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Autologous Adipose Tissue-Derived Mesenchymal Stem Cells Introduced by Biliary Stents or Local Immersion in Porcine Bile Duct Anastomoses

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Abstract

Background: Biliary complications (strictures and leaks), represent major limitations in living-donor liver transplantation. Mesenchymal stem cells (MSC) are a promising modality to prevent biliary complications owing to immunosuppressive and angiogenic properties. Our goal was to evaluate the safety of adipose-derived MSC delivery to biliary anastomoses in a porcine model. Secondary objectives were defining the optimal method of delivery (intraluminal vs extraluminal) and to investigate MSC engraftment, angiogenesis, and fibrosis.

Methods: Pigs were divided into three groups. Animals underwent adipose collection, MSC isolation, and expansion. Two weeks later, animals underwent bile duct transection, re-anastomosis, and stent insertion. Group 1 received plastic stents wrapped in unseeded vicryl mesh; Group 2 received stents wrapped in MSC-seeded mesh; Group 3 received unwrapped stents with the anastomosis immersed in an MSC-suspension. Animals were sacrificed one month after stent insertion when cholangiograms and biliary tissue were obtained. Serum was collected for liver biochemistries. Tissue was used for hematoxylin/eosin/trichrome staining and

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immunohistochemistry for MSC markers (CD44 and CD34) and a marker of neo-angiogenesis (CD31).

Results: There were no intra-operative complications. One pig died on post-operative day three due to acute cholangitis. All others recovered without complications. Cholangiography demonstrated no biliary leaks and minimal luminal narrowing. Surviving animals exhibited no symptoms, abnormal liver biochemistries, or clinically-significant biliary stricturing. Group 3 showed significantly greater CD44 and CD34 staining, indicating MSC engraftment. Fibrosis was reduced at the anastomotic site in Group 3 based on trichrome stain. CD31 staining of Group 3 was more pronounced, supporting enhanced neo-angiogenesis.

Conclusion: Adipose-derived MSC were safely applied to biliary anastomoses. MSC were locally engrafted within the bile duct and may have beneficial effects in terms of fibrosis and angiogenesis.

Keywords

Stricture; vicryl; mesh; angiogenesis; fibrosis

INTRODUCTION

Biliary strictures after liver transplantation are common and can lead to considerable morbidity, hospitalization rates, procedural burden, and cost^{1–6}. A 2017 analysis of the A2All cohort concluded that 41% of living-donor liver transplantation (LDLT) recipients with a duct-to-duct reconstruction had a biliary complication (leak or stricture) within a year⁶. Nobuhisa et al. summarized data from 14359 liver transplants and found that the overall incidence of biliary stricture was 12% among deceased donor liver transplantation (DDLT) patients and 19% among LDLT recipients⁷. In another well-matched cohort study, biliary complications occurred in 34% of the LDLT cohort versus 17% of the DDLT cohort⁸. However, living LDLT continues to provide an important surgical option for patients with end-stage liver disease (ESLD). Advantages of LDLT over DDLT include: 1.) decreased death on the waitlist; 2.) more timely transplantation for patients with low MELD scores; and 3.) provision of a high-quality donor graft⁹. However, these advantages are off-set by a higher rate of technical complications as compared with DDLT. A multi-institutional study found that a higher rate of retransplantation in the LDLT group (15.9% vs. 9.3%) was mostly due to biliary complications¹⁰. Early stricture formation is associated with surgical technique while late stricture formation is typically attributed to local inflammation caused by factors such as ischemia and bile leakage^{11–13}.

The gold standard treatment for late biliary strictures (endoscopic retrograde cholangiopancreatography with balloon dilatation and stent placement) has a success rate of approximately 50%^{14–16}. Therefore, biliary strictures are still regarded as the “Achilles heel” for LDLT, requiring long-term, repeated treatments and may ultimately impair the function of the graft and endanger the patient’s survival.

Mesenchymal stem cells (MSCs) have shown strong immunosuppressive and angiogenic properties while concurrently having a strong safety record in kidney, heart, and GI tract

disease applications^{17–23}. Misra et al. deposited MSCs into the adventitia of the outflow vein at the time of arteriovenous fistula creation. The retention of the delivered stem cells on day 21 was evidenced by serial PET images of 89Zr-labeled cells. In this setting, MSCs experimentally reduced venous stenosis formation, and this technology is now being proposed for use in a clinical trial²⁴. Based on previous positive clinical studies in these other areas, including immunosuppressed individuals, we hypothesized that adipose derived MSCs can be safely applied to common bile duct anastomoses and have the potential to reduce anastomotic complications such as bile leaks and anastomotic stricture formation.

