Iacobazzi, D., Rapetto, F., Albertario, A., Swim, M. M., Narayan, S., Skeffington, K., Salih, T., Alvino, V. V., Madeddu, P., Ghorbel, M. T., & Caputo, M. (2020). Wharton's Jelly-Mesenchymal Stem Cells-engineered conduit for pediatric translation in Heart Defect. *Tissue Engineering, Parts A, B, and C*.
<https://doi.org/10.1089/ten.TEA.2020.0088>

Peer reviewed version

Link to published version (if available):
[10.1089/ten.TEA.2020.0088](https://doi.org/10.1089/ten.TEA.2020.0088)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via

Mary Ann Liebert at <https://doi.org/10.1089/ten.TEA.2020.0088> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

**Wharton's Jelly-Mesenchymal Stem Cells- engineered conduit production for pediatric
Translation Congenital Heart Defects correction**

Dominga Iacobazzi, PhD; Filippo Rapetto, MD; Ambra Albertario, PhD; Megan M Swim, PhD; Srinivas Narayan, MD; Katie Skeffington, PhD; Tasneem Salih, MSc; Vincenza Valeria Alvino, MSc; Paolo Madeddu, MD; Mohamed T Ghorbel, PhD*, Massimo Caputo, MD*

Bristol Heart Institute, Translational Health Sciences, University of Bristol, Bristol Royal Infirmary, Bristol, UK.

* Co-senior authors.

Corresponding Authors

Dr Mohamed Ghorbel
Bristol Heart Institute,
University of Bristol, Bristol, UK
Electronic address: M.Ghorbel@bristol.ac.uk

Prof Massimo Caputo
Bristol Heart Institute,
University of Bristol, Bristol, UK
Electronic address: M.Caputo@bristol.ac.uk

Running title: WJ-MSCs-engineered graft for pediatric PA reconstruction

ABSTRACT

The materials available for the Right Ventricular Outflow Tract (RVOT) reconstruction in patients with Tetralogy of Fallot (TOF)/pulmonary atresia come with the severe limitation of long-term degeneration and lack of growth potential, causing right ventricular dysfunction, aneurysm formation and arrhythmias, thus necessitating several high-risk reoperations throughout patients' life.

In this study, we evaluated the capacity of Mesenchymal Stem Cells (MSCs) derived from the Wharton's Jelly (WJ-MSCs), the gelatinous inner portion of the umbilical cord, to grow and recellularize an extracellular matrix (ECM) graft in our optimised xeno-free, good manufacturing practice-compliant culture system. WJ-MSCs were phenotypically and functionally characterised by flow cytometry and multi-lineage differentiation capacity, respectively. The typical MSCs immunophenotype and functional characteristics were retained in our xeno-free culture system, as well as the capacity to grow and engraft onto a naturally occurring scaffold. Wharton's Jelly MSCs, from both human and swine source, showed excellent capacity to recellularize ECM graft producing a living cell-seeded construct.

In addition, we have provided an *in vivo* proof of concept of feasibility of the cellularised conduit, engineered with swine Wharton's Jelly MSCs, to be used in a novel porcine model of main pulmonary artery reconstruction, where it showed good integration within the host tissue.

Our study indicates that the addition of WJ-MSCs to the ECM scaffold can upgrade the material, converting it into a living tissue, with the potential to grow, repair and remodel the RVOT. These results could potentially represent a paradigm shift in paediatric cardiac intervention towards new modalities for effective and personalised surgical restoration of pulmonary artery and RVOT function in TOF/pulmonary atresia patients.

Key words: Congenital heart disease, right ventricular outflow tract reconstruction, stem cells, swine model, tissue engineering

INTRODUCTION

Surgery of Congenital Heart Defects (CHDs) has made fundamental achievements in the last decades, but there are still challenges related to the material used for correction (1). Premature failure of these grafts results in repeated operations during a patient's life (2). Amongst these conditions, Tetralogy of Fallot (TOF) is the most common complex CHD. In its most severe form, TOF can also include atresia of the pulmonary artery and valve, requiring a total replacement of the RVOT. Nevertheless, nearly all conduits/patches used for pulmonary artery and RVOT reconstruction require replacement because of lack of growth potential and long-term degeneration, causing right ventricular dysfunction, aneurysm formation and arrhythmias (3, 4).

Homograft have long been conduits of choice for RVOT reconstruction, however their limited availability, high costs and potential for allosensitization make them a less than ideal option (5-7). Alternate prosthetic conduits have been developed over the years, including Contegra (bovine jugular vein), porcine xenografts, or synthetic materials such as Dacron (polyethylene terephthalate) and Gore-Tex (polytetrafluoroethylene, PTFE). In particular, the latter one has been adopted as preferred choice by several institutions, due to its ready availability, low cost and lack of potential sensitization. However, the performance in terms of time to conduit explantation and development of conduit stenosis still remains comparable to the homograft, therefore there is no real clinical advantage other than cost/availability benefits (8).

