 ml of a solution containing 1.25 M sucrose in BM for 1 min and later transferred stepwise into decreasing sucrose solutions (0.6, 0.3 and 0.15 M sucrose in BM) for 30 s before and then washed twice in BM for 5 min.

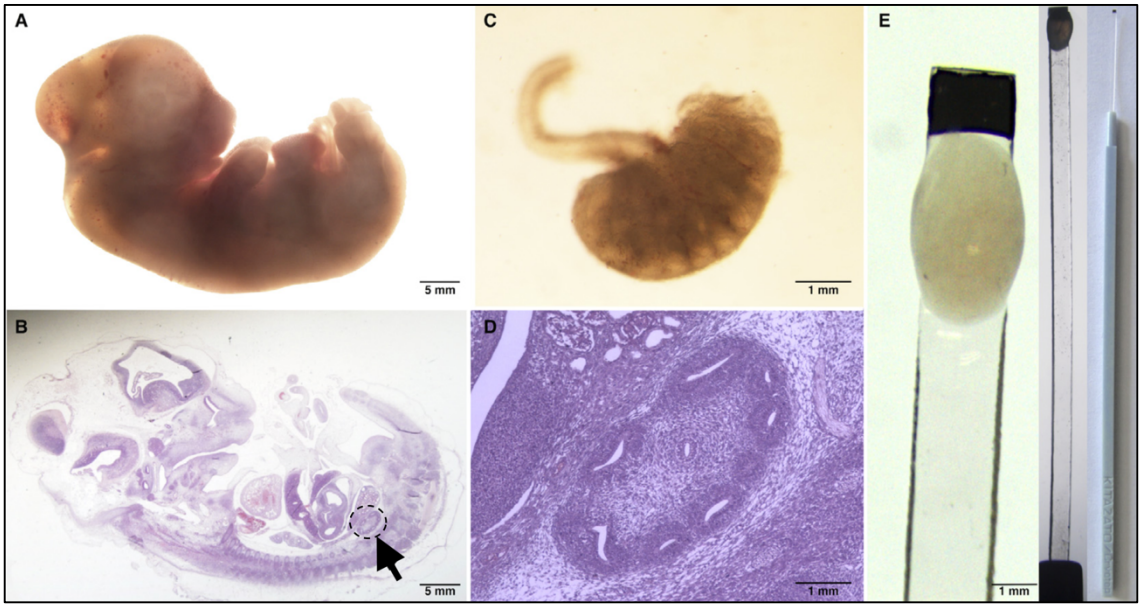

Figure 18. Histology of 15-day-old rabbit embryo and recovered metanephroi for vitrification. (A) 15-day-old embryo. (B) Micrographs (H&E) showing 15-day-old embryo. Black arrow indicates metanephroi allocation. (C) Detail of 15-day-old metanephros. (D) Micrographs (H&E) showing 15-day-old metanephros. (E) Detail of 15-day-old metanephros loaded in a Cryotop® device. Detail of metanephros loaded into the film strip of the Cryotop®.

5.3.4. Metanephroi transplantation surgery

After three months of storage in liquid nitrogen, the metanephroi were transplanted into recipients. Metanephroi were transplanted within 45 minutes after warming or collected (fresh). Recipients were sedated by intramuscular injection of 5 mg/kg of xylazine (Rompun, Bayer AG, Leverkusen, Germany) and anaesthetised by intravenous injection of 15 mg/kg ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France) into the marginal ear vein. During laparoscopy, 3 mg/kg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. Abdominal laparoscopy was performed as previously described [219]. Briefly, metanephroi were aspirated in an epidural catheter (Vygon corporate, Paterna, Valencia, Spain), introduced into the inguinal region with an epidural needle and then transplanted into a pouch created by the epidural needle in the retroperitoneal fat, adjacent to the renal vessels. Four metanephroi were transplanted into each host (one per pouch). After surgery, analgesia was administered for 3 days: 0.03 mg/kg of buprenorphine hydrochloride (Buprex®, Esteve, Barcelona, Spain) each 12 hours, and 0.2 mg/kg of meloxicam (Metacam®, 5 mg/mL; Norvet; Barcelona, Spain) every 24 h. In addition, all the recipients were treated with antibiotics: 4 mg/kg of gentamicin (Ganadexil®, Invesa, Barcelona, Spain) every 24 h for 3 days. No immunosuppression was given to recipients. Metanephroi transplantation was assessed in three sessions.

5.3.5. Metanephroi development and histomorphometry of the renal corpuscle

Twenty-one days after transplantation, hosts having received an allograft were euthanized and the new kidneys were retrieved. The new kidneys were individually weighed, fixed in formaldehyde solution, embedded in paraffin wax and stained as described above. The stained sections were examined with light microscopy for histological and histomorphometric analysis. In the histomorphometric measurements, 20 renal corpuscle and glomeruli on each sample were measured (area and perimeter) in each of the groups (control and experimental). Photomicrographs were taken with a total magnification of X1000. In addition, the glomerular tuft cellularity was estimated by counting the total number of nuclei of each glomerulus. Photomicrographs were measured using ImageJ analysis software (http://rsb.info.nih.gov/ij/). Kidneys originating from a 5-week-old rabbit (coeval with the metanephroi age) were used as controls.

5.3.6. Statistical analysis

The recovery rates after transplantation were analysed using the chi-square test. The weights of kidney precursors, renal corpuscle and glomeruli measurements (area and perimeter) and the glomerular tuft cellularity were compared using a general linear model, using the sample type (fresh and vitrified) as a fixed factor and replicate as random factor. The replicate was non-significant and was removed from the model. Significance was attributed to analyses where P is less than 0.05. All statistical analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, USA). Data were expressed as means ± standard error of means.

5.4. RESULTS

Two females were used as embryo donors for the recovery of metanephroi. A total of 3 recovered embryos were fixed for histological examination (Figure 18) and 17 were surgically dissected. After obtaining the metanephroi, 16 were transplanted directly (fresh group) and 18 after vitrification procedure (vitrified group) into 9 recipients. Twenty-one days after transplant, all the new kidneys recovered exhibited significant growth (Figure 19). In total, 9 metanephroi (50%) were successfully grown after vitrification. Similar rates were reached from fresh kidney precursors, as 7 metanephroi were obtained (43.7%). Transplanted kidney precursors, 3 weeks post-transplant, weighed 0.25±0.04 g and 0.37±0.05 g for vitrified and fresh kidney precursors, respectively, which was significantly less than the kidneys of control animals (0.78±0.07

g, P<0.001). Nevertheless, in all of them, new kidneys developed mature glomeruli (Figure 19 and Figure 20). The histomorphometry results as displayed in Table 5 show the significant increase in the renal corpuscle area and perimeter ($p < 0.05$) of the fresh and vitrified new kidneys when compared to the control group. Glomerular area showed a significant increase in vitrified group when compared with the control group ($p < 0.05$). Vitrification has no significant effect on glomerular perimeter, when compared to the corresponding values in the control. Nevertheless, in all kidney graft explants, there was a significant reduction in glomerular tuft cellularity when compared with the control group ($p < 0.05$).

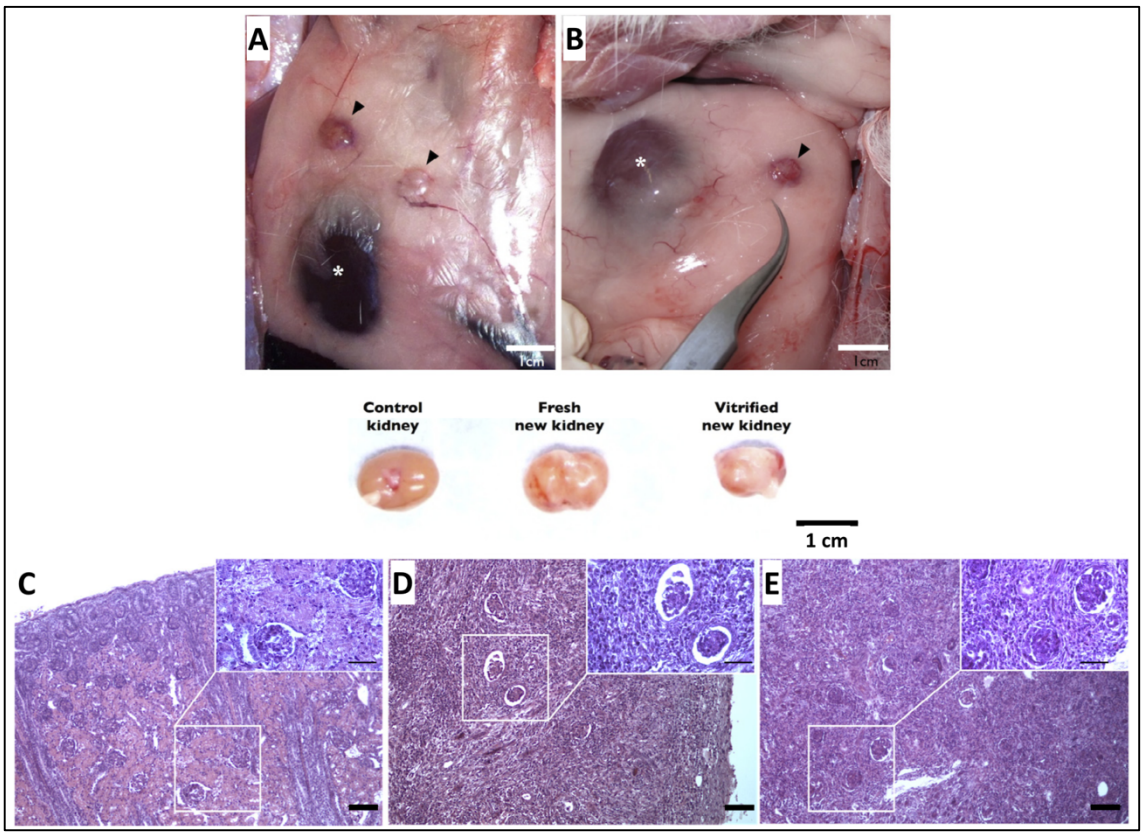

Figure 19. Successful development of new kidneys after allotransplantation of fresh and vitrified kidney precursors. (A) Macroscopic view of a fresh kidney precursor 3 weeks after transplantation. Black arrowheads indicate the new kidneys. Note massive growth and the blood vessels of a new kidney. White asterisk indicates the host kidney. Black arrowheads indicate the new kidneys. (B) Macroscopic view of a vitrified kidney precursor 3 weeks after transplantation. Black arrowhead indicates the new kidney and white asterisk indicates the host kidney. (C) Micrographs (H&E) showing glomeruli of the control kidney originating from a 5-week-old rabbit (coeval with the metanephroi age). (D) Micrograph (H&E) showing glomeruli of new kidney after allotransplant fresh kidney precursor. (E) Micrograph (H&E) showing glomeruli of new kidney after allotransplant vitrified kidney precursor. Scale bar: 1 cm (A and B); 0.1 mm (C, D and E).