In this study, we developed a CBD anastomosis model in pigs and tested the safety of bile duct anastomosis treatment with MSCs. Our secondary objective was to define the optimum method of MSC delivery to the bile duct: intraluminal vs extraluminal.

MATERIALS AND METHODS

Study Design

Nine female domestic white pigs were divided into three groups, with three animals in each group. Group 1 received plastic biliary stents wrapped with vicryl-mesh as the control group; Group 2 received plastic biliary stents wrapped with MSC-coated vicryl-mesh; Group 3 received bare, unwrapped, plastic biliary stents and the anastomosis sites were immersed with an MSC-suspension.

All pigs had an initial body weight of 43–46 kg. After the necessary quarantine and acclimatization period, adipose tissue was collected for the isolation and culture of mesenchymal stem cells. Group 1 animal cells were frozen for future experimentation. Groups 2 MSCs were isolated and expanded to passage four and then seeded onto a 2cm × 1cm vicryl-910 scaffold at a density of 2×10^6 cells/cm² (total 4×10^6 cells). Group 3 MSCs were isolated and expanded to passage 4 then suspended in 3 mL of lactated ringer's solution then slowly applied to the anastomosis site.

Day 0 was defined as the day of stent insertion and/or topical cell application. Serum was collected on Day 0, Day 7, and Day 30 for measuring of liver enzymes, bilirubin, and albumin; on the day of euthanasia (Day 30), cholangiograms and biliary tissue specimens were obtained. Biliary tissue specimens were cut in cross-section including three sites (proximal, anastomotic, and distal). Paraffin-embedded tissue slides were made for HE staining, Trichrome staining, and immunohistochemical detection of CD44 (a marker of MSCs), and CD31 (a marker of neo-angiogenesis). Quantification and statistical analysis were performed using ImageJ and Graph Pad Prism software, respectively.

Animal Care

Fasting was performed overnight prior to surgery. Intramuscular Telazole and Xylazine injections were performed for sedation, subcutaneous Buprophine for analgesia, intramuscular Excede and intravenous Cefazolin for infectious prophylaxis²⁵. Following surgery, animals were transferred to the intensive care unit for 24 hours and then returned to the standard housing and a regular diet. Pigs were examined once daily for the duration of the study.

Adipose Tissue Harvest

For adipose harvesting, animals were sedated with Telazol+Xylazine. Anesthesia was maintained using Isoflurane. Vital signs were monitored continuously throughout the surgical procedure. After preparation and draping, an 8–10 cm cutaneous incision was made between the pig's axillary space medial to anterior limb. Scissors were used to dissect the subcutaneous fat. Approximately 10g of adipose tissue was harvested from the subcutaneous site (Figure 1A–B). The subcutaneous wound was closed with buried absorbable sutures (3.0 vicryl and 4.0 PDS) in a subarticular pattern and then covered by Dermabond and an Ioban dressing protects the wound (Figure 1C).

MSC Isolation, Culture, and Seeding

MSC isolation, culture, and characterization were pursued as previously described²³. Briefly, porcine fat was washed thoroughly in sterile lactated ringers (LR) augmented with penicillin/streptomycin. Curved mayo scissors were used to mince the adipose tissue. The tissue was then digested at 39C using collagenase A (Worthington Biochemical, Lakewood NJ) for 70 minutes with solution swirling every 5–7 minutes. Collagenase was neutralized using an equal volume of 5% human platelet lysate (hPL, Mill Creek Life Sciences, Rochester, MN) enhanced media and spun down for 10 minutes at 1000 RPM. The floating fat layer and supernatant were siphoned off and pellets were resuspended in fresh hPL media. Suspended cells were serially strained using 70-micron and 40-micron filters and then plated into polystyrene flasks for expansion. Passaging was performed using 5 minute exposure to TrypLE (GIBCO Biosciences, Gaithersburg, MD) followed by neutralization using hPL Media and 5 minutes of centrifugation at 1000 RPM. The trypsinized cell pellet was resuspended in new media and seeded into new flasks at a density of 20,000 cells/cm². Incubation at all steps was at 39C. Porcine MSC characterization was performed via tri-lineage differentiation for Oil-Red-O (adipocyte differentiation), Alcian Blue (chondrocyte differentiation), and Alazarin Red (osteocyte differentiation). Growth kinetics were performed to compare porcine MSC growth to that of clinical grade human MSCs. Live-dead staining using Ethidium Bromide (Red/Dead) and Syto-13 (Green/Alive) evaluated viability of MSC cultures. Cytokine secretion was quantified using the Luminex Platform Human Cytokine Panel I and II. CD34 expression was evaluated in MSCs by real-time RT-PCR, Western blot, and immunofluorescence staining at the time of MSC harvest to evaluate baseline expression of this characteristic MSC marker.