Hence, alternative approaches, which meet the requirements of biocompatibility, non-immunogenicity and ready availability of an ideal scaffold, are needed.

We have previously presented Tissue Engineering (TE) as a promising approach to overcome the limitation of current treatments. By combining the use of a porcine decellularized small intestinal submucosa (SIS), a naturally occurring and a clinically approved extracellular matrix (ECM) scaffold, and perinatal autologous mesenchymal stem cells (MSCs), we have created cardiovascular grafts, in the form of small size conduits and cardiac patches, with demonstrated superior regenerative properties and cardiovascular performances *in vivo* compared to the acellular counterpart (9-11).

In the recent years, stem cells from foetal-associated tissues, such as placenta, amniotic fluid, amniotic membrane and umbilical cord have gained increasing popularity thanks to their

immediate availability at birth, which makes them ideal candidates for corrections of congenital pathologies (12-15).

Congenital anomalies of the heart and its vessels are among the diseases that can benefit from the therapy with umbilical cord derived MSCs. In particular, the Wharton's Jelly, the mucous proteoglycan-rich matrix surrounding the umbilical cord vessels, has been identified as the region of the umbilical cord homing the largest content of MSCs. These cells are accessible and endowed with extended plasticity, self-renewal capacity, long-term expansion and differentiation potential, as well as immunoprivileged properties (16-19). All these advantageous characteristics, in addition to the demonstrated capacity to form an optimal neo-matrix with excellent mechanical properties, make WJ-MSCs a promising and readily available cell source for cardio-vascular tissue engineering (20). Several experimental studies have been conducted so far to explore the feasibility of using WJ-MSCs to develop living materials for replacement of cardiovascular structures, mainly in the form of patches, heart valves and conduits (21-24). However, they have only provided an *in vitro* evidence of successful fabrication of biologically-active materials, with the exception of one pre-clinical case where a WJ-MSCs based heart valve has been validated in a large animal model (25).

With the present study, for the first time, we have further upgraded the *in vitro* manufacturing process of the WJ-MSCs- engineered vascular conduit to clinical grade, good manufacturing practices (GMP)- compliant standards, which would be essential for the first in human clinical trial. Pilot *in vivo* data suggest that the engineered WJ-MSCs SIS (CorMatrix®) can be safely implanted in the main pulmonary artery position of a porcine animal model, indicating potential suitability for TOF/pulmonary atresia surgical correction in infants.

METHODS

Ethics

Human umbilical cord samples were collected at St. Michael Hospital (Bristol, UK), under informed consent from the mother, in accordance with the licence approved by the Southwest Research Ethics Committee (11/HO107/4). The investigation conformed to the principals outlined in the Declaration of Helsinki.

Porcine umbilical cord samples were collected within 24 hours after birth from Landrace female piglets (average weight 1.5kg) sacrificed using schedule one, following the guidelines of the UK Home Office. Three to four-week-old female Landrace pigs (15 kg in body weight) were employed in the *in vivo* graft implantation studies under the UK Home Office ethical approval PPL 30/3019. Animals were treated in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health in 1996 and conforming to the “Animals (Scientific Procedures) Act” published in 1986.

Isolation and expansion of MSCs

Umbilical cord samples were obtained from natural labour or caesarean delivery of termed pregnancy women. After few washings in phosphate-buffered saline (PBS, Life Technologies), the umbilical cord was cut open and vessels were removed. ~~and~~ MSCs were isolated by mechanic dissociation of the cord by removing 2 -3 cm² segments from the inner gelatinous tissue. The finely minced tissues were distributed in a sterile petri dish and the culture medium poured slowly and gently, so as not to detach the fragments, which were then cultured in DMEM (Life Technologies) supplemented with 10% Hyclone Fetal Bovine Serum (FBS, Thermo Scientific) and FGF (2.5 ng/μL, Peprotech) at 37°C in a humidified 5%CO₂ incubator. Fresh medium was replaced after 5 days and then changed every 3 days until the visible out-growth WJ-MSCs were observed. Upon reaching 70-80% confluency, cells were detached using Trypsin-EDTA (0.05%, Life technologies) and passaged to new flasks.

The same protocol was applied to the isolation of WJ-MSCs under GMP conditions, whereby each culture medium component was replaced by the equivalent GMP product (GMP PBS, Sigma Aldrich; CTS StemPro MSC SFM DMEM, Life Technologies; Trypsin-EDTA, 0.05% Roche).