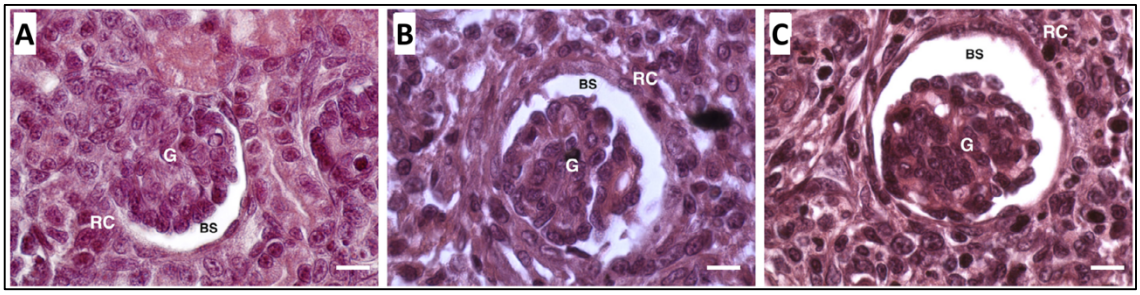

Figure 20. Representative photomicrograph of the renal corpuscles (H&E). G; Glomerulus. RC; Renal corpuscle. BS; Bowman´s space. (A) Renal corpuscle of the control kidney originating from a 5-week-old rabbit (coeval with the metanephroi age). (B) Renal corpuscle of a fresh kidney precursor 3 weeks after transplantation. (C) Renal corpuscle of vitrified kidney precursor 3 weeks after transplantation. Scale bar: 0.01 mm

Table 5. *Histomorphometric measurements of the renal corpuscles of kidneys developed after allotransplantation of vitrified and fresh metanephroi.*

Group	n	Renal corpuscle		Glomerulus		
		Area (μm^2)	**Perimeter** (μm)	**Area** (μm^2)	**Perimeter** (μm)	**Cell number**
Fresh	7	2985.9 ±109.51^{b}	196.9 ±3.81^{b}	2019.1 ±78.82^{ab}	169.5 ±3.48	39.9 ±1.47^{c}
Vitrified	8	3487.5 ±87.70^{a}	212.4 ±3.12^{a}	2388.7 ±65.13^{a}	177.5 ±2.88	46.7 ±1.21^{b}
Control	5	2778.2 ±105.60^{b}	188.5 ±3.75^{b}	2195.9 ±81.33^{b}	169.1 ±3.47	53.8 ±1.47^{a}

n: Number of new kidneys. Data are expressed as mean ± SD. a,b,c: Data in the same column with uncommon letters are different ($p < 0.05$).

5.5. DISCUSSION

This is the first study reporting that metanephroi survive after vitrification, underwent differentiation and growth, became vascularised by blood vessels of host origin and developed morphologically normal glomeruli. Only one previous study had examined metanephroi cryopreservation *in vitro*, suggesting that vitrification yielded more promising results, consistent with our findings [201]. The differences in methodology and evaluation methods (e.g. *in vitro* and *in vivo*) between Bottomley et al. [201] and our study make difficult any comparison. Vitrification, in which the liquids in a living system are converted into the glassy state at low temperatures, provides a potential alternative to freezing that can in principle avoid ice formation [9,196]. Specifically, tissues and organs are severely damaged by extracellular ice [9,187]. To date, small ovaries, blood vessels, heart valves, corneas and similar structures are the only

macroscopic structures with the capacity to recover, at least in part, after vitrification [9,192]. Presumably, the cause for our success was likely the combination of the vitrification device and the vitrification solution. Since its first reported application for embryo cryopreservation [189], the greatest improvement in the vitrification technology has been achieved by the use of newer vitrification containers aimed to minimise the volume of vitrification solution, which increase the speed of cooling and warming and the probability of vitrification success [191,235]. Moreover, we used a low-toxic vitrification solution well-suited for kidney cryopreservation [196,202]. In addition, as E15 rabbit kidney precursors are measured in a few millimetres, the adequate diffusion and equilibration of cryoprotective agents within the organ cells is not a hurdle and vitrification become feasible [201]. Furthermore, the metanephroi undergoing vitrification, warming and transplantation developed a blood supply originating from the host vasculature, not requiring immediate vascular anastomosis upon transplantation.

Our results on renal corpuscle histomorphometry further support earlier findings demonstrating that transplanted metanephroi have the ability to develop apparently normal glomeruli [136,138,144,155,162,172,212,239]. In this paper, we provide quantitative morphometric data that support these previous observations, but using vitrified metanephroi stored for 3 months. Although differences were observed in renal corpuscle area and perimeter between vitrified and control group, this can be explained by the fact that the metanephroi were not connected to the host's urinary system. Under this condition, unconnected metanephroi become hydronephrotic [156,158]. Furthermore, new kidneys originating from vitrified metanephroi exhibited similar renal glomerular morphometry, but with slightly less glomerular tuft cellularity than control. It may not be abnormal for a new kidney compared to kidneys from 5-weeks old animals, because the transplanted metanephroi have 30% of the organ mass compared to control. Moreover, such a small difference could be irrelevant or even normal after the developmental mismatch that takes place after the metanephro extraction of the embryo and its subsequent transplantation into an adult organism. Different authors have already shown that transplants of fresh kidney precursors are able to filter blood and produce urine [143,144,155,158,172], prolonging the life of anephric hosts [146,159]. In further work, we shall explore whether transplantation of vitrified embryonic kidneys may become a viable approach to renal replacement therapy, evaluating the haemodynamic capacity of transplanted vitrified metanephroi.

5.6. CONCLUSION

Having achieved these encouraging results, we suggest for the first time that it may be possible to create a long-term biobank of kidney precursors as an unlimited source of organs for transplantation, facilitating most of the problems of matching organs to recipients to reduce rejection, transporting the organs to where they need to go, and scheduling surgery at a time and a place that is best for both the patient and the transplant surgeon.

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VNIVERSITAT DE VALÈNCIA

DOCTORAL THESIS
Doctoral Programme in Medicine

DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

6. CHAPTER IV

FIRST STEPS TOWARDS ORGAN BANKS: VITRIFICATION OF RENAL PRIMORDIAL

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6.1. ABSTRACT

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand, by the risks of allograft loss rejection and immunosuppressive therapy toxicity and by limitations of organ preservation protocols, which is essential to organise staff and facilities, transport organs, and perform necessary laboratory tests. However, the cryopreservation of composite tissues poses technical challenges beyond those seen in the preservation of single tissue types or organs. The purpose of our study was to establish a protocol for long-term storing of renal primordia, that allow to generate new kidneys after its transplant into a syngeneic non-immunosuppressed host. With this aim, metanephroi from 16-days-old (E16) embryos were microdissected and vitrified following the minimum essential volume method, using the Cryotop® as a device and the VM3 vitrification solution. After 3 months of storage in liquid nitrogen (-196ºC), 20 metanephroi were warmed and transplanted using a minimally invasive laparoscopic surgery into the retroperitoneal fat of 5-month-old immune-competent rabbit hosts. In the same way, 22 fresh metanephroi were transplanted. Twenty-one days after transplantation, hosts were euthanized and developed kidneys were recovered and evaluated morphologically and histologically. The results showed that significant growth and fully differentiated mature glomeruli and tubule can be observed in all kidney graft explants recovered. In total, 5 metanephroi (25.0%) were successfully grown after vitrification. In the same way, 12 metanephroi (54.5%) were successfully grown in the fresh group. These encouraging results demonstrated that metanephroi not only survive vitrification, but they become vascularized and continue its development. These results suggest that it's possible to create a long-term biobank of kidney precursors as an unlimited source of organs for transplantation, opening new therapeutic possibilities for the patients with a chronic renal failure.

6.2. INTRODUCTION

In United States, approximately 100.000 individuals are waiting for a kidney transplant and more of 400.000 individuals are suffered some kind of end-stage renal disease requiring haemodialysis [56]. Nevertheless, the problem is even more severe than in the EEUU, being an universal problem affecting about 5-7% of the world population [240]. Allogeneic transplantation remains the only treatment that restores full physiological renal function, however it clinical application has been limited by the shortage of donors, the high surgical morbidity, the risks of allograft loss rejection and by the immunosuppressive therapy toxicity [2,3]. The use of xenotrasplants has been considered for years as a possible solution to the organ shortage, however the risks of xenograft loss rejection and zoonosis have limited the clinical application of this kind of

treatments [48,67,116,118]. However, recently and following this line, xenotransplantation of developed kidney precursor cells has provided a novel solution for these troubles [139,143,162]. Specifically, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties, and avoids its rejection from non-immunosuppressed hosts [138,139]. Furthermore, this embryonic kidneys (metanephroi) triggers the formation of a vascular system directly from host, attenuating any kind of rejection and encouraging its transplantation across de species barrier [138,144,155,212]. In addition, renal primordial do not require immediate vascular anastomosis upon transplantation, as is the case in vascularized organ [139,143,155–157,172].

However, even if in a most favourable future situation the organ supply and demand could be balanced using xenotransplants or lab-grown organs from regenerative medicine, the future of these treatments would still be compromised without proper cryoconservation treatments [9–11]. Long-term banking is essential to allow the physical distribution of the organs to patients in need and realize an adequate inventory control and quality assurance [9–11]. At present, two major groups of methods for cryopreservation can be defined: freezing and vitrification, which mainly differ in the composition of cryoprotectants and cooling rates [137,185,186]. In freezing, the ice crystal formation during cooling or warming steps becomes a major concern, a problem that has previously frustrated attempts to cryopreserve kidneys [192]. In contrast, in vitrification, the liquids in a living system are converted into the glassy state at low temperatures without ice crystal formation [241]. To date, small ovaries, blood vessels, heart valves, corneas and similar structures are the only macroscopic structures having the capacity to recover, at least in part, after vitrification [192], thus the long-term banking of human organs or their engineered substitutes for subsequent transplantation is an important long-sought objective that to date remains a challenge [9–11,187]. Fahy et al. [192] reported a case history of one rabbit kidney that survived vitrification and supported the life of a recipient animal for an indefinite period of time.

On the basis of our previous experience with early embryos and oocytes cryopreservation [242,243], we have described recently a method to cryopreserve the whole metanephroi organs and generate kidneys after its transplant into syngeneic non-immunosuppressed host [230]. Previously, to our best knowledge, only Bottomley et al. [201] evaluated the cryopreservation of metanephroi immediately after thawing, but only under *in vitro* conditions. In an effort to advance in organ cryopreservation knowledge and in the establishment of the time window in which rabbit metanephroi can implant and develop, this study was conducted to evaluate the developmental potential of 16-days-old (E16) rabbit metanpehroi after its vitrification and allogeneic transplantation into non-immunosuppressed hosts.

6.3. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

6.3.1. Animals and ethical statements

New Zealand females (5 months old) were used as embryo donors and metanephroi recipients. These animals came from the experimental farm of the Universidad Politécnica de Valencia, an accredited animal care facility (code: ES462500001091). All animals were kept in an alternating cycle of 16 h of light and 8 h of dark, with free access to food and water, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively.

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the "Universitat Politècnica de València" ethical committee prior to initiation of the study (code: 2015/VSC/PEA/00170). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. An authorisation certificate issued by the Valencian governmental administration to experiment on animals is held by XGD (code: 2815), FMJ (code: 2273) and JSV (code: 0690).

