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Texas, USA

DISSERTATION

**Use of Autologous Auricular Chondrocytes
For Lining Left Ventricular Assist Devices**

Zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

Vorgelegt der Medizinischen Fakultät Charité
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INDEX OF CONTENTS

1. INTRODUCTION
2. MATERIALS AND METHODS
 - 2.1. In Vitro Experiments
 - 2.1.1 *Tissue Harvesting*
 - 2.1.2 *Tissue Isolation*
 - 2.1.3 *Cell Culture*
 - 2.1.4 *Histology*
 - 2.1.5 *Immunocytochemistry*
 - 2.1.6 *Cell Seeding*
 - 2.1.7 *Cell Preconditioning*
 - 2.2. In Vivo Experiments
 - 2.2.1 *Animal Care*
 - 2.2.2 *LVAD Preparation*
 - 2.2.3 *LVAD Implantation*
 - 2.2.3.1. *Operative Procedure*
 - 2.2.3.2. *Postoperative Care*
 - 2.2.4 *Necropsy and Gross Observations*
 - 2.2.5 *Scanning Electron Microscopy and Transmission
Electron Microscopy*

3. RESULTS

3.1. In Vitro Experiments

3.1.1. Histology

3.1.2. Immunocytochemistry

3.1.3. Seeding Efficiency

3.1.4. Cumulative Cell Loss During Preconditioning

3.2. In Vivo Experiments

3.2.1. Necropsy and Gross Observations

3.2.2. SEM and TEM

4. DISCUSSION

5. ABSTRACT

6. REFERENCES

7. ACKNOWLEDGMENT

1. INTRODUCTION

Options for the surgical treatment of cardiovascular diseases include implantable cardiovascular devices such as stents, the total artificial heart, vascular grafts, heart valves, and ventricular assist devices. Still, cardiovascular devices implanted in the human body have been shown to be associated with complications indicating the need of improving their biocompatibility. Biocompatibility has been defined as *"the ability of a material to perform with an appropriate host response in a specific application"* (1). Cardiovascular devices, such as the left ventricular assist device, have been shown to be associated with thromboembolism, bleeding, and infection, which are characteristics of poor biocompatibility (2). Activation of the coagulation cascade caused by the artificial surfaces of such cardiovascular devices has been shown to cause thromboembolic events (3).

To improve the biocompatibility of implantable cardiovascular devices whose artificial surfaces will be contacting the blood stream, endothelial cells might be an optimal lining since they naturally occur in human blood vessels at the blood-contacting surface. Endothelial cells secrete--among others factors--chemotactic, growth, and nonthrombogenic factors such as prostacyclin and nitric oxide (NO).

Bordenave et al. suggested the seeding of endothelial cells onto vascular grafts because this metabolically active endothelial lining plays a major role in preventing in vivo blood thrombosis and because vascular grafts implanted into humans do not form an endothelial monolayer spontaneously (4).

Nikolaychik et al. have tested endothelial cell monolayers under dynamic conditions inside a beating ventricular prosthesis in vitro with the goal of producing a permanent biocompatible artificial cardiac prosthesis (5). They have measured an endothelial cell loss of 35% indicating denudation of the endothelial cell lining.

Scott-Burden et al., investigated smooth muscle cells genetically engineered with endothelial-Nitric Oxides Synthase seeded on the surfaces of left ventricular assist devices and implanted in a calf for 24 hours (6). Genetically engineered smooth muscle cells have been shown to secrete Nitric Oxide and to provide an adherent, nonthrombogenic autologous cell lining.

There are drawbacks, however, to using either endothelial cells or smooth muscle cells to line cardiovascular devices. Neither cell type is abundantly available nor easily accessible, harvested, and isolated. Moreover, endothelial cells have been observed to slough off easily from artificial surfaces (2). The process of harvesting, isolating, and cultivating both endothelial cells and smooth muscle cells from autologous vessels is invasive and time-consuming.