Flow Cytometry

Fluorescent-Activated Cell Sorting analysis was used to determine cell surface markers expression at early passage (2) and later (4-5). The protocol was performed as previously described by our group (10) (9). The following primary antibodies were used for human WJ-MSCs: CD105 (1:20, R&D System), CD90 (1:5, R&D System), CD45 (1:20, R&D System), CD31 (1:20, BD Pharmingen), CD34 (1:40, eBioscience), CD73 (1:50, BD Pharmingen), CD44 (1:20, BD Pharmingen), CD40 (1:20, BD Pharmingen), HLA-DR (1:20, BD Pharmingen), CD166 (1:100, BD Pharmingen), CD 79alpha (1:10, BD Pharmingen), CD29 (1:5, BD Pharmingen), CD14 (1:10 BD Pharmingen). Primary antibodies used against porcine WJ-MSCs were as follow: CD31 (1:10, Bio-Rad), CD44 (1:600, eBioscience), CD45 (1:25, Bio-Rad), CD73 (1:10, R&D Systems), CD90 (1:20, Biolegend), CD105 (1:5, LSBio). Analysis was performed on a NovoCyt flow cytometer (ACEA Bioscience, Inc) using NovoExpress (ACEA Bioscience, Inc) for data collection and FlowJo (TreeStar, Ashland) for analysis.

In vitro multilineage differentiation

In vitro multi-lineage differentiation into osteocytes, adipocytes and chondrocytes was performed with MSCs at passage between 3 and 5. Cells were cultured in alpha-MEM medium with specific StemXVivo supplement kits (R&D system) for different time points. Osteogenic differentiation was assessed after 3 weeks of culture using Alizarin Red (Sigma-Aldrich) to detect calcium deposition. Oil Red O staining (Sigma-Aldrich) was used to detect lipid accumulation of cells undergoing 2 weeks of adipogenic differentiation. Alcian Blue staining (Sigma-Aldrich) was used to determine chondrogenic cartilage formation after 3 weeks of cell culture.

In vitro functional studies

All *in vitro* experiments assessing differences throughout expansion were set up with cells at P2, P4, P6, P8, P10 and P12 (P4 to P12 for senescence assay) under the same experimental settings. CFU-F (colony forming unit-fibroblast) assay was performed by seeding cells at very low density (100 cells/cm²) in a culture petri dish. After 2 weeks, the dishes were stained with 3% Crystal violet (Sigma) and the number of colonies was counted. For proliferation assessment, the BrdU immunofluorescence assay (Roche) was used, according to the manufacturer's instructions. The β -galactosidase Senescence Detection kit (Calbiochem) was used as a biomarker of senescence in

MSCs. The percentage of senescent cells was represented by the number of β -galactosidase stained cells in total population. All in vitro assays were performed in triplicate wells and in 6 independent experiments.

Graft cellularization and maturation in a bioreactor

Decellularized porcine small intestinal submucosa (CorMatrix® Cardiovascular, Inc, USA) was seeded with WJ-MSCs (p2 to p4) at a density of 2.5×10^5 /cm² and maintained under static conditions for 3 days. The engineered-graft was then stitched to the rotating arm of an InBreath bioreactor (Harvard Apparatus, USA) as to fashion a conduit-shape with the cells facing the outer side of the graft. The rotation was set at 1.5 rpm for the first 24 hours, and then at 2 rpm for 7 days, after which the viability of the seeded cells was assessed by a fluorescent viability/cytotoxicity assay (Life technologies) according to the manufacturer's instruction. Seeded grafts were imaged on a Zeiss Axio Observer.Z1 with Zen Blue software (Zeiss).

Mechanical testing of the engineered graft

Acellular and cell-seeded SIS pieces were analysed for mechanical properties using an Instron 3343B machine (Instron, UK) with a 100 N load cell and pneumatic grips. Crosshead speed was 100 mm/min. Samples were measured for tensile stress at break using a Bluehill software (Instron, UK).

In vivo implantation of the porcine WJ-MSCs- cellularized conduit in piglet main Pulmonary Artery

Two Landrace female pigs (3-4 weeks old) of 15-20 kg were employed in this study. The SIS was engineered with porcine WJ-MSCs, whose isolation, characterization and graft engineering were performed following the same protocol used for the human WJ-MSCs. Surgical procedures were performed under general anaesthesia (Ketamine/Midazolam/ Dexmedetomidine, Isoflurane) and neuromuscular blockade (Pancuronium Bromide), and the heart was accessed by median sternotomy. Cardiopulmonary bypass (CBP) was then established by cannulating the inferior and superior vena cava and the ascending aorta. On the beating heart 8 mm of the main pulmonary artery was excised just above the pulmonary valve up to the pulmonary artery bifurcation and the tissue engineered conduit implanted as an interposition graft. Animal cardiac functions were monthly monitored with Doppler Echocardiography and, after 6 months from

implantation, were scanned with a magnetic resonance (MRI) and euthanized. Pulmonary arteries were dissected from the heart and fixed in 4% PFA or fresh-frozen in liquid nitrogen.