Group 2 MSC Seeding Procedure

A 2cm × 1cm strip of vicryl-910 woven mesh was cut from a larger sheet using sterile scissors. This scaffold was placed into sterile 50ml container with 40mLs of LR solution containing penicillin and streptomycin and placed in a rotator for 24-hours at 39C. The scaffold was then removed and placed into a 50-ml polypropylene culture tube containing 40mL of 5% hPL Media and strictly controlled dosage of autologous MSCs. The free floating MSC dosage was optimized using a cell counter. Each 4cm² stent contained 500,000 cells/cm² for a total of 2e10⁶ cells (Supplementary Figure 1). The culture tube was placed in an incubator at 39C on a 10–15-degree tilt for 4 days. At the end of the culture period the scaffold was removed and placed in LR solution and delivered to the operating

room 20 minutes prior to implantation. Visual confirmation of seeding was performed by staining a separately seeded scaffold with Ethidium Bromide (EtBr) and Syto-13 live-dead staining.

Group 3 MSC Processing Procedure

Cells were cultured to passage 3 until the operative day. Culture flasks were trypsinized using 10cc of TrypLE for 5 minutes at 39°C. The cell solution was neutralized using equal amounts of hPL 5% hPL Media. Neutralized solution was spun down at 1000 RPM for 5 minutes. Pelleted cells were resuspended in 1mL of fresh LR and counted using Trypan Blue staining on an automatic cell counter. Cells were reduced to a concentration of 2×10^6 cells per 3 mL of LR. Three mLs of suspended cells were transported to the OR for autologous application. The number of free floating cells applied to the bile duct (in the extraluminal group) was identical to the dose delivered within the MSC loaded matrix.

Common Bile Duct (CBD) Anastomosis & MSC Application

Group 1 and 2 CBD Surgery: The CBD was identified and transected centrally. The posterior wall was sutured with a running 7-0 PDS. A plastic biliary stent (0.4cm × 3cm, Figure 3A), with or without vicryl-mesh wrap (Figure 3B), was inserted into the bile duct lumen (Figure 3C). The anterior wall of the CBD was closed with a running 7-0 PDS suture. The fascia was closed with a running 1-0 PDS suture. The abdomen was closed in 2 layers. PDS and interrupted Ethibon was used to close the fascial layer and the peritoneum. Subcutaneous tissue was approximated using 2-0 vicryl. Skin was closed with 4-0 Monocryl in a continuous fashion and a wound dressing was applied. After surgery, all animals received continuous monitoring of vital signs until recovered from anesthesia and fully ambulatory.

Group 3 CBD Surgery: After identifying and transecting the CBD, the distal and proximal ends of the CBD were clamped. The MSC suspension was applied to immerse the transected bile duct for 10 minutes (Figure 3D). After inserting a bare, unwrapped, biliary stent and suturing the CBD, the fascia around the CBD was sutured into a pocket in which additional MSC suspension was added before the pocket was closed (Figure 3E-F). Thus, the anastomotic site of the CBD was completely immersed in the MSC-suspension.

Staining, Immunohistochemistry, and Immunofluorescence

Masson's Trichrome and Picro Sirius Red staining was performed in the standard fashion. For immunohistochemistry, paraffin-embedded tissue sections were antigen unmasked with citrate. After quenching of endogenous peroxidase, sections were blocked, and incubated with primary antibody against CD44, CD34, CD31, CD68, transforming growth factor beta (TGFβ), vascular cell adhesion molecule 1 (VCAM-1), or alpha-smooth muscle actin (SMA) overnight @ 4°C. The remaining steps were carried out using species-appropriate biotinylated secondary antibodies and an immunoperoxidase detection Kit (Vector Laboratories). Image J software was utilized to quantify the positive stained areas. For immunofluorescence, after fixation with paraformaldehyde and permeabilization with Triton X-100, cells were blocked with FBS, incubated with primary antibodies and then with fluorescence-conjugated secondary antibodies. Cells were washed and nuclei were stained

with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Coverslips were mounted onto glass slides and observed under confocal microscopy (Carl Zeiss, Jena, Germany). Images were acquired using Zeiss LSM software.