In contrast, auricular chondrocytes are abundant, readily accessible, and able to be easily and efficiently harvested. One potential source of chondrocytes, auricular cartilage, can be harvested by a minimally invasive technique that preserves cell viability, decreases surgical time, and postoperative complications (7). *In vivo*, chondrocytes are naturally nourished by diffusion and produce substantial amounts of extracellular matrix components (8). Therefore, cultured chondrocytes may be more likely to remain viable and adherent to artificial surfaces.

We investigated *in vitro* the isolation and culture of auricular cartilage as a more efficient, less invasive means of obtaining autologous tissue for lining the luminal surfaces of implantable left ventricular assist devices (LVAD's) and *in vivo* the adherence of cultured auricular cartilage cells (chondrocytes) to the luminal surfaces of a LVAD in a calf model.

2. MATERIALS AND METHODS

2.1. In Vitro Experiments

2.1.1. Tissue Harvesting

Six weeks before the *in vivo* experiments began, a 2-mm-diameter tissue sample was harvested from the ear of a 4-month-old longhorn-crossbreed calf weighing 70 kg (4B Livestock, Midway, Texas) by punch biopsy with a trephine (Nasco, Fort Atkinson, WI) (Figure 1). The biopsy was performed under sterile conditions and local anesthesia. The tissue sample was immediately placed in sterile cell culture medium containing antibiotics (Table 1) and transferred to a sterile hood. The sample was kept at 4°C for a minimal amount of time to keep the tissue viable.

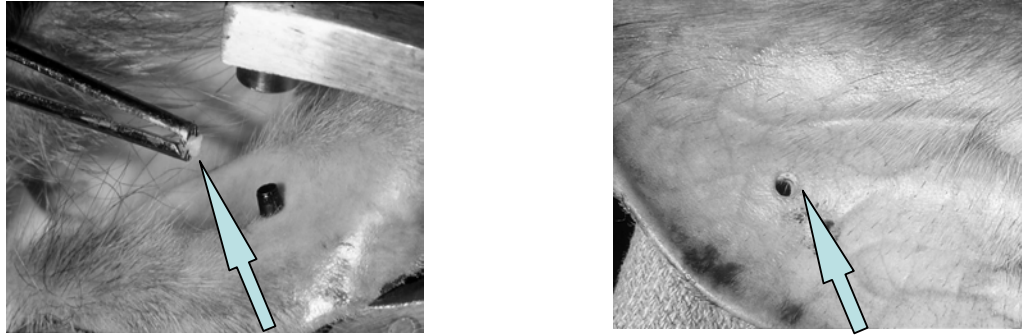


FIGURE 1 Harvest of bovine auricular cartilage from the ear of the tissue-donor calf. (Left) A 2-mm-diameter biopsy sample (arrow) of auricular cartilage being held by a pickup. The trephine used to obtain the sample is shown still in the ear. (Right) Biopsy site (arrow) at the ear after the trephine was removed.

2.1.2. *Tissue Isolation*

Under the sterile hood, auricular elastic cartilage was removed from the surrounding dermal tissue of the biopsy sample by microdissection. The isolated cartilage was placed in 10- x 10-mm tissue culture dishes with 2% antimycotic-antibiotic phosphate-buffered saline (Table 2) and incubated at 37°C for 10 minutes. Multiple small pieces of the cartilage were placed in 6-well tissue culture plates in 500 µl of modified RPMI cell culture medium and incubated at 37°C.

Table 1. Composition of Modified RPMI 1640 Cell Culture Medium

RPMI 1640 medium (90%)
Fetal bovine serum (10%)*
Sodium pyruvate (1X) †
Minimum essential amino acids (500 µl)
L-Glutamine (2mmol/L)
Penicillin G
Streptomycin
HEPES (1X)

*Equitech-Bio, Inc., Kerrville, TX.

†100 mM MEM sodium pyruvate solution; GIBCO-BRL, Rockville, MD.

‡Sigma, St. Louis, MO.