6.3.2. Metanephroi recovery and vitrification

Metanephroi were surgically dissected from E16 rabbit embryos under a dissecting microscope using previously described techniques [219,230] and vitrified within 1 h following the minimum essential volume method [242], using the Cryotop [238] as device and the VM3 [196,202] vitrification solution. Metanephroi were first exposed for 3 min to 2.5 ml equilibration solution containing 1.7% w/v ethylene glycol (EG), 1.3% w/v formamide, 2.2% w/v dimethyl sulphoxide (DMSO), 0.7% w/v PVP K12 (polyvinylpyrrolidone of Mr 5000Da) and 0.1% w/v final concentrations of commercially available SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium (BM: DPBS + 20% foetal bovine serum, FBS). Then, the metanephroi were submerged into 2.5 ml of solution containing 4.7% w/v EG, 3.6% w/v formamide, 6.2% w/v DMSO, 1.9% w/v PVP K12 and 0.3% w/v final concentrations of ice blockers in BM for 1 min. Finally, the metanephroi were submerged into 2.5 ml of vitrification solution consisting of 16.84% w/v EG, 12.86% w/v formamide, 22.3% w/v DMSO, 7% w/v PVP K12 and 1% w/v final concentrations of ice blockers in BM before being loaded into the Cryotop devices and

directly plunged into liquid nitrogen within 1 min. All manipulations were performed at room temperature (25 ± 1 °C) and all the media were used at room temperature, except for the first warming solution, which was used at 37.5 °C.

6.3.3. Metanephroi transplantation surgery

After 3 months of storage in liquid nitrogen, the metanephroi were warmed and transplanted into recipients. For warming, metanephroi were submerged into 2.5 ml of a solution containing 1.25 M sucrose in BM for 1 min and later transferred stepwise into decreasing sucrose solutions (0.6, 0.3 and 0.15 M sucrose in BM) for 30 s, then washed twice in BM for 5 min. The allotransplantation was performed using a minimally invasive laparoscopic technique, as described previously [219], within 45 min after metanephroi were warmed or collected (fresh). Only one endoscope trocar was inserted into the abdominal cavity. Then, an epidural needle was inserted into the inguinal region. After identify a renal vessel in the retroperitoneal fat, a hole was performed adjacent to the vessel. Then, kidney precursor was aspirated in a Cystofix® epidural catheter, and the catheter was introduced through a Cystofix® needle into the inguinal region, releasing the metanephroi into the performed holes. Between 3 to 4 kidney precursors were transplanted in each host without immunosuppression (one metanephroi per hole). After surgery, analgesia and antibiotic treatments were administered. No immunosuppression was given to recipients. Metanephroi transplantation was assessed in three sessions.

6.3.4. Metanephroi development and histomorphometry of the renal corpuscle

The animals receiving implants were euthanatized at 3 weeks after transplantation. New kidneys were then removed, fixed in formaldehyde solution and embedded in paraffin wax. New kidneys were cut into 5-µm histological sections and stained with haematoxylin and eosin. Kidneys from a 5-weeks old rabbit were used as controls. The stained sections were examined with light microscopy for histological and histomorphometric analysis according with Marco-Jiménez et al. [230]. In the histomorphometric measurements, 20 renal corpuscle and glomeruli on each sample were measured (area and perimeter) in each of the experimental groups. Photomicrographs were taken at total magnification of x1000. In addition, the glomerular tuft cellularity was estimated by counting the total number of nuclei of each glomerulus. Photomicrographs were measured using ImageJ analysis software (public domain http://rsb.info.nih.gov/ij/). Kidneys originating from a 5-week-old rabbit (coeval with the metanephroi age) were used as controls.

6.3.5. Statistical analysis

The recovery rate after transplantation (new kidneys recovered/total metanephroi transplanted) was analysed using the chi-square test. The renal corpuscle and glomeruli measurements (area and perimeter), and the glomerular tuft cellularity were compared using a general linear model, including the sample type (fresh and vitrified) as a fixed factor and replicate as random factor. The replicate was non-significant and was removed from the model. Significance was attributed to analyses where p is less than 0.05. All statistical analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, USA). Data were expressed as means ± standard error of means.

6.4. RESULTS

Three females were used as embryo donors for the metanephroi recovery. A total of 22 recovered embryos were surgically dissected. After obtaining the metanephroi, 22 were transplanted directly (fresh group) and 20 after vitrification (vitrified group) into 11 recipients. Twenty-one days after transplant, the new kidneys were recovered and examined. In total, 5 vitrified metanephros (25.0%) and 12 fresh metanephroi (54.5%) were successfully grown, although with statistically different efficiencies ($p<0.05$). In all of them, new kidneys developed mature glomeruli (Figure 21), whose histomorphometry analysis (Table 6) shown a comparable area and perimeter of renal corpuscle and glomerulus between the vitrified new kidneys and control samples. Renal corpuscular and glomerular area and perimeter showed a significant increase in fresh group when compared with the control and vitrified group ($p < 0.05$). However, glomerular tuft cellularity was reduced significantly ($p < 0.05$) in all the kidney graft explants when compared with the control group.

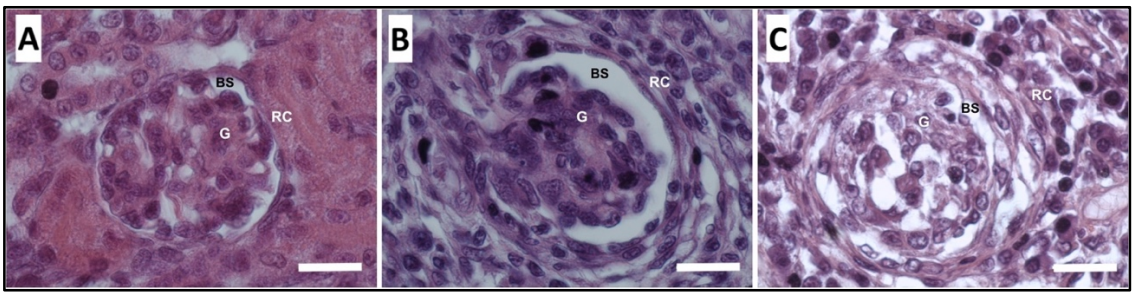

Figure 21. Representative photomicrograph of the renal corpuscles. G; Glomerulus. RC; Renal Corpuscle. BS; Bowman's space. (A) Renal corpuscle of the control kidney originating from 5-week-old rabbit (coeval with metanephroi age). (B) Renal corpuscle of a fresh kidney precursor 3 weeks after transplantation. (C) Renal corpuscle of vitrified kidney precursor 3 weeks after transplantation. Scale bar: 0.02 mm.

***Table 6**. Histomorphometric quantification of renal corpuscle and glomerulus of new kidneys developed after vitrified and fresh metanephroi allotransplantation.*

Group	n	Renal corpuscle		Glomerulus		
		Area (μm^2)	Perimeter (μm)	Area (μm^2)	Perimeter (μm)	Cells number
Fresh	12	4487.7 ±183.79[a]	278.7 ±6.27[a]	3019.1 ±146.63[a]	238.6 ±5.71[a]	43.4 ±1.69[b]
Vitrified	5	3453.2 ±378.90[b]	208.2 ±15.59[b]	2323.2 ±293.27[b]	175.4 ±14.19[b]	27,7 ±3.97[c]
Control	5	2778.2 ±131.28[b]	188.6 ±5.71[b]	2221.5 ±101.58[b]	169.2 ±4.91[b]	53.8 ±1.45[a]

n: Number of new kidneys. Data are expressed as mean ± SD. a,b,c: Data in the same column with uncommon letters are different ($p < 0.05$).

6.5. DISCUSSION

This study reports that E16 rabbit metanephroi survives vitrification, underwent differentiation and growth, became vascularised and developed morphologically and histomorphometric normal glomeruli. These results are according with the only previous study that uses *in vivo* methodology to test the metanephroi viability after a vitrification procedure [230]. Bottomley et al. [201] also evaluated metanephroi viability after cryopreservation procedures, but the *in vitro* methodology used in this study difficult the comparison with our study. In this study, the effect of different cryopreservation procedures (slow freezing vs vitrification) was compared, suggesting that vitrification yielded more promising results [201], which is consistent with our findings.Presumably, the cause for our success was likely attributable to the vitrification technique that avoids the ice crystal formation [188,241], the use of a lox-toxic vitrification solution well-suited for renal tissue preservation [196,202], and the use of the Cryotop® device [238]. This vitrification container was aimed to minimise the volume of vitrification solution and thereby increase the speed of cooling and warming rate, which facilitates the vitrification procedure [191,235].In addition, as Marco-Jimenez et al. reports, the small size of metanephroi could allow an adequate diffusion and equilibration of cryoprotectants within the organ cells [230]. In concordance, to date, only small ovaries, blood vessels, heart valves, corneas and similar structures are the unique macroscopic structures with the capacity to recover, at least in part, after vitrification [192]. Furthermore, vascularized tissues and organs are severely damaged by extracellular ice during cryopreservation [244]. In contrast with its mature counterpart, kidney precursors are avascular and developed a blood supply originating from the host vasculature [138,145,162].

According to the porpoise of this study to establish the time window in which rabbit metanephroi can implant and develop into new kidneys, we demonstrated that, although with less efficiency than in previous E15 evidences [230], E16 vitrified metanephroi also develop new kidneys after its transplantation in non-immunosuppressed hosts. As similar efficiencies rates were observed between E15 and E16 fresh metanephroi, differences in its vitrified counterparts could be due to the larger size of E16 metanephroi, which could hinder the spread of cryoprotectants into the tissue and thus the vitrification process. This study demonstrated that developed kidneys from E16 vitrified metanephroi exhibited similar renal glomerular morphometry compared with control kidneys. These results further support earlier findings demonstrating that transplanted metanephroi have the ability to develop apparently normal glomeruli [136,138,144,155,162,172,212,239]. In agreement with or previous results [230], less glomerular cell number were observed for the fresh and vitrified developed metanephroi. It could be normal because, as cryopreservation under extreme low temperature allows to achieve the “cryogenic suspension of life” and the metabolic arrest [137,185,186], warmed metanephroi could need an extra time in order to reactivate its developmental program. A similar explanation can be provided for the fresh metanephroi, as their extraction from the embryo could incur a developmental delay. Therefore, these differences could be irrelevant. Different authors have already shown that transplants of fresh kidney precursors are able to filter blood and produce urine [143,144,155,158,172], prolonging the life of anephric hosts [146,159]. It would be interesting in future studies to demonstrate the same capacities in vitrified metanephros. These results encourage the creation of a long-term biobank of kidney precursors, as an unlimited source of organs for transplantation with therapeutic potential.

6.6. CONCLUSION

Here we demonstrate that long-term metanephroi cyobanking is possible, which is to date one of the most important health challenges of the twenty-first century regarding the transplantation of mature organs. If the organ supply and demand could be balanced using embryonic kidneys, this cryopreservation approach can dissociate the time of the organ retrieval and its transplantation, ensuring the therapeutic potential of this field.