RT-PCR

Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed with 500 ng RNA using oligo (dT) primer and SuperScript III. PCR was performed in a volume of 20 μ l using Sybr Green Master Mix and the 7500 Real-Time PCR System (Applied Biosystems).

Immunoprecipitation and Western Blotting

Cell lysates were sonicated for 15 sec and centrifuged at 14,000g for 15 mins. 5–20 μ g of protein was loaded onto 4–20% Tris-Glycine gels, electrophoresed, and transferred onto nitrocellulose membranes (Scientific Laboratory Supplies) for blotting. The membrane was blocked, incubated with primary antibody against CD34, rinsed with TBST, and incubated with HRP-conjugated secondary antibody. After washing, the membrane was exposed to luminol (Santa Cruz Biotechnology) and developed.

RESULTS

MSC Characterization

Clinical grade human MSCs have been extensively characterized using tri-lineage differentiation as well as flow cytometry²³. The porcine MSC harvest procedure (Figure 1) is identical to that for humans, with the exception that a larger quantity of fat is harvested from pigs. We have found that the commercially available flow cytometry antibodies for animal MSC identification are substandard and yield inconsistent results. Hence, we performed tri-lineage differentiation studies (the current gold standard), to show that our harvest procedure yields bone fide MSCs (Supplemental Figure 2). The stains used were: Oil-Red-O (adipocyte differentiation), Alcian Blue (chondrocyte differentiation), and Alazarin Red (osteocyte differentiation). Growth kinetics data showed that porcine MSCs have statistically the same growth characteristics as clinical grade human MSCs (Figure 2A). Luminex cytokine quantification (Supplemental Figure 4). demonstrated robust secretion of a variety of inflammatory and anti-inflammatory cytokines as well as angiogenic growth factors, including C-X-C Motif Chemokine Ligand 1 (CXCL1), Basic Fibroblast Growth Factor (FGFb), Interferon Gamma (IFN γ), Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Platelet Derived Growth Factor Subunit A (PDGFA), and Vascular Endothelial Growth Factor (VEGF). Live-dead staining using Ethidium Bromide (Red/Dead) and Syto-13 (Green/Alive) confirmed viability of MSC cultures (Figure 2B). We also performed RT-PCR (Figure 2C), Western blotting (Figure 2D), and immunofluorescence staining (Figure 2E) for CD34 at the time of MSC harvest, demonstrating robust expression of this characteristic MSC protein.

Surgical Outcomes (Table 1)

The initial weight of experimental animals varied between 40 to 46 kg. We found that a minimum initial weight of 43–46 kg is needed to obtain sufficient subcutaneous fat for MSC culture 2–3 weeks prior to autologous implantation. Pigs weighing less than 43 kg have a very thin subcutaneous fat layer that requires a large subcutaneous dissection. Similar to humans, the bile duct diameter of animals varied between 0.4 cm to 1.0 cm. An intravenous infusion tube (diameter $\times$ length $\approx$ 0.35cm $\times$ 3.0cm) served as an appropriately sized biliary stent. No abnormalities were observed in postoperative liver function tests.

One pig from group 2 died on the third postoperative day due to acute cholangitis. Necropsy of the deceased animal showed that the vicryl mesh had retained a large amount of biliary sludge within the CBD lumen. Other animals had an uncomplicated recovery. The vicryl mesh dissolved in all other cases and did not accumulate biliary sludge during the 30-day observation period.

Cholangiography

Animals were sacrificed 30 days following the CBD anastomosis surgery. Postoperative cholangiography was obtained to evaluate for strictures, leaks, or obstruction. The animals had no obvious strictures or leaks at the anastomosis site although some animals showed varying degrees of anastomotic narrowing without clinical symptoms or abnormal liver function (Figure 4A–H).