Table 2. Composition of 2% Antimycotic-Antibiotic Phosphate-Buffered Saline Solution

Dulbecco's phosphate-buffered saline (1X)*
Penicillin G sodium (10,000 U/ml)
Streptomycin sulfate (10,000 µg/ml)
Amphotericin B (25 µg/ml)

*Sigma, St. Louis, MO.

2.1.3. Cell Culture

Chondrocytes growing from the auricular cartilage were cultured under sterile conditions and maintained in a humidified 5% CO₂ incubator at 37°C. Every 12 hours, drops of medium were added to each culture well. After 5-7 days, when chondrocytes became adherent to the culture dishes, cartilage pieces were removed. The chondrocytes were then passaged as follows. First, they were trypsinized in a solution of 0.5 g trypsin and 0.2 g EDTA (Sigma, St. Louis, MO). Then, when the cells became detached, fetal bovine serum was added to inactivate the trypsin. The resulting single cell suspension was then transferred to tissue culture plates to establish a monolayer of chondrocytes. Thereafter, the cell culture medium was changed every 48 hours. Chondrocytes were passaged twice, and cells of the second passage were used for subsequent experiments.

2.1.4. Histology

Fine (1- μ m-thick), paraffin-embedded sections of pure elastic cartilage tissue were stained with Verhoff-van Gieson stain to visualize elastic fibers, Masson's trichrome stain to visualize collagen fibers, and hematoxylin and eosin to assess tissue and cell morphology.

2.1.5. *Immunocytochemistry*

A portion of the second-passage chondrocytes from each culture plate was plated on slides and fixed in 1% formalin for immunocytochemistry using a double-antibody labeling technique. Paraffin-embedded sections of pure elastic cartilage tissue immunostained in the same way served as a control. The primary antibody used was collagen type II (NCL-Coll-Iip; Novocastra); the secondary antibody used was a biotinylated immunoglobulin specific for the primary antibody (Vectastain[®] Elite IgG; Vector Laboratories, Burlington, CA). In brief, deparaffinized and hydrated cartilage tissue sections and cells were incubated in 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. All specimens were then incubated with normal mouse serum for 20 minutes to block nonspecific binding sites. Next, all specimens were exposed to a 1:50 diluted solution of the primary antibody for 1 hour. After repeated washes, all specimens were incubated with the secondary antibody for 30 minutes. Next, all specimens were exposed to avidin DH and biotinylated enzyme (Vectastain[®] Elite ABC reagent) for 30 minutes and then to diaminobenzidine as a peroxidase substrate for 30 seconds. Finally, all specimens were counterstained with hematoxylin for 1 minute. Control incubations were performed in the absence of the primary antibody.

2.1.6. Cell Seeding

Four implantable pneumatic LVAD's (HeartMate®; Thermo Cardiosystems, Inc., Woburn, MA) were used for the *in vitro* cell seeding experiments. The HeartMate® has two luminal artificial surfaces: a flexible diaphragm made of a "biomer" (i.e., polyurethane flocced with polyester microfibrils) and a metal housing made of sintered titanium microspheres⁶. One of the four LVAD's used in the *in vitro* experiments was later used in the *in vivo* experiments described below.

Seven days before the *in vivo* experiments began, each of the four LVAD's was seeded with a total of 3×10^7 autologous cells under sterile conditions. The inlet and outlet conduits of each LVAD were closed, and the LVAD was placed in a humidified 5% CO₂ incubator at 37°C. Each LVAD was tilted 15 degrees every 2 hours for 24 hours during cell seeding to ensure complete cell coverage of surfaces. After seeding was completed, three samples of cell culture medium were collected from each LVAD and assessed for the number of nonadherent cells using a Coulter counter (Coulter, Hialeah, FL). The seeding procedure and the consecutive assessment of seeding efficiency were repeated to ensure complete cell coverage of both luminal surfaces of the LVAD. Each LVAD was then incubated for 4 days in the same incubator. Cell culture medium supplemented with 50 µg/ml of sodium ascorbate (Sigma, St. Louis, MO) was changed every 48 hours to promote extracellular matrix synthesis in order to maximize the adherence of cells to both artificial surfaces. After each medium change, samples were collected to assess the

number of nonadherent cells as before and to calculate seeding efficiency based on the number of initially seeded cells.