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DOCTORAL THESIS
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DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

7. CHAPTER V

SUCCESSFUL DEVELOPMENT OF VITRIFIED EMBRYONIC KIDNEYS AFTER ITS LAPAROSCOPIC TRANSPLANTATION INTO NON-IMMUNOSUPPRESSED HOSTS

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7.1. ABSTRACT

Transplantation from living or deceased donors has been limited by donor availability that is opposed to the increasing demand and by the risk of allograft loss rejection and immunosuppressive therapy toxicity. In recent years, xenotransplantation of metanephroi has offered a novel solution for the limited supply of human donor organs. However, even if in a most favourable and idyllic situation the organ availability and its demand could be balanced using xenotransplantation of embryonic organs, the future of this treatment would still be compromised without proper long-term storage procedures. Thus, based on the ongoing long-term storage necessities, this study was designed to investigate the effect of two specific "time windows" of the metanephroi development (E15 and E16) on their *in vivo* developmental capacity. Their potential to develop morphologically normal glomeruli following vitrification and allotransplantation into non-immunosuppressive hosts was assessed. Metanephroi originating from E15 and E16 rabbit embryos were vitrified using the M22 solution and the Cryotop® as a device. After three months of storage in liquid nitrogen, metanephroi were transplanted into non-immunosuppressed adult hosts by laparoscopy surgery. Twenty-one days after allotransplantation, 6 (32%) and 7 (35%) "new kidneys" were recovered from the E15 and E16 vitrified metanephroi, respectively. All the "new kidneys" recovered exhibited significant growth and mature glomeruli. However, histomorphometry analysis revealed that "new kidneys" developed from E16 metanephroi exhibit a greater degree of maturity compared with E15 metanephroi. Results obtained in the present study point out that, in rabbit model, vitrified E16 metanephroi can be stored in liquid nitrogen, achieving good *in vivo* developmental capacity and morphologically normal glomeruli after its laparoscopy transplantation into non-immunosuppressed hosts.

7.2. INTRODUCTION

In end-stage renal disease, the kidneys no longer adequately clarify the blood of wastes and remove the excess fluids [245]. Then, organ transplantation provides the ideal method to restore full physiological organ functions [2]. However, transplantation from living or deceased donors has been limited by donor availability that is opposed to the increasing demand [2,3]. For this reason, patients with end-stage renal disease are usually forced to receive dialysis treatment to support the lost kidney function. However, none of these treatments is a well-suited remedy for the end-stage renal disease. It does not replace the kidneys' role in metabolism regulation, endocrine function or homeostasis, including the control of blood pressure, electrolyte balance, acid-base balance, etc. [246–251]. Furthermore, dialysis is an expensive treatment, supposing a cost of 1518 million € in countries like Spain or 1.2 billion in United Kingdom [33,252,253]. In other countries like Canada the estimated annual per-patient costs

were $56,000 for peritoneal dialysis, $71,000 to $90,000 for home haemodialysis and $95,000 to $107,000 for in-centre or satellite unit haemodialysis [254]. Furthermore, the dialysis centre capacity is limited and resources make impossible to provide more frequent or longer treatment sessions to patients [254–256]. In addition, the diet changes that patients must make, and the need to attend to receive dialysis treatment, affect negatively the life quality of patients and deplete its health [257]. For this, many patients define dialysis as more life to his years, but no more years to his life [258]. Paradoxically, for these reasons that evidence the superior benefits of kidney transplantation over dialysis, and the growing incidence of end-stage renal disease, have led to an exponential increase in the need for kidney transplantation worldwide [259]. In addition, it is known that five-year survival was considerably better after living-donor kidney transplantation (94%) or after cadaveric-kidney transplantation (76%) than on chronic dialysis (60%) [260]. These facts cause that the lack of these organs becomes in a very serious problem of global health for patients in waiting lists. Only in United States, the length of waiting list has doubled over the past decade, reaching around 100.000 patients and a median waiting time of 4.5 years [261]. Thus, as long as the patient does not get an organ it will be dependent on dialysis, which have a higher risk for morbidity and mortality, dying nearly 5000 patients every year in this country [261]. The long- term mortality risk was 68% lower in the case of the patient receives a transplant when compared with patients remaining on the waiting list [262]. So taking into account all this information, seeking alternative solutions to this grave problem is indispensable.

In this point, regenerative medicine has garnered great attention in the last years because it has the potential to generate new organs for transplantation. Particularly, human kidney exhibits a remarkable complexity, coupled with the presence of at least 30 different specialized cells that have to be able to function together, for which their proper spatial distribution is indispensable [69,72]. Thus, the need for recapitulation the three-dimensionally integrated kidney structure is the reason because cell therapies with individual cells fail in restoring kidney function [69]. However, there is an open line of research that retakes the field of xenografts as a possible solution to the organ shortage. Transplantation of embryonic kidneys into non-immunosuppressed adult hosts has showed that these primordia can mature and growth as if they had not been extracted from the embryo. These embryonic kidneys or metanephroi are able to attract the formation of a vascular system from host (angiogenesis), undergoing maturation and exhibiting functional renal properties [139,143,145]. Dekel et al. [143] transplanted embryonic kidneys from both human and pig origins into mice, demonstrating that metanephroi were differentiated into functional nephrons evidenced by the dilute urine that they produce. However, it’s known that new renal tissue developed from metanephroi not only provide an excretion function, but also an endocrine function, synthesising renal hormones such as renin and erythropoietin [156,157]. Furthermore, if metanephroi are transplanted beside bladders developed from cloacas, and if cloacal-

developed bladder was connected to the host ureters, new metanephroi-developed kidney produces and excretes urine through the recipient ureter, avoiding hydronephrosis and allowing the nascent kidney to continue their growth [158]. These findings, together with the scientific capability to produce specific pathogen-free animals [263], suggest than metanephroi transplantation could be a possible solution for kidney need [136,139,143].

Our group has been working on the development of a novel technique that allows the metanephroi transplantation procedure thought a minimal invasive procedure, presenting recently an effective way by laparoscopy [219]. To our best knowledge, all the previous studies were performed through open surgery, a fact that has limited the experiments on larger animals and should be clinically avoided. However, even if in a most favourable and idyllic situation the organ availability and its demand could be balanced using embryonic xeno-transplants, the future of this treatment would still be compromised without proper cryopreservation procedures [9–11]. Effective cryopreservation can permit long-term storage of the transplantable organ, dissociating the recuperation and transplantation time, and allows an adequate inventory control and quality assurance thought the pertinent laboratory analysis. To date, only small structures such as small ovaries, heart valves or corneas are the only macroscopic structures that retain the capacity to recover, at least in part, after vitrification [9,192]. A historical case was reported by Fahy et al. [192], who describe how one whole rabbit kidney survive and supported indefinitely the life of a recipient animal after a vitrification procedure using the M22 vitrification medium.

Based on this knowledge, we recently described a method to cryopreserve metanephroi effectively using the VM3 vitrification solution and the Cryotop® as device [230,232]. Previously to our works, only Bottomley et al. [201] examined the cryopreservation of metanephroi, but only under *in vitro* conditions. In an effort to advance in the organ cryopreservation knowledge, this study investigates the effect of two specific “time windows” of the rabbit metanephroi (E15 and E16) on their *in vivo* developmental capacity. Their potential to develop morphologically normal glomeruli following vitrification and allotransplantation into non-immunosuppressive hosts was assessed.

7.3. MATERIAL AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

7.3.1. Animals and ethical statements

New Zealand females (5 months old) were used as embryo donors and metanephroi recipients. These animals came from the experimental farm of the Universidad Politécnica de Valencia, an accredited animal care facility (code: ES462500001091). All animals were kept in an alternating cycle of 16 h of light and 8 h of dark, with free access to food and water, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively.

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the "Universitat Politècnica de València" ethical committee prior to initiation of the study (code: 2015/VSC/PEA/00170). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. An authorisation certificate issued by the Valencian governmental administration to experiment on animals is held by XGD (code: 2815), FMJ (code: 2273) and JSV (code: 0690).

7.3.2. Metanephroi recovery and vitrification

Metanephroi were surgically dissected from a 15 (E15) and 16 (E16)-day-old rabbit embryos under a dissecting microscope, using previously described techniques [219,230,232] and vitrified within 1 h following the minimum essential volume method, using the Cryotop® [238] as device and the M22 vitrification solution [192,196].

Metanephroi were first exposed for 3 min to 2.5 ml equilibration solution containing 1.68% w/v ethylene glycol (EG), 1.28% w/v formamide, 2.23% w/v dimethyl sulphoxide (DMSO), 0.3% w/v N-methylformamide (NMF), 0.4% w/v 3-methoxy-1,2-propanediol (MP), 0.28% w/v PVP K12 (polyvinylpyrrolidone of Mr 5000Da) and 0.1% and 0.2% w/v final concentrations respectively of commercially available SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium (BM): Dulbecco's Phosphate Buffered Saline (DPBS) + 20% foetal bovine serum (FBS). Then, the metanephros were submerged into 2.5 ml of solution containing 4,82% w/v EG, 3.68% w/v formamide, 6.38% w/v DMSO, 0.86% w/v NMF, 1.14% w/v MP, 0.8% w/v PVP K12 and 0.29% w/v with 0.57% w/v final concentrations respectively of SuperCool X-1000 and SuperCool Z-1000 ice blockers in BM for 1 min. Finally, metanephroi were submerged into 2.5 ml of vitrification solution consisting of 16.84% w/v EG, 12.86% w/v formamide, 22.31% w/v DMSO, 3% w/v NMF, 4% w/v MP, 2.8% w/v PVP K12 and 1% w/v with 2% w/v final concentrations respectively of SuperCool X-1000 and SuperCool Z-1000 ice blockers in BM before being loaded into Cryotop devices and directly plunged into liquid nitrogen

within 1 min. All manipulations were performed at room temperature (25 ± 1°C) and all the media were used at room temperature, except for the first warming solution, which was used at 37.5°C.

7.3.3. Metanephroi transplantation surgery

After 3 months of cryostorage in liquid nitrogen, metanephroi were warmed and transplanted into recipients. For warming, metanephroi were submerged into 2.5 ml of a solution containing 1.25 M sucrose in BM for 1 min, being later transferred stepwise into decreasing sucrose solutions (0.6, 0.3 and 0.15 M sucrose in BM) for 30 s each, then washed twice in BM for 5 min. After that, metanephroi were transplanted within 45 min after warming or collected (fresh) by abdominal laparoscopy [219]. Briefly, only one endoscope trocar was inserted into the abdominal cavity. Then, an epidural 17G needle (for E15 metanephroi) or Cistofix® needle (for E16 metanephroi) was inserted into the inguinal region. After identify a renal vessel in the retroperitoneal fat, a hole was performed adjacent to the vessel. Then, kidney precursor was aspirated in appropriate catheter, and the catheter was introduced through the corresponding needle to release the metanephroi into the performed holes. Between 3 to 4 kidney precursors were transplanted in each host without immunosuppression (one metanephros per hole). After surgery, analgesia and antibiotic treatments were administered for 3 days [168]. No immunosuppression was given to recipients. Metanephroi transplantation was assessed in three sessions.

7.3.4. Metanephroi development and histomorphometry of the renal corpuscle

The transplanted animals were euthanatized at 3 weeks after transplantation. New kidneys were then retrieved, fixed in 4% paraformaldehyde solution and embedded in paraffin wax. New kidneys were cut into 5-µm histological sections and stained with haematoxylin and eosin. Kidneys from a 5-weeks (approximately 36 days) old rabbit were used as controls. The stained sections were examined with light microscopy for histological and histomorphometric analysis, according with previous works [219,230,232]. In the histomorphometric measurements, 20 renal corpuscle and glomeruli on each sample were measured (area and perimeter) in each of the groups (control and experimental). Photomicrographs were taken at total magnification of x1000. In addition, the glomerular tuft cellularity was estimated by counting the total number of nuclei of each glomerulus. Photomicrographs were measured using ImageJ analysis software (free software http://rsb.info.nih.gov/ij/). Kidneys originating from a 5-week-old rabbit (coeval with the metanephroi age) were used as controls.