Histology

In the anastomotic site, animals in Group 3 (extraluminal MSCs) showed significantly greater CD44 staining in the wall of the bile duct compared to either Group 2 (intraluminal MSCs) or the control group (Figure 5A–C). Correspondingly, the anastomotic sites of Group 3 also showed the strongest CD34 staining (Figure 5D–F). High power images of CD31 staining (Figure 5G–I) and subsequent quantification of CD31 positive area (Figure 5J) confirm increased neoangiogenesis in Group 3 (extraluminal MSCs). As expected, CD44 and CD34 also showed the strongest staining at the anastomotic site compared to proximal or distal sites (Figure 6A–F). Collectively, these results suggest that MSCs were successfully and safely engrafted and retained at the anastomotic site with extraluminal MSC application and that MSC engraftment promotes neoangiogenesis.

Trichrome staining revealed that Group 3 also had significantly less fibrosis at the anastomotic sites (Figure 7A–C). High power images of Trichrome staining (Figure 7D–F) and subsequent quantification of Trichrome positive area (Figure 7G) confirm reduced fibrosis in Group 3 (extraluminal MSCs). Concordantly, levels of the fibrogenic growth factor, TGF β , the myofibroblast activation marker, SMA, the macrophage marker, CD68, and the pro-inflammatory adhesion molecule, VCAM-1, were also all reduced at the anastomotic sites in Group 3 (Supplemental Figure 3). The reduction in fibrosis and inflammation correlates with both MSC engraftment and increased neo-angiogenesis, providing proof-of-concept that MSCs may ultimately have a role in preventing fibrotic strictures from forming at biliary anastomoses.

DISCUSSION

LDLT has expanded considerably in the past decade and, as experience and surgical technique have evolved, outcomes have also improved. These advancements have partially addressed the critical shortfall of available organs for those awaiting liver transplantation. Nonetheless, biliary complications, including bile leaks and biliary strictures, continue to be important drivers of morbidity and cost following LDLT. The development of these common complications remains biologically enigmatic and clinically problematic. Current treatment strategies require multiple invasive procedures and frequent hospitalizations. Therefore, the current porcine *in vivo* study outlines a novel protocol for the use of autologous, adipose-derived MSCs, applied either intraluminally on bio-stents or extraluminally in solution. Porcine MSCs were extensively characterized and found to be similar to clinical grade, human MSCs in terms of differentiation capacity and growth kinetics. MSCs secreted a variety of inflammatory and anti-inflammatory cytokines as well as angiogenic growth factors. We find that extraluminal application of an MSC solution to biliary anastomoses was safe and did not lead to cholangitis, biliary obstruction, strictures, or leaks. We also demonstrate that MSCs applied extraluminally are able to engraft within the wall of the bile duct, prevent anastomotic fibrosis / inflammation, and promote neoangiogenesis of the biliary plexus. In contrast, intraluminal vicryl mesh-wrapped stents were associated with technical challenges, did not affect fibrosis and angiogenesis, and led to one death related to cholangitis. To our knowledge, this is the first study to evaluate the safety of MSCs as a potential approach to reinforce biliary anastomoses, promote angiogenesis and healing, and prevent biliary complications following LDLT.

We found that 43–46 kg domestic pigs were most suitable for this study. This range of weight is ideal since it provides sufficient subcutaneous fat to obtain high quality stem cells. In domestic pigs below this weight, the subcutaneous adipose layer was too thin to provide adequate cells without a large area of subcutaneous dissection. We felt that this large dissection may affect the recovery of animals and potentially the quality of MSC culture. However, excessive weight, greater than 46kg, increases the difficulty of transport and experimental costs.

In this study, the diameter of the bile ducts of the domestic pigs varied widely, ranging from 0.4–1.0 cm, which is a similar range to that encountered in humans. All stent insertions were performed successfully, despite some technical challenges associated with insertion of the vicryl-wrapped stents. Most of the pigs have varying degrees of anastomotic narrowing based on the results of cholangiography on day 30. However, these narrowings were not accompanied by clinical symptoms or alterations of liver biochemistries and thus did not constitute clinically-significant strictures.