2.1.7. Cell Preconditioning

Because preconditioning a.) Promotes good cell adherence once an LVAD is implanted and perfusion initiated and b.) Cell loss reaches a plateau after 12 hours of preconditioning, each seeded LVAD was subjected to an *in vitro* preconditioning regimen for 12 hours that exposed the cell lining to flow conditions(9). In brief, 12 hours before the *in vivo* experiments began, each LVAD was incorporated under sterile conditions into an *in vitro* flow loop (Figure 2). The *in vitro* flow loop was connected to a pneumatic drive console operated at 70 beats per minute and an ejection fraction of 30%. It has been demonstrated that cell loss reached a plateau after 12 hours of preconditioning a smooth muscle cell layer on LVAD surfaces (9). During the 12-hour preconditioning period, 18 samples of cell culture medium were collected (three each at 0, 0.3, 3, 5, 7 and 9 hours) to assess the amount of cell loss and to calculate preconditioning efficiency based on the number of initially seeded cells.

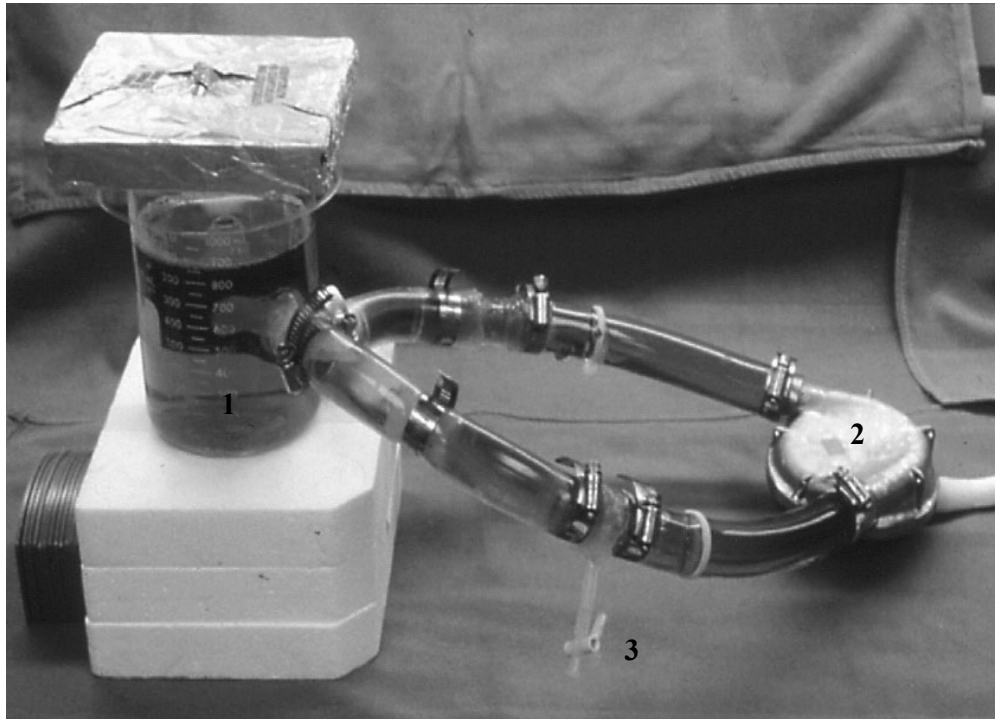


FIGURE 2 *In vitro* flow loop used for preconditioning of seeded cells. The loop consisted of a reservoir filled with cell culture medium (1) and the cell-seeded LVAD (2). Samples of cell culture medium emptied into the reservoir via a stop cock (3) were collected from the reservoir at several time points during the 12-hour preconditioning period as described in Materials and Methods. The *in vitro* flow loop was connected to a pneumatic drive console (not shown).