7.3.5. Statistical analysis

The recovery rate (transplantation efficiency = recovered kidneys/transplanted metanephroi) were evaluated using a probit-link function with binomial error distribution, including the sample type (fresh and vitrified), the embryonic day (E15 and E16) and experimental session (1^{st}, 2^{nd} or 3^{rd}) as a fixed factors. The renal corpuscle and glomeruli measured (area and perimeter), and the glomerular tuft cellularity were compared using a general linear model, including the sample type (fresh and vitrified) as a fixed factor and replicate as random factor. The replicate was non-significant and was removed from the model. Differences of $p<0.05$ were considered significant. Data are shown as means ± standard error means. All analyses, were performed with SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, US).

7.4. RESULTS

Six rabbit females were used as embryo donors. Three of the females were euthanatized 15 days since artificial insemination (to obtained E15 metanephroi) and the rest were euthanatized after 16 days since artificial insemination (to obtained E16 metanephroi). In total, 35 metanephroi from E15 embryos and 42 metanephroi from E16 embryos were carefully micro-dissected. After this, 19 vitrified and 16 fresh metanephroi from E15 embryos and 22 vitrified and 20 fresh metanephroi from E16 embryos were transplanted into 20 host. Twenty-one days after transplantation procedure, the “new kidneys” were recovered and analysed (Figure 22).

In total, 6 (32%) vitrified and 7 (44%) fresh metanephroi from 15-days-old embryos and 7 (35%) vitrified and 12 (55%) fresh metanephroi from 16-days-old were successfully grown. No significant effects were observed nor for embryonic day (E15 vs E16, $p=0.533$) nor for metanephroi origin (fresh vs vitrified, $p=0.161$). All the new kidneys underwent differentiation and developed histological mature glomeruli (Figure 22). Histomorphometry analysis showed similar values between vitrified and fresh E16 metanephroi and fresh E15 metanephroi (Table 7). All the measurements from E16 metanephroi were statistically higher than those in E15 metanephroi (Table 7). Similar glomerular tuft cellularity was observed between the new kidneys developed from vitrified E15 and E16 metanephroi (Table 7).

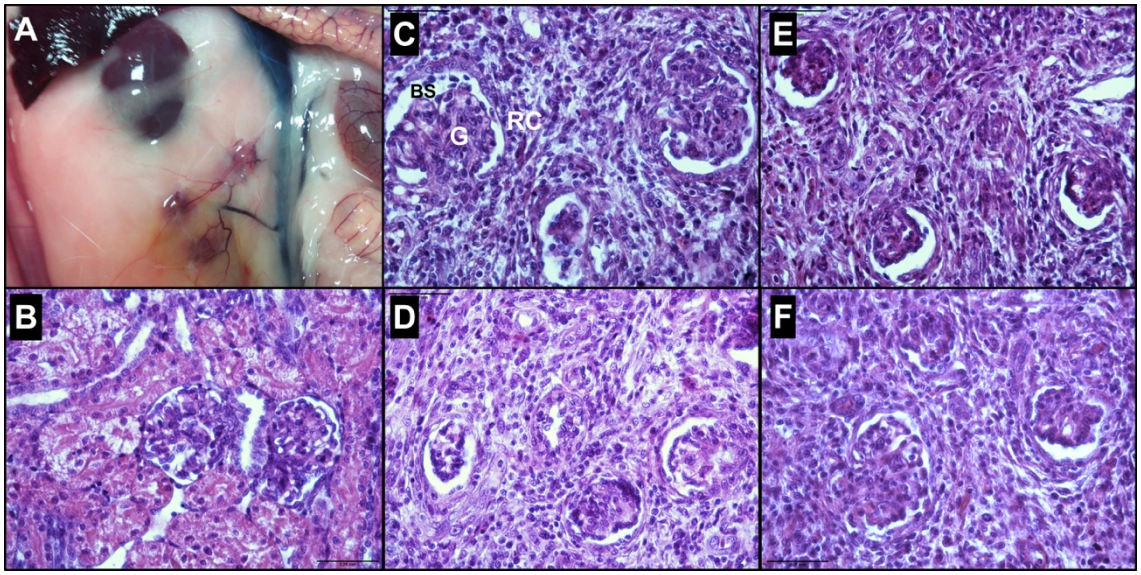

Figure 22. Developed metanephroi and representative photomicrographs of its renal corpuscles. (A) New-kidney developed from metanephroi. Representative photomicrograph of the renal corpuscles (400x): G; Glomerulus. RC; Renal Corpuscle. BS; Bowman's space. (B) Renal corpuscle of the control kidney originating from 5-week-old rabbit (coeval with metanephroi age). (C) Renal corpuscle of a fresh 15 days kidney precursor 3 weeks after transplantation. (D) Renal corpuscle of vitrified 15 days kidney precursor 3 weeks after transplantation. (E) Renal corpuscle of a fresh 16 days kidney precursor 3 weeks after transplantation. (F) Renal corpuscle of vitrified 16 days kidney precursor 3 weeks after transplantation.

Table 7. *Histomorphometric quantification of renal corpuscle of kidneys developed after allotransplantation of vitrified and fresh metanephroi.*

Embryonic age	Group	n	RENAL CORPUSCLE		GLOMERULUS		
			Area (µm2)	Perimeter (µm)	Area (µm2)	Perimeter (µm)	Cell number
	Control	4	2778 ± 199.8^{c}	188.5 ± 5.53^{c}	2221 ± 152.1^{c}	169.1 ± 4.96^{c}	54 ± 2^{a}
E15	Fresh	7	3222 ± 200.8^{c}	200.5 ± 5.53^{c}	2267 ± 154.3^{c}	167.9 ± 4.96^{c}	42 ± 2^{c}
	Vitrified	6	3834 ± 214.2^{b}	223.1 ± 5.93^{b}	2726 ± 162.9^{b}	189.3 ± 5.32^{b}	50 ± 2^{a,b}
E16	Fresh	12	6164 ± 233.8^{a}	283.4 ± 6.34^{a}	4498 ± 176.7^{a}	242.4 ± 5.69^{a}	47 ± 2^{b}
	Vitrified	7	5716 ± 229.2^{a}	271.3 ± 6.30^{a}	4045 ± 174.3^{a}	231.5 ± 5.66^{a}	52 ± 2^{a}

n: Number of new kidneys or control kidneys. Data are expressed as mean ± SD. a,b,c: Data in the same column with uncommon letters are different ($p < 0.05$).

7.5. DISCUSSION

This study evaluated the effect of the metanephroi age on their *in vivo* developmental capacity after a long-term storage period. After transplant, both E15 and E16 metanephroi underwent differentiation and growth, became vascularised by host blood vessels, and developed histological and morphologically mature glomeruli. Previously to our recent studies [137,219,232], only one group had evaluated the feasibility of the metanephroi cryoconservation, but under *in vitro* conditions [201]. Vitrification was presented as the more promising technique instead of slow-freezing [201].

Cryobiology is the science that studies the effects of low temperatures on living organism and its aim is to achieve cells become immortals at low temperatures though stopping its metabolism [137]. One of the major problems to obtain this goal is the need to avoid the ice crystal formation that causes cell death [185,188,264]. Presumably, the cause for our success can be attributable to the vitrification technique, in which the liquids in a living system are turned into the glassy state without ice crystal formation [9,188]. In this study, we evaluate the vitrification solution called M22, which had already demonstrated its effectiveness in the preservation of renal tissue [192,196,202]. In addition, we combined this solution with the Cryotop® device [238], which minimise the volume to be vitrified, facilitating the rapid transfer of heat to liquid nitrogen that allows the glassy transition to the vitrified state [191,235]. In our previous works, we had already observed that the small size of metanephroi could allow an adequate diffusion and equilibration of cryoprotectants within the organ cells, which could explain also the effectiveness in the vitrification procedure [230,232]. This was in concordance with the fact that to date, only small biological structures had been vitrified and recovered later [9,192]. Another point in favour of the metanephroi survival after it be thawed is that this embryonic organ is avascular, not requiring immediate anastomosis as in the case of mature vascularized adult organs [69,137]. We previously demonstrated that the time window in which rabbit metanephroi can implant and develop into new kidneys are E15 and E16 [219]. In our previous metanephroi vitrification studies, based on VM3 vitrification solution, E15 metanephroi exhibited better growth rate than E16 metanephroi. The result could be explained due to the larger size of the E16 ones, which could hinder the cryoprotectant diffusion and heat transfer. However, in the present study, no differences were observed between both metanephroi ages. A plausible explanation could be that M22 vitrification medium includes components that might help in the diffusion of cryoprotective agents into the whole metanephroi structure [192,196,202].

On the other hand, this study demonstrated a clear effect of metanephroi age on the renal histomorphometry. Specifically, developed new kidneys from E16 vitrified metanephroi exhibited similar renal glomerular histomorphometry than its fresh

counterparts. Furthermore, glomerulus cell density was maintained in E16 vitrified metanephroi compared with control. In contrast, E15 vitrified metanephroi exhibited different histomorphometric parameters compared with their fresh counterparts. Nevertheless, results indicate that vitrification procedure not hurdle the development capacity of metanephroi. In general, all the histomorphometric parameters relating to new kidneys are higher than control samples. In part, these results could be explained by the fact that these new renal structures were not connected to the host's urinary system. Under this condition, unconnected metanephroi become hydronephrotic, accumulating the liquid inside the new kidney [158]. In this paper we demonstrated than metanephroi vitrification using M22 medium support the developmental capability of both E15 and E16 rabbit metanephroi. However, although we have focused the study solely from the point of the cryobiological effect, it is important to say that some authors have already shown that transplants of fresh kidney precursors are able to filter blood and produce urine [143,155,158,172,173], as well as to synthetize renal hormones such as renin and erythropoietin [156,157,163]. In the future, it would be interesting demonstrate the same capacities in developed kidneys from vitrified metanephroi. In the case of clinical translation, this study reinforces the idea that it is possible to create a biobank of renal precursors, a fact that would facilitate inventory control and give the necessary time to guarantee the safety of the transplant.

7.6. CONCLUSION

The present study shows that storing vitrified metanephroi in liquid nitrogen is an effective long-term storage option that maintains metanephroi developmental viability, allowing a good developmental capacity and the development of morphologically normal glomeruli. Further studies are needed in order to support our results and understand the potential future implication of this technique in the clinical practice.

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VNIVERSITAT DE VALÈNCIA

DOCTORAL THESIS

Doctoral Programme in Medicine

DEVELOPMENT OF EMBRYONIC KIDNEY BIOBANKS AS AN UNLIMITED GRAFTS SOURCE FOR RENAL REPLACEMENT THERAPY

8. GENERAL DISCUSSION

The aim of this thesis was to use the rabbit as animal model to further in the establishment of strategies that turns the metanephroi transplantation in an ever closer clinical reality. First, we show that metanephroi transplantation can be achieved through a minimally invasive laparoscopic approach, which also was used to determine the ideal time point to transplant rabbit metanephroi without immunosuppression treatment. Second, we have proved that the addition of sildenafil citrate during metanephroi transplantation can stimulate neoangiogenesis in developing kidneys, increasing its growth and enhancing its glomerular maturity and filtration. Third, we demonstrate that metanephroi banking is feasible by using vitrification approaches, which preserve its viability and developmental potential. Together, these findings should encourage the design of proper studies to support the clinical translation of the metanephroi transplantation to treat patients with end-stage renal disease (ESRD).