Histological analysis

For paraffin embedding, samples were fixed in 4% PFA, washed in PBS, moved into cassettes (Histosette I, Simport), processed in a Thermo Excelsior AS, and embedded in a Thermo HistoStar machine. A Sandon Finesse 325 (Thermo) microtome was used to cut 5 μ m sections which were floated onto Menzel-Glaser SuperFrost Plus slides (Thermo). Slides were stored at 37°C overnight to dry completely before staining. Hematoxylin and eosin (H&E) and van Gieson's (EVG) stains were performed using a Shandon Varistain 24-4 (Thermo). Slides were removed from machine and mounted with DPX (distyrene, a plasticizer, in toluene-xylene; Sigma) with 24 x 50 mm cover glass (VWR). Slides were imaged on a Zeiss Axio Observer.Z1 with Zen Blue software (Zeiss). Von Kossa staining was carried out with a Silver plating kit (In Vitro Diagnostic Medical Device) for detection of microcalcification.

Fluorescent immunohistochemistry

Paraffin-embedded sections were deparaffinized by two changes of xylene and rehydrated through an alcohol gradient. A heated antigen retrieval with 10mM citrate buffer pH 6.0 was performed. Samples were blocked with 10% goat serum (Sigma-Aldrich) in PBS for 30 minutes at RT and incubated with the unconjugated primary antibodies (α -SMA, 1:100, SIGMA; Isolectin B4-Biotin 1:100, Life Technologies) overnight at 4°C. Fluorophore-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546, 1:400, Life Technologies) were incubated on the sections for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI (1:1000; Sigma-Aldrich) for 10 minutes at room temperature. Slides were mounted with Hardset mounting medium (Vectashield). Images were taken with a Zeiss Observer.Z1 fluorescent microscope.

Scanning Electron Microscopy (SEM)

PFA-fixed samples were washed in 0.1 M phosphate buffer and then fixed in osmium tetroxide in phosphate buffer. After washing in phosphate buffer, samples were dehydrated through an alcohol gradient ending with three changes of absolute alcohol. Samples were dried in a critical point dryer

(Leica EM CPD300). They were finally coated at 100 mA for 30 s using EMITECH K575X sputter coater and observed using a Quanta 400 FEI scanning electron microscope.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Samples were analyzed by Student's t test or One-way ANOVA followed by Tukey's HSD post hoc test. Results were considered significant if $p \leq 0.05$.

RESULTS

Isolation and characterization of human Wharton's Jelly- derived Mesenchymal Stem Cells (WJ-MSCs)

After vessels removal from the human umbilical cord received upon delivery, Wharton's Jelly segments were obtained by mechanical dissociation of the cord and placed into culture flasks. Outgrowing human WJ-MSCs from the adhering tissues were observed in 7 to 12 days (P0), after which time they proliferated rapidly in the expansion medium, acquiring a higher degree of morphological homogeneity throughout passages (**Figure 1**). Flow cytometry immunoprofiling demonstrated that all preparations exhibited the main typical MSCs surface markers (CD29, CD44, CD73, CD90, CD105) and lacked expression of the hematopoietic (CD34), leucocytes (HLA-DR, CD45), monocytes (CD14), B cell (CD79alpha), and endothelial cells markers (CD31) (**Figure 2 a, b**). The gold standard for functional characterization of MSCs is the ability to differentiate towards more than one cell type. Our results confirmed that WJ-MSCs have the potential to differentiate into adipogenic, osteogenic and chondrogenic phenotype, as demonstrated by Oil Red O, Alizarin Red and Alcian Blue staining respectively (**Figure 2c**).