2.2. In Vivo Experiments

2.2.1. *Animal Care*

The tissue- donor calf used in the *in vivo* experiments received humane care in compliance with the *Principles of Laboratory Animal Care* prepared by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

2.2.2. *LVAD Preparation*

Immediately before implantation, one of the four seeded LVAD's used in the *in vitro* experiments was disconnected from its preconditioning *in vitro* flow loop and discharged of cell culture medium. To eliminate any remaining cell culture medium, the LVAD was rinsed twice with pure RPMI medium and washed three times with 37°C phosphate-buffered saline. Once the LVAD was filled with PBS, its inflow and outflow conduits were capped and its external surface sterilized by rinsing twice with 70% ethanol. The LVAD was then transported under sterile conditions to the operating room for immediate implantation.

2.2.3. *LVAD Implantation*

2.2.3.1. Operative Procedure. Once prepared, the seeded LVAD was implanted into the tissue-donor calf under cardiopulmonary bypass using a standard procedure described previously (10). In brief, an abdominal incision was made through which the LVAD was placed. The LVAD's percutaneous driveline was tunneled subcutaneously and exteriorized high on the left flank. The inflow and outflow conduits were passed through separate 1- to 2-cm-long incisions in the anterior left hemidiaphragm. A 20-mm-diameter low-porosity Dacron outlet graft was preclotted using autologous serum and blood and then anastomosed end-to-side at the descending thoracic aorta. The sewing ring of the LVAD was sutured to the left ventricular apex, using interrupted #2-0 braided polyester sutures with Teflon felt pledgets. A small crux incision was made in the apex, and a coring knife was inserted into the left ventricular cavity. A full-thickness circular segment of the apical myocardium was then excised. The pump inlet tube was inserted into the left ventricle. Air was removed from the LVAD via a needle inserted into the Dacron graft. Protamine sulfate was administered intravenously to antagonize heparin. The LVAD was kept in automatic mode at all times while implanted.

2.2.3.1. *Postoperative Care.* Dextrose 5% in Ringer's lactate solution was infused intravenously as necessary to maintain central venous pressure (CVP). Potassium chloride was added to the intravenous infusion as indicated by serial measurements of serum potassium. Sodium cefonicid 1 g was administered daily for 4 days to prevent infection. Butorphanol 10 mg was given i.m. every 4-8 hours for pain. No anticoagulative therapy was administered.

2.2.4. *Necropsy and Gross Observations*

Seven days after LVAD implantation, the calf was euthanized (by i.v. administration of 3 mg/kg heparin and then 1 ml/kg beuthanasia-D) and necropsied. The LVAD and pertinent organs (e.g., heart, lungs, liver, rumen, spleen, kidney, adrenal glands, and brain) were examined grossly and photographed. The organs were evaluated for the presence of emboli, ischemia, and infarction.

**2.2.5. *Scanning Electron Microscopy and Transmission Electron
Microscopy***

The LVAD was disassembled, inspected, photographed, and subjected to scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In brief, both artificial surfaces of the LVAD's, along with the attached cellular lining, were immediately fixed in Millonig's phosphate buffer supplemented with 3% glutaraldehyde and incubated for several days at 4°C. SEM samples were further fixed in 1% osmium tetroxide for 1 hour at room temperature. The samples were then rinsed with Millonig's phosphate buffer and gradually dehydrated with ethanol (9).

For SEM, samples of the diaphragm and the titanium housing of the LVAD were coated with gold using a Denton Vacuum 502A Cold Sputter Module. The samples were then placed in a digital scanning electron microscope (Zeiss Model 960) to visualize the cell-lined sintered titanium and textured polyurethane surfaces.

For TEM, samples of the LVAD's diaphragm and housing were infiltrated with resin and ethanol, embedded in the resin overnight, cut with a diamond knife to a thickness of 60-80 nm, pulse-stained in uranyl acetate and lead citrate, and finally viewed under a transmission electron microscope (JEOL Model 1200EX).