Today, it is well recognized that allotransplantation of mature organs cannot be the solution for all patients suffering from an end-stage organ disease. With almost all countries having growing waiting lists, transplantation meets only 10% of the global need [12,265]. Although both living donation and deceased-donor donation go hand in hand, no country in the world has sufficient organs to meet the needs of its citizens [265]. According to the last WHO report, 36 % kidney transplants and 19 % liver transplants currently performed worldwide were from living donor [12]. Moreover, heart transplant from living donors also occurs when one patient undergoes heart-lung transplantation and their heart is given, as part of a “domino” procedure, to a heart transplant recipient [3]. However, living donation also has been a valid option for patients in need of pancreas [266], lung [267] and intestine [268], in which an organ segment able to support life is retrieved and transplanted. Besides, new approaches such as kidney paired donation are trying to increase transplantation rates, in which a transplant candidate with an incompatible live donor joins a registry of other incompatible pairs in order to find potentially compatible solutions [269]. Nevertheless, despite these desperate attempts to increase the number of available organs, poor access to transplantation is almost inevitable for most of the world’s population [12,265]. Therefore, alternative solutions to organ transplantation, either from living or deceased donor, are in need for patients with end-stage organ diseases. Regenerative medicine (RM) is looking for different technologies to generate organs on demand, either through *in vitro* or *in vivo* strategies. Particularly, embryonic kidneys transplantation has long been seen as a possible solution to the ESRD, with particular advantages from other RM options, as reviewed elsewhere [67–69]. Metanephroi, extracted at the appropriate time and properly implanted, are able to continue its growth, giving rise to host-vascularized renal structures with life-sustaining excretory and endocrine functionality [136,138,165,140,146,147,155–159]. Classically, omentum has been considered the preferential site for transplantation, allowing undisturbed expansion of a growing organ primordium that results vascularized entirely by host

vessels [136]. However, transplantation surgery has been performed through open laparotomy mainly in rodent models experiences, probably due to the lack of an easy procedure to develop preclinical trials in higher species. In order to overcome this limitation, and adapted from our previous embryo transfer technic [168], here we describe a feasible and minimally invasive laparoscopic approach to transplant metanephroi into the rabbit host omentum with high efficiency. This technique is performed using a single port instrument (5 mm endoscope), whose incision does not require suture. Laparoscopic approaches showed similar grafts outcomes and survival rates compared to open surgery [171], but includes some benefits such as decreased postoperative pain, quicker return to normal activity, fewer postoperative complications, and better host immune response [168]. Because of their intermediate size between rodents and farm animals, rabbit model was well-suited for laparoscopic approach modelling, but this procedure has also been validated in small ruminants for the first time in our laboratory [137]. With slight adaptations, the laparoscopic technique described has allowed us to carry out a pilot study to transplant metanephroi into goats with good quality, control, and safety for both researchers and animals (Figure 23). This study provides a valuable test of the procedure feasibility for impulse both the preclinical and subsequent clinical trials.

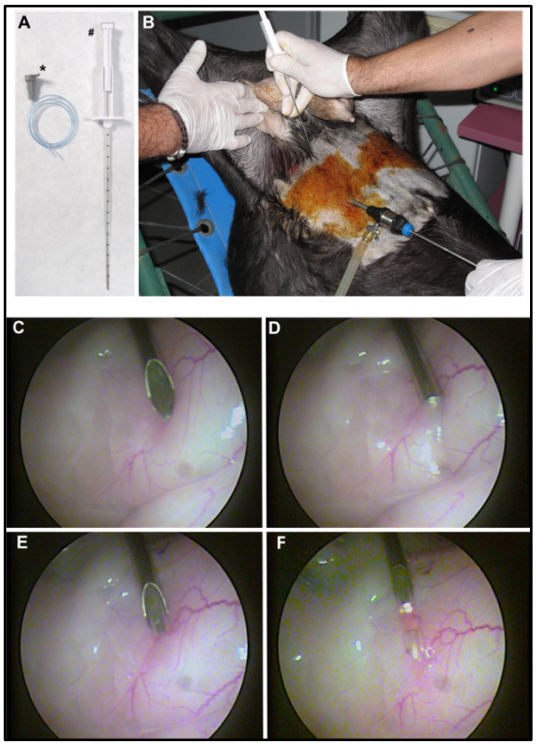

Figure 23. Laparoscopic metanephroi transplantation in goats [137]. (A-B) Metanephroi are aspirated into a orogastric feeding catheter (*), which were inserted through a Tru-cut® biopsy needle (#) into the inguinal region. (B) Laparoscopic surgery (external view). (C-F) Laparoscopic surgery (internal view). (C) Localization of vascularized bed for metanephroi transplantation. (D) Hole for transplantation is created. (E) The catheter is inserted into the hole. (F) Metanephros is released into the performed hole.

However, to ensure the therapeutic potential of the transplanted metanephroi, the resultant new kidneys must reach a renal mass able to produce concentrated urine and sustain life. Interestingly, both weight and clearances of the nascent kidneys can be enhanced by compensatory renal growth following reduction of host native renal mass, which stimulus is accompanied by changes in the expressions of several growth factors [155]. Therefore, it is of especial importance to investigate whether growth factors could be used to enhance the growth and function of developing metanephroi. Hammerman's group demonstrated that clearance rates can be increased using combinations of growth factors such as IGF-1 and VEGF to pre-treat (prior implantation) and post-treat metanephroi (during ureteroureterostomy), although none triggered a weight increase in developing metanephroi [144,172,173]. In this thesis, we demonstrate that both metanephroi neovascularization and growth, as well as glomerular filtration, can be enhanced using low doses (10 μM) of sildenafil citrate (SC) during metanephroi transplantation. SC can exert its growth-promoting actions up-regulating angiogenic systems that improves renal haemodynamics, and inducing renoprotective effect through anti-inflammatory, anti-oxidant, and anti-apoptotic mechanisms [172,175–178]. Of note, although VEGF is also produced by developing kidneys and is crucial for renal vascularization [172], treatments with low SC doses resulted in more robust angiogenesis-related properties than that produced by a saturating VEGF concentrations [223]. Moreover, it has been reported that SC exerts a dilatation of afferent arterioles, promoting an increase in the glomerular filtration process [224], which are consistent with our results. Pursuing our same goal, Cohen et al. reported a novel approach for the enhancement of growth and differentiation of transplanted embryonic tissues by its coimplantation with more undifferentiated cells or the use of soluble factors secreted by embryonic fibroblasts [133]. Similarly, preliminary results from our laboratory also demonstrated that metanephroi growth is promoted if they were grafted embedded in an adipose-derived stem cells suspension [270]. It is thought that these cells act as trophic mediators through paracrine effects [271]. Therefore, although SC treatment could be one of the most promising factor used during metanephroi transplantation, it should be tested in combination with other emerging approaches to allow the standardization of a procedure that enables the formation of enlarged new kidneys with a life-sustaining function.

Today, we assume that metanephroi retrieved at the proper time during embryogenesis differentiate along defined organ-committed lines [138,272]. However, what seems to hamper this established developmental program before a mature state is reached, is the presence of an hydronephrotic state. Because the ureter of nascent kidneys was buried under the fat of the omentum with no egress for the urine, hydronephrosis was the result. Hydronephrotic developing metanephroi become dysplastic and stops its growth [158,164]. However, if metanephroi are transplanted beside cloacas, and the cloacal-developed bladder is connected to the host ureters, the developing kidneys can excrete

urine thought the recipient ureter, avoiding hydronephrosis [158]. Of note, we have demonstrated that these cloacal-metanephric structures also can be laparoscopically allotransplanted into non-immunosuppressed rabbit hosts, being able to grow with the same efficiency as individual metanephroi without triggering an immunological response (Figure 24).

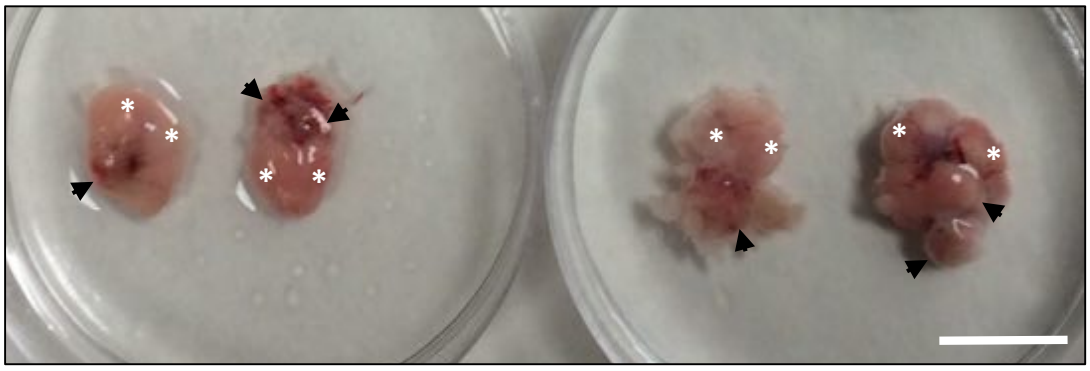

Figure 24. Rabbit metanephroi-cloaca structures laparoscopically allotransplanted into omentum. Three weeks after transplantation, shows developed metanephroi (arrows) and a liquid-filled compartments (asterisks), which may correspond to the ureters or the bladders. Scale bar: 1 cm.

Although this strategy allows to obtain enlarged developing kidneys that prolonged the lifespan of anephric rats, its long-term life-sustaining ability is restricted to hours [158]. In contrast, a more extensive prolongation of life has been demonstrated if individual metanephroi were treated with growth factors before its transplantation and during ureteroureterostomy in anephric rats [159]. Therefore, although the transplantation of metanephroi attached to the cloaca can solve the hydronephrosis, it seems that the use of growth-promoting factors such as SC, VEGF and IGF-1 is essential to achieve life-compatible levels of metanephroi functionality. Glomerular filtration rates in growth factor treated metanephroi are approximately 6% of normal on average, which represents a 250-fold increase over non-treated metanephroi [144,159,174]. Taking into account that hemodialysis provides 10% of normal glomerular filtration, these values approximates a level of renal function that would be expected to preserve life [144]. Moreover, because previous studies showed a correlation between the total mass of the developed metanephroi and the survival of anephric rats [146], the transplantation of several metanephroi is thought to be another non-exclusive approach to achieve sufficient renal mass to support life. This strategy is similar to that used in kidney transplantation from paediatric donors, in which both kidneys are transplanted in bloc into adult recipients to guarantee acceptable glomerular filtration rates [228,229].If all of these approaches are combined to develop a standardized strategy that allows the new kidney to grow and maintain life, the feasibility of metanephroi transplantation for clinical application could be guaranteed.

Whatever the mechanisms used, we must go towards the generation of a life-sustaining renal structure. To this end, our ability to *in vivo* determine the renal function through non-invasive techniques, and in real time, will be decisive to verify the effectiveness of the applied procedures, allowing us also to elucidate the optimal point to undertake a connection surgery to the host's excretory system. Based on the study reported by Dekel et al. [142], we developed a pilot study to detect any developing renal activity through scintigraphy [273]. Briefly, ^{99}technetium-2,3-dimercaptosuccinic acid (^{99}Tc-DMSA), a radioisotope known to be extracted and secreted by functional renal tissue, was used as a renal activity tracer. Images were acquired using a portable gamma-camera (Sentinella® S102, Oncovision S.A., Valencia, Spain), which demonstrate the suitability of this strategy to display whether metanephroi survive, growth and function (Figure 25). This technique could be used to monitor the expansion and function of the renal primordium both in preclinical as in clinical trials.