Incorporation of WJ-MSCs in a clinical-grade scaffold and maturation of the tissue engineered conduit in a dynamic bioreactor

The feasibility of using WJ-MSCs as a cell source for cardiovascular tissue engineering was next investigated. We used an optimised method, previously developed and described by our group, to seed WJ-MSCs onto the SIS and grow the resulting cell-seeded construct under static then dynamic conditions. These latter were reproduced by accommodating the graft in a conduit shape fashion into a bioreactor. The generated flow, provided by rotating stirrers, ensures that nutrients and O₂ are homogenously diffused to the three-dimensional construct, thus priming the cells to the dynamic condition they would be exposed to *in vivo*. Human WJ-MSCs showed excellent cell attachment and growth as demonstrated by the calcein positive staining of the viable cells and the H&E histological staining of the nuclei that evenly populated the seeded scaffold (**Figure 3 a v, vi**). Scanning electron microscopy further showed the progressive tissue formation, with a confluent cell layer on the SIS surface. (**Figure 3 a viii**). In seeded conduits, a higher signal intensity of collagen was detected, as compared to unseeded conduit, meaning that WJ-MSCs were able to synthesize their own extracellular matrix components (**Figure 3 a iii, vii**). We confirmed

that the multicell layer maintained its original phenotype as showed by the expression of the MSCs marker CD44, after the culture of the conduit under the flow provided by the bioreactor (**Figure 3 b**). No difference was found among the mechanical properties of the WJ-MSCs-engineered constructs, in terms of strength and stiffness, as demonstrated by comparable values of Ultimate tensile strength and Young's modulus of elasticity (**Figure 3 c**).

Optimization of a GMP-grade culture system of the human WJ-MSCs engineered conduit for clinical translation in cardiac paediatric surgery

For the clinical translation of the WJ-MSCs- derived engineered vascular graft, we have next set up a standard operating procedure (SOP) to expand human WJ-MSCs under xeno-free, GMP-compliant conditions, an essential requirement for clinical applications to exclude the risk of transmission of unknown infectious agents.

Phenotypic analysis of WJ-MSCs cultured under GMP compliant conditions revealed that cells expressed the typical MSCs markers and lacked immune, hematopoietic and endothelial markers, with these features being retained after a cycle of freezing and thawing, a step that might be required to meet the surgical schedule (**Figure 4 a**). In addition, the multipotent differentiation capacity was conserved, after culture in adipogenic, osteogenic and chondrogenic-supplemented media in both fresh (**Figure 4 b**) and frozen cell culture (data not shown).

Moreover, cells functional behaviour was not affected by the change of medium composition. BrdU incorporation and CFU assay revealed that human WJ-MSCs cultured under GMP conditions exhibit a rather superior growth rate compared to standard, not xeno-free culture system, with this being statistically significant at P6 and P2/P8 respectively (**Figure 4 c, d**). The incidence of senescence was significantly lower at higher passages (P10, P12) in the GMP-condition compared to the standard (**Figure 4 e**). The cells proliferative capacity and engraftment on the SIS in GMP conditions was likewise retained, which is paramount for the clinical-grade realization of the engineered vascular graft using MSCs derived from Wharton's Jelly tissue (**Figure 4 f**).

Translation of the human WJ-MSCs-engineered conduit manufacturing process to the swine product

The standard operative protocol for isolation and expansion of human WJ-MSCs has been successfully adapted to obtain MSCs from porcine umbilical cord, to be used for the autologous implantation into the swine model. Flow cytometry of all cell lines analysed confirmed the typical MSCs phenotypical features and functional differentiation capacity of the swine WJ-MSCs of the swine WJ-MSCs (**Figure 5 a-c**). The ability of the swine cells to adhere and proliferate on the SIS, resulting into a mechanically sound tissue engineered conduit, was also confirmed (**Figure 5 d, e**).

In vivo integration and remodelling of swine WJ-MSCs -engineered conduit within the host pulmonary artery tissue

To assess the *in vivo* safety and efficacy of the engineered vascular conduit produced *in vitro* (**Figure 6 a, i-ii**), we have developed a new piglet model consisting of main pulmonary artery reconstruction (**Figure 6 a, iii**) with the WJ-MSCs derived conduit or the acellular counterpart. Pilot echocardiography data show that at 6 months follow up, the conduits were patent with no stenosis, rupture, or deformation (**Figure 6 b**).

Macroscopic observation of the explanted WJ-MSCs- seeded conduit showed smooth luminal surfaces without signs of thrombosis and tissue degradation (**Figure 6 a, iv, v**). Histological H&E images show extensive nucleation throughout the structure of seeded and unseeded grafts (**Figure 6 c**). Interestingly, however, a higher content of elastin, the major extracellular matrix component of the pulmonary artery, was visible in the seeded graft (**Figure 6 d**). No sign of calcification was detected either in the explanted seeded or acellular grafts (**Figure 6 e**). Moreover, only in the cellularised conduit, we could detect a highly organised layer of smooth muscle cells populating the tunica media, and a newly formed layer of endothelial cells, resembling the structure of the native pulmonary artery (**Figure 6 f**). In line with this latter finding, a confluent layer of endothelial cells could be detected through scanning electron microscopy of the luminal side of the cellularised graft, while this appeared patchy in the acellular graft (**Figure 6 g**).