3. RESULTS

3.1. In Vitro Experiments

3.1.1. Histology

Isolated auricular cartilage tissue stained positive for elastic and collagen fibers, indicating the presence of pure elastic cartilage. Tissue culture cells derived from isolated auricular cartilage tissue at zero passage stained positive for elastic and collagen fibers, indicating the presence of chondrocytes (Figure 3).

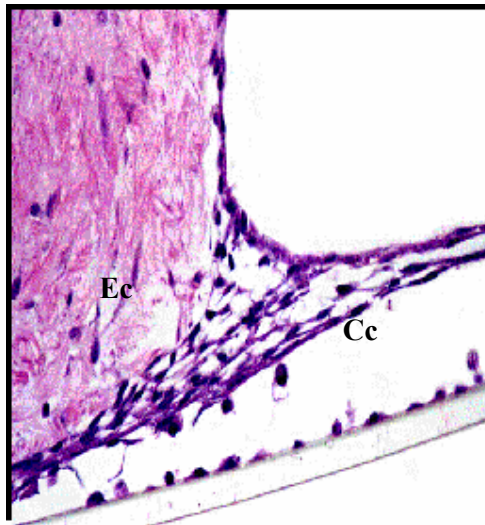


FIGURE 3 Chondrocytes (Cc) growing from a piece of elastic cartilage (Ec) at zero passage. Tissue culture cells (Cc) derived from isolated auricular cartilage tissue (Ec) at zero passage stained positive for elastic and collagen fibers, indicating the presence of chondrocytes.

3.1.2. *Immunocytochemistry*

Elastic cartilage tissue and cells at second passage stained positive for collagen II, indicating the presence of chondrocytes (Figure 4). Cells treated with cell culture medium supplemented with sodium ascorbate showed much stronger staining of collagen II, indicating improved collagen synthesis.

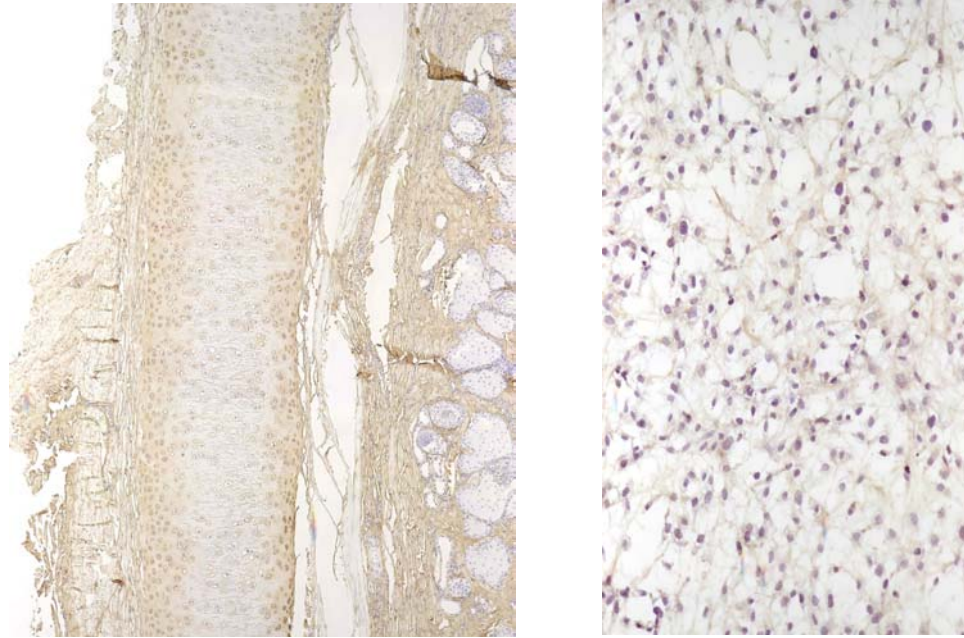


FIGURE 4 Immunocytochemistry of elastic cartilage (left) and chondrocytes in tissue culture of second passage (right). Positive staining for collagen II indicated the presence of chondrocytes.