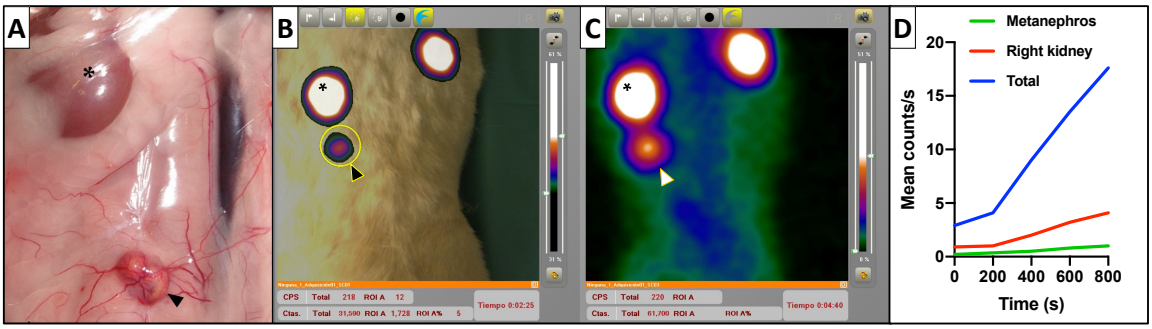

Figure 25. Renal scintigraphy (renography). (A) Developing kidney from an allotransplanted metanephros (arrow), and rabbit host kidney (asterisk). (B-C) Images illustrating functional assessment of developing metanephric transplants. (D) Time-activity curves (renograms).

In the case of human renal allotransplantation from deceased donors, there is an unavoidable delay between the time organ harvest and the time of implantation, which can be minimized in the case of a living donation. Theoretically, metanephroi could be harvested immediately prior to implantation. However, it would be best if metanephroi could be stored for a period of time prior to transplantation that permits their distribution to distant sites and allows time to plan the transplant procedure and perform the pertinent sanitary tests [10,11]. Although no direct analogy is possible between storage of metanephroi and adult kidneys, comparisons can be made. Most if not all tissues suffer functional injury even after short periods of ischemia and, once a critical duration of ischemia is reached, cell injury and death results [9]. In adult allograft, the risk of delayed graft function is minimized by keeping the duration of warm ischemia (time between lack of blood and the beginning of cold storage) below 20 min, and by keeping the time in cold ischemia (time in cold storage prior transplantation) below 30

h [274]. In the case of metanephroi, it has been demonstrated that its developmental potential is preserved after 15 min of warm ischemia (time between removal from embryos, dissection, and placement in cold storage) followed by 3 days of cold ischemia (time in cold storage prior transplantation) [274]. However, if metanephroi could be cryopreserved immediately after its recovery from embryos, the extremely low-temperature conditions can be used to initiate the metabolic arrest and prevent further ischemic damage [9]. Previously, some groups demonstrated that metanephroi cryopreservation could be feasible due to its small size, which allows a great heat exchange and diffusion of cryoprotectant solutions into the tissue [200,201]. However, none of them demonstrated *in vivo* the viability of metanephroi following its vitrification and subsequent transplantation. To date, we provide in this thesis the first success in the long-term metanephroi banking, which demonstrated that warmed renal primordia are able to grow and undergo differentiation following its allotransplantation into non-immunosuppressed rabbit hosts. Developed renal structures following cryopreservation exhibited mature glomeruli and hydronephrosis, which proved its filtering capacities. In addition, here we extend our previous data [275] showing that proper endocrine function in nascent kidneys is not impaired by metanephroi vitrification, as renin and erythropoietin gene expression levels were indistinguishable regardless of the metanephroi origin and comparable to control kidneys (Figure 26). Such results are consistent with previous literature showing the endocrine potential of the metanephroi-developed kidneys [156,157,163,165].

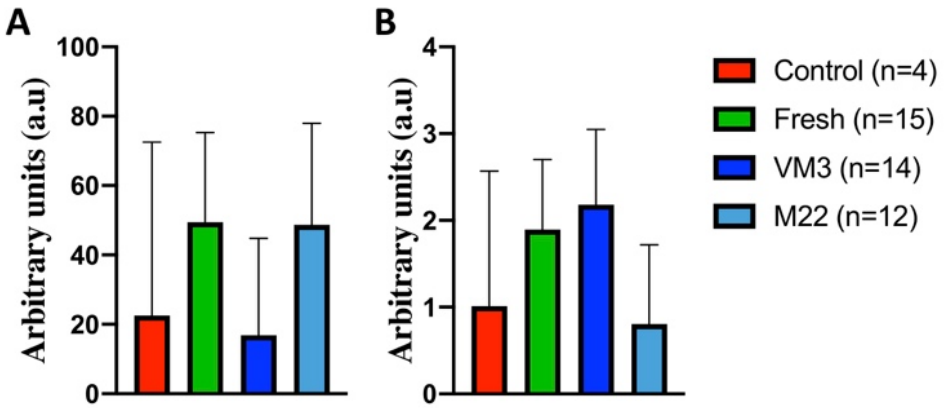

Figure 26. RT-qPCR analysis of renin (A) and (B) erythropoietin transcript expression in coetaneous control kidneys with those developed from fresh and vitrified metanephroi. The experimental group (control vs fresh vs VM3 vs M22) not have a significant effect on the renin (p= 0.64) and erythropoietin (p=0.52) expression. The metanephroi age (E15 vs E16) not have a significant effect on the renin (p= 0.32) and erythropoietin (p=0.62) expression, so the data has been shown according to the experimental group. Control: Coetaneous kidneys developed from undisturbed metanephroi. Fresh: Kidneys developed from transplanted metanephroi immediately after its dissection. VM3: Kidneys developed from transplanted metanephroi following vitrification using the VM3 medium. M22: Kidneys developed from transplanted metanephroi following vitrification using the M22 medium.

The triumph achieved in the long-term metanephroi cryopreservation could be attributable to some reasons: (i) the use of vitrification instead of slow-freezing strategies, in which ice nucleation and expansion is the single most critical factor that severely restricts the tissue survival [9,187]; (ii) the choice of vitrification mediums, which are low toxic and well-suited for renal tissue preservation, and include two novel antinucleating substances called “ice blockers” that dramatically reduces the probability of ice formation [187,192,196]; (iii) the use of cryotop® devices, which based on the “minimum drop size” strategy extremely minimize the volume of the sample and thus increase the cooling rates and the probability of vitrification [235,276]; and (iv) the reduced size of the renal primordia, that consent a good penetration and diffusion of the cryoprotectants [201], allowing to achieve high rates (> 80%) of cell survival (Figure 27).

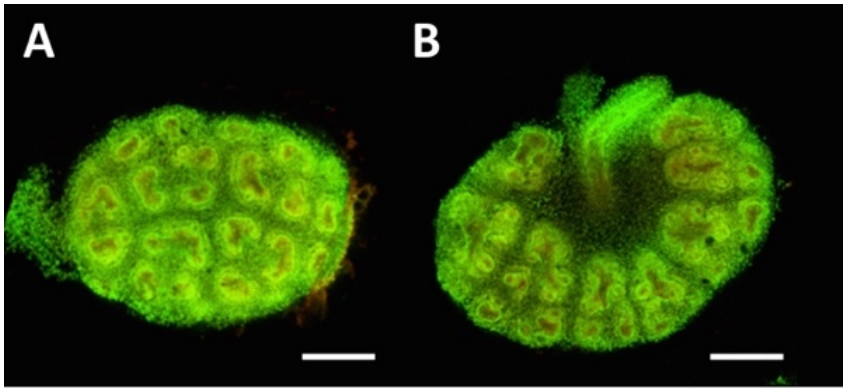

Figure 27. Metanephroi viability analysis through confocal microscope [137]. Cell viability was evaluated using fluorescent staining: SYBR-14 (live) and propidium iodide (dead). (A) Fresh metanephros. (B) Vitrified-warmed metanephros.

If the organ supply and demand could be balanced using embryonic kidneys, this cryopreservation approach can dissociate the time of the organ retrieval and its transplantation, allowing its distribution to patients in need to any desired location and guaranteeing an adequate inventory control and quality assurance [9–11]. This is today one of the most important health challenges of the twenty-first century regarding the transplantation of mature organs from donors [9].

However, if the metanephroi transplantation become a therapeutic reality in the future, crossing the species barrier would be necessary. Each year more than 73 million abortions from unintended pregnancies occur [277]. However, although the procurement of cadaveric human embryonic tissue for therapeutic treatments is legally permitted, regulated, and supported by medical responsibility [278], the intricate donation process coupled with ethical concerns results in a scarce availability of

transplantable embryonic tissues [278,279]. Besides, when the tissues are given, they exhibit inevitably intrinsic differences between each patient and gestation week, hindering the standardization of quality procedures [279]. Therefore, although proof of concept already exists for human metanephroi allotransplantation [143], the precise organ-development timing and structural integrity required to transplant metanephroi successfully, makes allogeneic sources not feasible in the long run. Fortunately, compared with adult kidney transplants, transplanted metanephroi exhibited a decreased immunogenicity, requiring lower immunosuppression to survive [143]. Hence, metanephroi xenotransplantation can be successfully carried out across a concordant xenogeneic barrier or highly disparate barrier if hosts are immunosuppressed [136,138,143,162,239]. Our experience with metanephroi xenotransplantation, previously reported [137] and now increased, supposes a total of 133 mouse metanephroi (ranging from E13.5 to E15.5) transplanted into rabbit hosts, 156 pig metanephroi (ranging from E26 to E35) transplanted into rabbit hosts, and 75 rabbit metanephroi (E15 and E16) transplanted into goat hosts. In summary, only 12 (9.0%) mouse-to-rabbit, 21 (13.5%) pig-to-rabbit and 3 (4.0%) rabbit-to-goat metanephroi grew despite hosts were treated with methylprednisolone, an effective inductor of a systemic immunosuppressed state both in rabbits [280] and goats [281]. Two could be the reasons that explain this low rate of metanephroi growth: (i) an insufficient immunosuppression regimen, or (ii) an excessive time between transplantation and post-mortem recovery, which can lead to hydronephrosis and regression of the transplant. In the first case, other immunosuppression therapies, which have been used in our group to successfully allotransplant mature organs (i.e. tacrolimus [282]), could provide alone or in combination more suitable immunosuppression regimens to sustain the metanephroi xenogeneic transplantation. In the second case, the use of trophic factors or growth-promoting substances, such as SC, may exert a positive effect on the metanephroi growth, which also can be monitored *in vivo* using the previously described renal scintigraphy technique.

Following the aim of transfer the metanephroi transplant to the clinic, plausible translational standpoints must be established. Here we present novel approaches that should bridge this still experimental path to the real cases of patients. In the present thesis, we have attempted to document our knowledge, as well as ignorance, about the therapeutic potential of the metanephroi transplantation to solve the precarious situation of the ESRD (see Annex I). Having in hands the new tools described throughout this thesis, future preclinical studies become more feasible, which makes the clinical translation of the technique one step closer. We must not forget that renal transplantation required more than half a century to reach its full clinical application. Hence, metanephroi transplantation should be viewed with the same hope.