DISCUSSION

In the present study, we showed the Wharton's Jelly as suitable source of MSCs for tissue-engineering cardiovascular replacement grafts. Furthermore, our pilot data provided an *in vivo* proof of concept that the WJ-MSCs engineered graft can be safely implanted in the main Pulmonary Artery position of a porcine animal model reproducing part of the clinical scenario of Tetralogy of Fallot/pulmonary atresia surgical correction in infants. Umbilical cord is becoming increasingly popular for regenerative medicine applications required soon after birth (12, 13). Particularly, the Wharton's Jelly is the home of a large number of MSCs that can be easily harvested and expanded. These cells exhibit higher plasticity, self-renewal capacity, differentiation potential and immunoprivileged properties than MSCs from other sources (16-18). Importantly, the availability of the umbilical cord at birth has the great advantage of providing an ethically acceptable source of cells that can be immediately harvested and expanded in order to provide a therapeutic tool for congenital defects that require a surgical intervention few weeks or months after birth, like TOF/pulmonary atresia correction.

While most of previous studies have demonstrated *in vitro* successful fabrication of biologically-active materials using WJ-MSCs (21-24), our study showed *in vivo* successful use of WJ-MSCs-based construct in a large animal model of main Pulmonary Artery reconstruction surgery. To our knowledge, only one previous pre-clinical study has demonstrated the implantation of WJ-MSCs based heart valve in a large animal model (25). Precisely, WJ-MSCs were seeded on biodegradable matrices to generate living trileaflet heart valves that have been successfully implanted in sheep models up to 20 weeks showing good functional performance and structural characteristics (25).

We have used the SIS, as the ECM scaffold to support the growth and maturation of WJ-MSCs into a living tissue. SIS (Cormatrix®) has received approval by both the Food and Drug Administration and European authorities for cardiac tissue repair and is being used in various clinical applications of cardiovascular surgery, thanks to its excellent physical properties, including resistance to deformation, easy handling and shaping, suture retention, good absorbability, and lack of immunogenicity [Web Ref. 1]. However, recent pre-clinical studies using the SIS fashioned as a trileaflet valved conduit in a pig model of thoracic aorta replacement showed that this model failed to remodel in a structural and anatomical manner, leading to early fibrosis and calcification (26). Similar concerns were raised by studies using SIS-ECM for valve

repair in children (27). Nevertheless, our group has previously demonstrated that, compared to the acellular products, the presence of the MSCs on the SIS favoured the remodelling and regeneration of the graft in piglet models of RVOT reconstruction and Left Pulmonary Artery replacement, respectively (10, 11). The animal model developed in this study, whereby the Main Pulmonary Artery is replaced by the engineered conduit, allowed us to optimize a surgical procedure suitable for pre-clinical and pediatric clinical application, while providing a proof of concept of the safety of our WJ-MSCs-seeded SIS.

The porcine species was chosen for its widely accepted recognition as a suitable animal model in cardiovascular research, owing to its similarity to humans in terms of cardiovascular anatomy and physiology (28, 29). Therefore, the ECM scaffold engineered with porcine WJ-MSCs was used for the *in vivo* study. The use of an allogeneic cellular product avoids the risks of cross-species immunogenic responses and of an increased susceptibility to infections that would derive from immunosuppressive therapy, with the ultimate effect of overshadowing any potential benefit related to the product.

The surrogate cell product we have produced for the pre-clinical study was validated through extensive characterization of the cells phenotype, function and capacity to integrate and give rise to a living tissue, equivalent to the human counterpart. The results of our pilot *in vivo* study indicates the safety of a decellularized scaffold repopulated with Wharton's Jelly- derived cells to reconstruct the main pulmonary artery and its capacity to grow and remodel in a large animal model. Compared to the acellular counterpart, the seeded conduit displayed a remarkably organised layer of Smooth Muscle Cells, which are most likely endogenous cells recruited from the recipient tissue as our previous study investigating the fate of the implanted donor cells showed that no cells were detected in the explanted graft at 4 months post-operation (9, 10).

Furthermore, the new endothelialisation observed on the luminal side of the graft supports the paracrine activity typically associated to MSCs in driving vascular cells recruitment from the adjacent vascular tissue, which we also reported in our previous studies where a high content of paracrine and trophic factors released by MSCs was detected in the culture medium (9, 10).

While a pre-clinical controlled randomized efficacy/safety study based on the proposed technology is currently ongoing, we have pre-emptively established a successful manufacturing process, from WJ-MSCs isolation throughout the production of the graft, in GMP-compatible conditions, strictly

required for the clinical application of cell based medical produces. The results obtained from a systematic comparison between WJ-MSCs cultured under standard procedures, involving the use of xenogenic supplements, and GMP conditions showed that the optimised xeno-free, GMP compliant culture system did not compromise the quality of the cells and the final product, suggesting that WJ-MSCs can easily fulfil the need of clinical scale production. This is a remarkable result, considering that initial cell adhesion and growth in xeno-free cultures still remains a major problem for many cell types when switching from animal-derived products.