Misra and colleagues previously demonstrated that adventitial transplantation of human adipose tissue-derived MSCs to the outflow vein of mice with arteriovenous fistula reduces venous neointimal hyperplasia²⁴. Furthermore, they demonstrated prolonged retention of MSCs at the adventitia. In our analogous study, we introduced MSCs to the anastomotic site of the CBD via an intraluminal bio-stent and by local extraluminal immersion. We found that local immersion-introduced MSCs were safely applied to CBD anastomoses and there

were no complications in the Group 3 animals. Similar to the Misra study, we noted long-term retention of MSCs in the CBD wall at day 30 based on CD44 staining. Presumably, these retained MSCs rejuvenate the damaged tissue via their secretome leading to the activation of endogenous wound-healing responses and tissue self-renewal. It is important to note that the post-transplant setting will be associated with systemic immunosuppression, raising the question of whether MSC function will be altered in this setting. Two recent studies have addressed this question in immunosuppressed patients with Crohn disease^{22,23}. Collectively, these examples represent over 220 immunosuppressed patients with successful application of MSCs for fistulas. In these studies, there were no untoward effects noted, perfect MSC function and no change in systemic immune response. We acknowledge that it is impossible to predict with certainty the effect of a post-liver transplant immunosuppression regimen on our system. However, the preponderance of the available evidence suggests that MSC function will remain intact.

Intraluminal application of MSCs via vicryl mesh-wrapped biliary stents was technically challenging and was associated with the only animal death in our study. It is difficult to say if this was directly related to the breakdown products of the mesh, the secretory activity of intraluminal MSCs, or both. There was a second pig in Group 2 that had mild vomiting within 1-week after surgery, but eventually recovered. In contrast, the recovery of all pigs in Group 3 was unremarkable. Hence, while local, extraluminal immersion of the biliary anastomosis appears to be a safe delivery method, additional studies will be required to identify appropriate biodegradable materials that are compatible with intraluminal delivery. Histological staining of biliary specimens in Group 2 also did not show evidence of long-term retention of MSCs. We speculate that this may be due to the toxicity of the intraluminal environment and/or early shedding of the vicryl mesh. The successful deployment of biliary bio-stents would represent an important advance since non-invasive delivery via endoscopic retrograde cholangiopancreatography (ERCP) could ultimately expand the potential indications for biliary MSCs.

CD44 and CD31 stains suggest that extraluminally applied MSCs are engrafted and retained in a perivascular distribution within the biliary plexus of the bile duct wall and are accompanied by enhanced staining for markers of neoangiogenesis. This is consistent with previous studies of the perivascular localization of MSCs and their role in vasculogenesis and vessel maintenance²⁶, which is presumably driven by the angiogenic secretome of MSCs. Therefore, topical administration of MSCs to the CBD anastomosis site during surgery appears to be a satisfactory method for introducing MSCs into the bile duct wall. Given the frequent occurrence of biliary complications after LDLT, and since LDLT scheduling is planned in advance, one reasonable model would be to harvest autologous adipose tissue and cultivate MSCs prior to surgery and perform local immersion of the anastomotic site during LDLT. This method is also simpler than the biostent method and appears to also be safer in this initial study. Nonetheless, endoscopic delivery of MSC-coated stents remains an attractive future direction to treat biliary strictures through minimally invasive methods though will require an alternative approach compared to vicryl mesh. Recent advances in biocompatible 3D-printed biliary stents may provide an avenue for future patient applications²⁷. This approach could be a promising treatment for patients who have ischemic biliary strictures after liver transplantation and even strictures related to

primary sclerosing cholangitis or biliary malignancy. In conclusion, our pilot study shows a reliable method to create CBD anastomoses in a porcine model. We demonstrate that local, extraluminal delivery of autologous MSCs is safe and that the cells remain engrafted near the peribiliary plexus at 30 days. Furthermore, the topically delivered MSCs lead to reduced fibrotic scarring based on trichrome staining and promoted neoangiogenesis based on CD31 staining. Given the mounting evidence demonstrating the safety and efficacy of MSC applications in various clinical settings^{28–30}, this study represents an encouraging first step toward biliary applications of MSCs and a potential strategy toward reduction of anastomotic biliary strictures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

FGFb	basic fibroblast growth factor
CBD	common bile duct
CXCL1	C-X-C motif chemokine ligand 1
DDLT	deceased donor liver transplantation
ERCP	endoscopic retrograde cholangiopancreatography
ESLD	end-stage liver disease
IFNg	interferon gamma
IL-4	interleukin 4
IL-6	interleukin 6
IL-7	interleukin 7
LDLT	living-donor liver transplantation
MSC	mesenchymal stem cells
PDGFA	platelet derived growth factor subunit A
VEGF	vascular endothelial growth factor

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Figure 2:

Characterization of Porcine MSCs. (A) The growth kinetics graphs compare the growth rate of Human MSCs cultured at 37°C against Porcine MSCs cultured at 39°C or 37°C. Three seeding densities were used: 20k cells/cm² (high density-left), 10k cells/cm² (medium density-middle), and 5k cells/cm² (low density-right). At the 72hr time point, porcine cells cultured at 39°C grew at a rate similar to human MSCs at 37°C when seeded at 20k cells/cm² $p < 0.0001$ (95% CI: -13.52 to 9.605). When cultured at 37°C and/or seeded at concentrations less than 20k cells/cm², porcine MSCs grew slower than human MSCs. Seeding at a concentration of 5k cells/cm² resulted in porcine MSCs failing to reach 90% confluence even after 10 days of culture. (B) For live-dead staining, porcine MSCs were seeded onto a vicryl-910 scaffold at 10x (left) and 20x (right) magnification. Live-dead staining is performed using Ethidium Bromide (Red/Dead) and Syto-13 (Green/Alive). Blue strands represent scaffold fibers that are tightly bundled and oriented in a perpendicular woven pattern. (C) CD34 expression was evaluated in MSCs by real-time RT-PCR, compared to GAPDH. (D) CD34 protein was measured by Western blot in MSCs at passage numbers 3 and 4. (E) CD34 expression was evaluated using immunofluorescence staining for CD34 (green) and nuclear DAPI (blue). Inset shows high power merged images.

Figure 3:

(A, B, C) Stent insertion for group 1 and 2. (D, E, F) Application of MSC-suspension for group 3. A plastic stent, made from an intravenous infusion tube (Diameter×Length≈0.4cm×3cm), with or without vicryl-mesh, was inserted into the bile duct lumen. The distal and proximal ends of the CBD were clamped, the bile was cleaned. After inserting a bare stent, the fascia around the CBD was sutured and designed into a “pocket” in which the anastomotic site of CBD was totally immersed in MSCs-suspension.

Figure 4:

Cholangiograms on day 30. Blue arrows indicate the anastomotic site of CBD. (A, C, H) biliary anastomotic narrowing. (E, F) No biliary narrowing. (B, D, G) biliary anastomotic narrowing.

Figure 5: CD44, CD34, and CD31 staining of paraffin-embedded slides at the anastomotic site. A, D, G - Group 1 (control); B, E, H - Group 2 (intraluminal MSCs); C, F, I - Group 3 (extraluminal MSCs). (J) Quantification of the CD31 positive area showing more staining with Extraluminal MSCs, * $p < 0.05$

Figure 6:
CD44 and CD34 staining of paraffin-embedded slides at different sites in Group 3
(extraluminal MSCs).

Figure 7: Trichrome staining of paraffin-embedded slides at the anastomotic site. A, D - Group 1 (control); B, E - Group 2 (intraluminal MSCs); C, F - Group 3 (extraluminal MSCs). The graph shows the quantified fibrosis score according to the measurement of collagen staining.

Outcome of indwelling stents and MSCs, *Diameter × Length, Dilatation of the distal bile duct

Table 1:

Pig No.	Group	Weight (kg) D0, D7, D30	Dose of MSCs Delivered to Surgical Site	CBD Diameter(cm) Stent Size(cm) *	Postoperative Follow Up Cholangiograms
1	Control	40,42,48	NA	0.4, 0.35×2.5	Asymptomatic recovery, normal liver function, biliary narrowing
2		43,45,52	NA	0.4,0.35×2.5	Asymptomatic recovery, normal liver function, biliary narrowing
3		45,47,55	NA	0.5,0.35×3.0	Asymptomatic recovery, normal liver function, biliary narrowing
4	Intraluminal MSCs	45,47,58	4×10 ⁶ Cells	1.0,0.35×3.5	Asymptomatic recovery, normal liver function, biliary narrowing
5		46,48, NA	4×10 ⁶ Cells	0.6,0.25×2.5	Died 3 days after stent insertion due to cholangitis
6	Extraluminal MSCs	44,46,52	4×10 ⁶ Cells	0.8,0.35×3.0	Vomiting within 1-week, normal liver function, no biliary narrowing
7		45,47,53	4×10 ⁶ Cells	1.0,0.35×3.0	Asymptomatic recovery, normal liver function, no biliary narrowing
8		43,44,50	4×10 ⁶ Cells	0.5,0.35×2.5	Asymptomatic recovery, normal liver function, biliary narrowing
9		46,47,54	4×10 ⁶ Cells	0.6,0.35×3.0	Asymptomatic recovery, normal liver function, biliary narrowing