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9. CONCLUSIONS

CONCLUSIONS

The conclusions of this thesis are:

→ The laparoscopic metanephroi transplantation is a feasible alternative to the current open surgeries, offering a minimally invasive and efficient approach that should facilitate the design of trials with larger mammals as a prior step to clinical application.

→ The addition of sildenafil citrate during the metanephroi transplantation exerts a growth-promoting action, enhancing both the graft size and glomerular filtration of the resultant renal structures.

→ The long-term metanephroi banking its possible by using ice-free cryopreservation techniques based upon vitrification, which can dissociate the time between the organ retrieval and its transplantation, allowing time to allocate the graft where necessary and guarantee an adequate inventory control and safety assurance.

→ Each of these endpoints has been considered successful after obtaining functional renal structures from allotransplanted metanephroi without immunosuppressive treatment. Therefore, having these new strategies in our hands, the therapeutic potential of the metanephroi transplantation should become a closer clinical reality.

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10. TESTIMONY: A PATIENT WITH KIDNEY FAILURE

LA FELICIDAD DE VOLVER A VOLAR

José Jordá ha perdido las alas en dos ocasiones

Ximo García Domínguez

Balones, 24 de octubre de 2020

LA FELICIDAD DE VOLVER A VOLAR

José Jordá ha perdido las alas en dos ocasiones

Ximo García Domínguez

¿Y ahora qué? Es la pregunta que se hizo José Jordá cuando abandonó el Hospital General de Alicante el 27 de enero de 1995, tras recibir uno de los golpes más duros de su vida: sus riñones habían dejado de funcionar.

Por aquel entonces, José tenía tan solo 24 años y, tras haber terminado la carrera de educación primaria unos meses atrás, compaginaba sus clases como monitor de futbol sala y baloncesto con una intensa actividad deportiva profesional. Aquel día de invierno, acudió al hospital por una molesta rigidez en los dedos de las manos y los gemelos de las piernas, algo que asoció a la intensa actividad deportiva que practicaba. Sin embargo, lo que reveló la primera analítica fue solo el principio de un mal presagio, que se fue haciendo cada vez más real a medida que se sucedían las diferentes pruebas: José sufría insuficiencia renal idiopática en etapa avanzada. Solo un trasplante renal le devolvería la vida que, sin previo aviso, acababa de dejar atrás.

Desafortunadamente, la pionera técnica del trasplante renal, establecida en 1954 por Joseph Murray, ganador del Premio Nobel, tenía un factor limitante: la disponibilidad de órganos. Se estima que más de 5 millones de personas podrían necesitar un trasplante renal, según un estudio publicado en la prestigiosa revista *Lancet.* Sin embargo, la Organización Mundial de la Salud registra menos de 100.000 intervenciones anuales. Hasta que hubiese un riñón disponible, habría que esperar. “Estaba convencido de que todo aquello era una broma, no podía ser verdad”, recuerda José. “¿De verdad esto va conmigo?”, se preguntaba. No quedaría lugar para las dudas cuando pocos días después acudió a su primera cita con “la máquina” (así la llama) de hemodiálisis. Con esta nueva compañera pasaría más de 12 horas semanales. “A pesar de que te mantiene con vida, te corta las alas”, cuenta José. “Son horas en las que intentas no pensar, solo quieres que pase lo más rápido posible e intentar no marearte”, explica. Así empezaría una de las etapas más duras que José puede recordar, en la que amigos y familiares fueron las piezas clave para que, de entre todas las opciones que le pasaban por la cabeza, siempre escogiese seguir adelante. Privado de toda función renal, José carecía de la capacidad para eliminar el exceso de líquidos corporales, por lo que se veía obligado a escupir la mayor parte del agua que utilizaba para mojarse la boca. No obstante, en dichas circunstancias, hasta el agua contenida en los alimentos podía convertirse en un problema. Más de 4 litros de líquido acumulado le eran retirados en cada sesión de diálisis, lo que aliviaba el daño fisiológico que ello implicaba para el resto de órganos y sistemas. José tuvo que renunciar a su vida para luchar por ella. Sin embargo, fue durante las sesiones de diálisis cuando José conoció a Marisa, la enfermera que acabaría convirtiéndose en su mujer y su principal apoyo para poder volar, incluso sin alas. Paradójicamente, una de cal y otra de arena.

Hasta que, el 28 de febrero de 1996, a las 11 de la noche, sonó el teléfono: José era uno de los candidatos para el próximo trasplante de riñón. Tras realizarse las pruebas de compatibilidad, y una noche sin dormir, José recorría a las 9 de la mañana los últimos metros camino de quirófano, hacía una operación de casi 4 horas que le devolvería "las alas" a golpe de bisturí. "Solo quería despertarme ya", pensaba. Cuando lo hizo, vio que junto a su familia le esperaba un apetecible zumo de frutas. "¿Puedo beberme eso?", se extrañó. Al rato, tuvo la agradable percepción que le liberaría de "la maquina": el catéter, con el que se encontraba sondado, drenaba orina… Su orina. Aquel fue el primer síntoma de cotidianidad que José experimento después de mucho tiempo y, poco a poco, los días se le llenaron de vida. Ahora, José tiene casi 50 años, es padre de dos hijos, y disfruta como maestro en un colegio de Torrevieja (Alicante) desde hace más de 20 años. **Aquel nuevo órgano era un contenedor de vida, unas nuevas alas.**

José no tuvo que volver a entrar por la puerta de urgencias hasta un año después. Pero cuando lo hizo, su temperatura corporal sobrepasaba los 40ºC, víctima de un rechazo agudo. Aunque aquel episodio fue revertido, le hizo asimilar que aquellas alas con forma de habichuela no durarían para siempre… Pero no fue lo único que se le pasó por la cabeza. Decidió que, si volvía a caer enfermo, quería estar lejos de casa para evitar el sufrimiento de su familia. No quería generar más enfermos por el camino. Afortunadamente, no tuvo que volver a planteárselo durante muchos años.

El primer signo del fallo renal se manifestó hace escaso tiempo. Hoy en día, se sabe que la enfermedad renal está profundamente asociada a un aumento de los factores que promueven la coagulación de la sangre, incrementando así en gran medida el riesgo de sufrir trombosis. Rondaba el 2015 cuando José sufrió un episodio de tromboembolia pulmonar que lo obligó a marcar el teléfono de emergencia por falta de oxígeno. Aquella noche la pasó en la Unidad de Cuidados Intensivos, pero no sería la última. Las altas dosis de heparina utilizadas para tratar la patología, desencadenaron una hemorragia interna que provocó la pérdida de más de 3 litro de sangre cuando José fue intervenido de urgencia. "Si la próxima hemoglobina no le sale bien, avisad a su mujer y decirle que no hemos podido hacer nada más", logró escuchar José a duras penas con los ojos cerrados. Afortunadamente, aquella hemoglobina salió como se esperaba (o, mejor dicho, se deseaba). El segundo aviso tardó tres años más en llegar. Preso de un profundo agotamiento físico, José tuvo que empezar a dar sus clases desde la silla. Varias trasfusiones de sangre no bastaron para acabar con una anemia "de caballo", cuyo origen no confundía a los médicos… Había muchas formas de dar una notica, pero ninguna era buena para aquella ocasión: el riñón se estaba parando. Eran finales de septiembre del año pasado, cuando José fue cateterizado y entró de nuevo en la sala de hemodiálisis. Allí no encontró viejos amigos, salvo uno: "la maquina". José había perdido las alas de nuevo.

"En la sala de diálisis, reconozco que me daba cierta vergüenza comentar con los demás pacientes que mi riñón había tardado en fallar casi 24 años, en contra de los 5-10 que se pronosticaron", reconoce José agradecido. Por suerte, a diferencia de la última vez, los trámites para quedar a la espera de un nuevo trasplante habían empezado unos meses antes que la diálisis. Por ello, cuando recibió una llamada con el típico número largo del hospital, para que acudiese a la consulta de coordinación de trasplantes a mediados del pasado diciembre, un rayo de esperanza iluminó el alma de José. Sin embargo, aquel día, aún con la sonrisa congelada en los labios, José trataba de asimilar las palabras que acababa de oír: no habría nuevo trasplante. Dada la escasez de órganos, existe un riguroso protocolo de donación y trasplante que garantiza la mayor efectividad de la donación. Según éste protocolo, el estado de salud de José no cumplía con los criterios de inclusión en la lista de espera para un trasplante renal. Estaba fuera de juego.

Hoy por hoy, personas como José están condenadas de por vida a seguir encadenadas a la máquina de diálisis. Vivas, pero sin vivir, sin poder volar. Se trata de una forma de sumarle más años a la vida, pero no más vida a los años. “En esta situación, la cabeza te juega malas pasadas”, reconoce José. “El apoyo constante de mi familia y mis amigos es lo que me ayuda a seguir adelante: ellos son quien me empujan”, afirma. Aunque en algunos momentos haya estado más cerca de la muerte, José es a día de hoy una persona luchadora cuyas ganas de vivir lo mantienen con vida. Afirma que la esperanza es lo último que se pierde, y que ninguna causa está perdida mientras haya gente luchando por resolverla. En este sentido, la Universitat Politècnica de València y el Hospital Politècnic i Universitari La Fe, apuestan por el trasplante laparoscópico de riñones embrionarios (metanefros) como una posible solución para garantizar un suministro ilimitado de órganos para el trasplante clínico. “Los avances son sorprendentes y los resultados esperanzadores”, comenta José ilusionado. “Además, del primer trasplante salí con 47 grapas, nada que ver con una intervención laparoscópica”, explica. Además, asegura que estaría dispuesto a recibir un trasplante de metanefros si esta técnica llegase a contemplarse clínicamente.

Por otro lado, tras haber perdido la función del primer y único trasplante, José es consciente de que solo fue una solución temporal. “Encontrar una solución alternativa que sea efectiva y definitiva supondría un antes y un después para la gente que dependemos de la diálisis”, anhela José. Sin embargo, el trasplante de metanefros se encuentra todavía en su fase de investigación preclínica, desde donde avanza muy lentamente debido a la falta de recursos. Hasta la fecha, la investigación ha sido financiada en su mayor parte por la Asociación para la Lucha Contra las Enfermedades Renales (ALCER), de la que José es socio desde hace casi 25 años. Aunque no le gusta mezclar la sanidad con la economía, José considera que el gasto sanitario de las sesiones de diálisis cae en saco roto. “La investigación es una inversión a largo plazo; debería potenciarse la búsqueda de nuevas alternativas que permitan sustituir el elevado coste de las diálisis, pero también incrementar nuestra calidad de vida”, opina José.

A la espera de nuevas alternativas al trasplante para restablecer la función renal, aquella vida en la que José podía disfrutar de beber tranquilamente un vaso de agua queda únicamente relegada al recuerdo. Mientras tanto, aunque de momento sobrevive junto a “la maquina” de diálisis, José no pierde la esperanza de que en algún momento le den vía libre para poder optar de nuevo a un trasplante. Y que salga bien. Y recuperar las alas. Otra vez.

José Vicente Jordá Vicens
Balones, 18 de noviembre de 1970

Declara que el presente escrito es un fiel reflejo de su historia personal,
y autoriza su publicación en la tesis doctoral de Ximo García Domínguez.

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With Greg Fahy, the father of the vitrification.