Limitations

Although the Proof of Concept of suitability of the Wharton's Jelly-Mesenchymal Stem Cells-engineered conduit for TOF correction showed favourable outcome, a full-scale study is needed to prove the efficacy and superiority of our tissue engineered product compared to the currently used acellular SIS-ECM. A controlled randomized efficacy/safety study based on this technology is currently ongoing.

CONCLUSIONS

Our study demonstrates that the functionalization of the SIS scaffold with WJ-MSCs can upgrade the material, converting it into a living tissue, with the potential to grow, remodel and repair when implanted into an in vivo porcine model of pulmonary trunk replacement. Furthermore, we have established a GMP-compliant protocol for a human WJ-MSCs-derived vascular graft for its clinical translation for the first-in-human trial in neonates and infants born with TOF/pulmonary atresia.

Author contributions

DI, MTG, MC conceived and designed the research. DI, MTG, FR, AA, MMS, KS, and TS performed experiments. DI, VA, SN, PM, MTG, MC analyzed data and interpreted results of experiments. DI, MTG and MC prepared and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Dr Huidong Jia for his technical advice and Dr Andrew Herman and Lorena Sueiro Ballesteros from the University of Bristol Flow Cytometry Facility for their

technical assistance. We are grateful to the staff of the University of Bristol Large Animal Surgical Facility for their help.

Funding

This study was supported by grants from the Sir Jules Thorn Charitable Trust, the Enid Linder Foundation and the British Heart Foundation.

Conflicts of Interest

The authors have no conflicts of interest to disclosure.

REFERENCES

1. Erikssen G, Liestol K, Seem E, Birkeland S, Saatvedt KJ, Hoel TN, et al. Achievements in congenital heart defect surgery: a prospective, 40-year study of 7038 patients. *Circulation* **131**, 337, 2015.
2. Raissadati A, Nieminen H, Haukka J, Sairanen H, Jokinen E. Late Causes of Death After Pediatric Cardiac Surgery: A 60-Year Population-Based Study. *J Am Coll Cardiol* **68**, 487, 2016.
3. Udink ten Cate FE, Sreeram N, Brockmeier K. The pathophysiologic aspects and clinical implications of electrocardiographic parameters of ventricular conduction delay in repaired tetralogy of Fallot. *J Electrocardiol* **47**, 618, 2014.
4. Davlouros PA, Kilner PJ, Hornung TS, Li W, Francis JM, Moon JC, et al. Right ventricular function in adults with repaired tetralogy of Fallot assessed with cardiovascular magnetic resonance imaging: detrimental role of right ventricular outflow aneurysms or akinesia and adverse right-to-left ventricular interaction. *J Am Coll Cardiol* **40**, 2044, 2002.
5. Selamet Tierney ES, Gersony WM, Altmann K, Solowiejczyk DE, Bevilacqua LM, Khan C, et al. Pulmonary position cryopreserved homografts: durability in pediatric Ross and non-Ross patients. *J Thorac Cardiovasc Surg* **130**, 282, 2005.
6. Tweddell JS, Pelech AN, Frommelt PC, Mussatto KA, Wyman JD, Fedderly RT, et al. Factors affecting longevity of homograft valves used in right ventricular outflow tract reconstruction for congenital heart disease. *Circulation* **102**, III130, 2000.
7. Baskett RJ, Nanton MA, Warren AE, Ross DB. Human leukocyte antigen-DR and ABO mismatch are associated with accelerated homograft valve failure in children: implications for therapeutic interventions. *J Thorac Cardiovasc Surg* **126**, 232, 2003.
8. Mercer CW, West SC, Sharma MS, Yoshida M, Morell VO. Polytetrafluoroethylene conduits versus homografts for right ventricular outflow tract reconstruction in infants and young children: An institutional experience. *J Thorac Cardiovasc Surg* **155**, 2082, 2018.
9. Iacobazzi D, Swim MM, Albertario A, Caputo M, Ghorbel MT. Thymus-Derived Mesenchymal Stem Cells for Tissue Engineering Clinical-Grade Cardiovascular Grafts. *Tissue Eng Part A* **24**, 794, 2018.
10. Albertario A, Swim MM, Ahmed EM, Iacobazzi D, Yeong M, Madeddu P, et al. Successful Reconstruction of the Right Ventricular Outflow Tract by Implantation of Thymus Stem Cell Engineered Graft in Growing Swine. *JACC Basic Transl Sci* **4**, 364, 2019.