3.1.3. *Seeding Efficiency*

The luminal surfaces of the four LVAD's, consisting of sintered titanium and polyurethane, were seeded twice with autologous auricular chondrocytes during the first seeding procedure and the second seeding procedure. The first seeding efficiency was $92.66 \pm 10.08\%$ and the second seeding efficiency was $98.14 \pm 0.93\%$. The calculated total seeding efficiency was $95.11\% \pm 4.23\%$ (n = 4) (Figure 5).

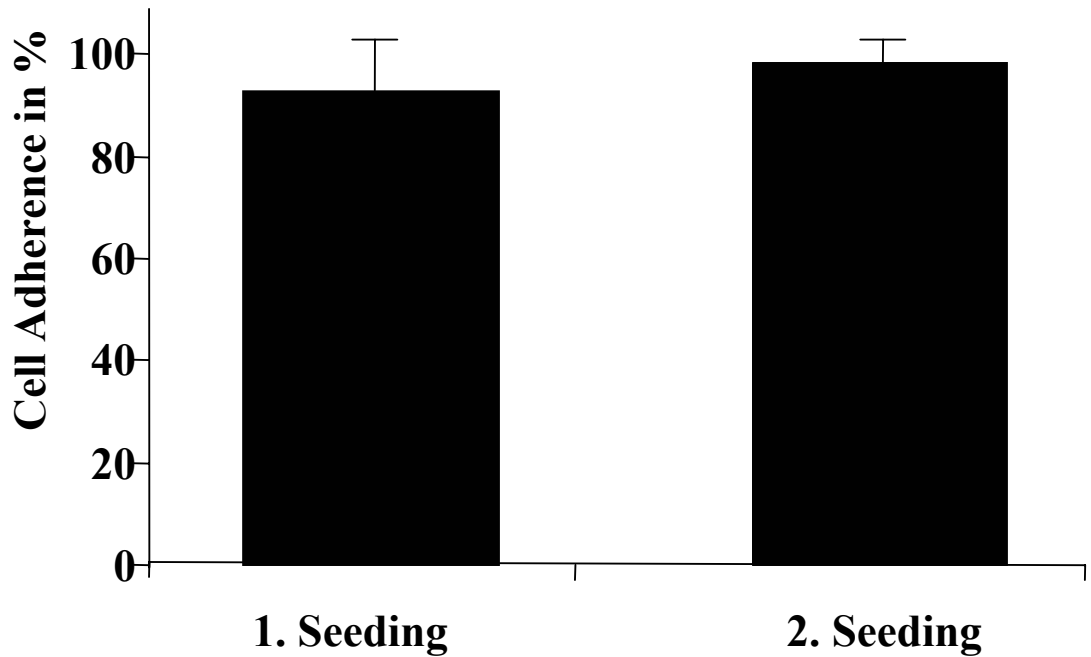


FIGURE 5 Seeding efficiency. The first seeding efficiency of four LVAD's was $92.66 \pm 10.08\%$ and the second seeding efficiency of four LVAD's was $98.14 \pm 0.93\%$. Total seeding efficiency of four LVAD's was $95.11 \pm 4.23\%$.

3.1.4. Cumulative Cell Loss During Preconditioning

During the 12 hours of preconditioning under flow conditions *in vitro*, the average cumulative cell loss was $11.45 \pm 0.21\%$ ($n = 4$). The average cumulative cell loss was $2.22 \pm 0.32\%$ after 30 minutes, $5.13 \pm 0.15\%$ after 3 hours, $7.47 \pm 0.20\%$ after 5 hours, and $9.78 \pm 0.35\%$ after 7 hours (Figure 6).