11. Ghorbel MT, Jia H, Swim MM, Iacobazzi D, Albertario A, Zebele C, et al. Reconstruction of the pulmonary artery by a novel biodegradable conduit engineered with perinatal stem cell-derived vascular smooth muscle cells enables physiological vascular growth in a large animal model of congenital heart disease. *Biomaterials* **217**, 119284, 2019.
12. Bongso A, Fong CY, Gauthaman K. Taking stem cells to the clinic: Major challenges. *J Cell Biochem* **105**, 1352, 2008.
13. Pappa KI, Anagnou NP. Novel sources of fetal stem cells: where do they fit on the developmental continuum? *Regen Med* **4**, 423, 2009.
14. Loukogeorgakis SP, De Coppi P. Stem cells from amniotic fluid--Potential for regenerative medicine. *Best Pract Res Clin Obstet Gynaecol* **31**, 45, 2016.
15. Pipino C, Shangaris P, Resca E, Zia S, Deprest J, Sebire NJ, et al. Placenta as a reservoir of stem cells: an underutilized resource? *Br Med Bull* **105**, 43, 2013.
16. Bongso A, Fong CY. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's jelly of the human umbilical cord. *Stem Cell Rev Rep* **9**, 226, 2013.
17. Davies JE, Walker JT, Keating A. Concise Review: Wharton's Jelly: The Rich, but Enigmatic, Source of Mesenchymal Stromal Cells. *Stem Cells Transl Med* **6**, 1620, 2017.
18. Kim DW, Staples M, Shinozuka K, Pantcheva P, Kang SD, Borlongan CV. Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications. *Int J Mol Sci* **14**, 11692, 2013.
19. Wu M, Zhang R, Zou Q, Chen Y, Zhou M, Li X, et al. Comparison of the Biological Characteristics of Mesenchymal Stem Cells Derived from the Human Placenta and Umbilical Cord. *Sci Rep* **8**, 5014, 2018.
20. Breymann C, Schmidt D, Hoerstrup SP. Umbilical cord cells as a source of cardiovascular tissue engineering. *Stem Cell Rev* **2**, 87, 2006.
21. Schmidt D, Mol A, Neuenschwander S, Breymann C, Gossi M, Zund G, et al. Living patches engineered from human umbilical cord derived fibroblasts and endothelial progenitor cells. *Eur J Cardiothorac Surg* **27**, 795, 2005.
22. Kenar H, Kose GT, Toner M, Kaplan DL, Hasirci V. A 3D aligned microfibrillar myocardial tissue construct cultured under transient perfusion. *Biomaterials* **32**, 5320, 2011.

23. Schmidt D, Mol A, Breymann C, Achermann J, Odermatt B, Gossi M, et al. Living autologous heart valves engineered from human prenatally harvested progenitors. *Circulation* **114**, I125, 2006.
24. Schmidt D, Mol A, Odermatt B, Neuenschwander S, Breymann C, Gossi M, et al. Engineering of biologically active living heart valve leaflets using human umbilical cord-derived progenitor cells. *Tissue Eng* **12**, 3223, 2006.
25. Shinoka T, Ma PX, Shum-Tim D, Breuer CK, Cusick RA, Zund G, et al. Tissue-engineered heart valves. Autologous valve leaflet replacement study in a lamb model. *Circulation* **94**, II164, 1996.
26. Mosala Nezhad Z, Poncelet A, de Kerchove L, Fervaille C, Banse X, Bollen X, et al. CorMatrix valved conduit in a porcine model: long-term remodelling and biomechanical characterization. *Interact Cardiovasc Thorac Surg* **24**, 90, 2017.
27. Zaidi AH, Nathan M, Emani S, Baird C, del Nido PJ, Gauvreau K, et al. Preliminary experience with porcine intestinal submucosa (CorMatrix) for valve reconstruction in congenital heart disease: histologic evaluation of explanted valves. *J Thorac Cardiovasc Surg* **148**, 2216, 2014.
28. Suzuki Y, Yeung AC, Ikeno F. The representative porcine model for human cardiovascular disease. *J Biomed Biotechnol* **2011**, 195483, 2011.
29. Crisostomo V, Sun F, Maynar M, Baez-Diaz C, Blanco V, Garcia-Lindo M, et al. Common swine models of cardiovascular disease for research and training. *Lab Anim (NY)* **45**, 67, 2016.

Web References

1. Xue A, Sarkeshik A, Boyd WD, Perry P. *Tricuspid Valve Replacement Using CorMatrix® Extracellular Matrix Cylindrical Construct*. **2019**. Available from URL: doi:10.25373/ctsnet.9793169.