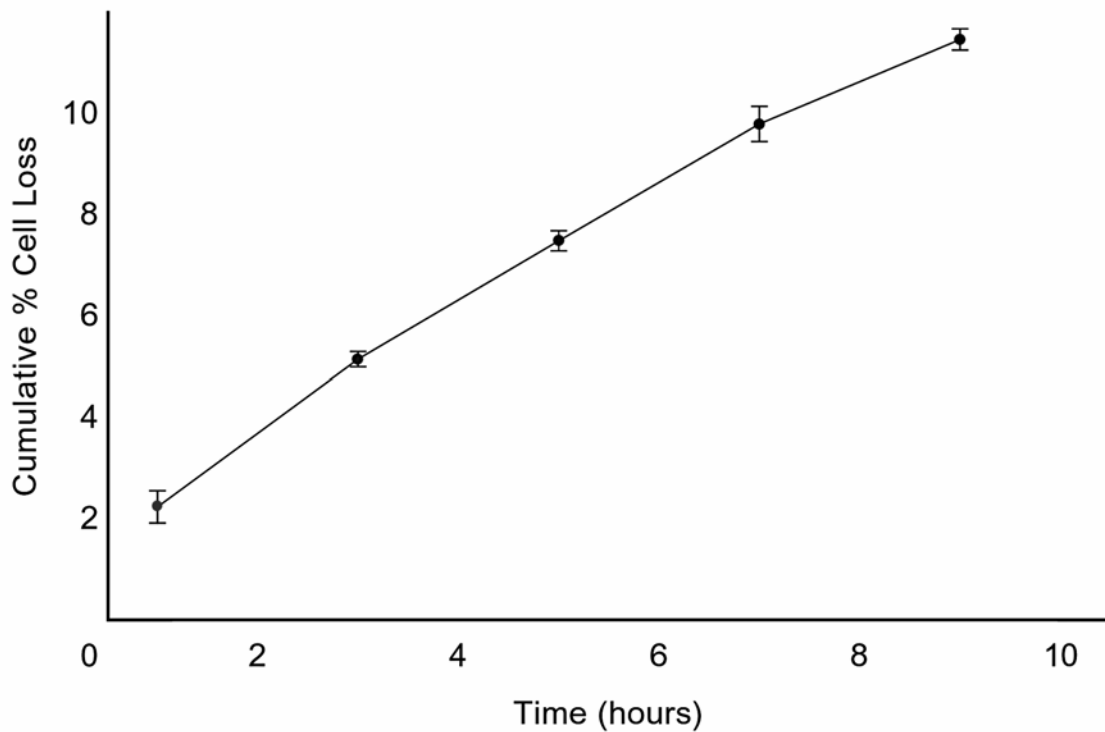


FIGURE 6 Cumulative cell loss ($n = 4$). The cumulative cell loss during preconditioning under flow conditions on the *in vitro* flow loop was $2.22 \pm 0.32\%$ after 30 minutes, $5.13 \pm 0.15\%$ after 3 hours, $7.47 \pm 0.20\%$ after 5 hours, $9.78 \pm 0.35\%$ after 7 hours and 11.45 ± 0.21 after 9 hours.

3.2. In Vivo Experiments

3.2.1. Necropsy and Gross Observations

Gross examination of the luminal surfaces of the LVAD revealed an intact cell layer and complete coverage of both artificial surfaces after 7 days of implantation (Figure 7). There was no evidence of infection or thrombus on either luminal surface, within the graft, or within the aortic anastomoses. Lungs, liver, rumen, spleen, kidney, rete mirabile, adrenal glands, and brain showed no gross and no histological evidence of emboli, ischemia, or infarction.



FIGURE 7 Gross appearance of the implanted LVAD's biomaterial surfaces after 7 days of implantation *in vivo*. (Left) Textured polyurethane surface; (right) sintered titanium surface. The luminal surfaces of the LVAD are completely covered with an intact cell layer.

3.2.2. *SEM and TEM*

SEM revealed an extensive amount of extracellular matrix components and an intact, well-incorporated cellular lining on the sintered titanium and polyurethane surfaces of the implanted LVAD (Figure 8). TEM revealed a well-established monolayer of chondrocytes (Figure 9). No endothelial cells were seen.

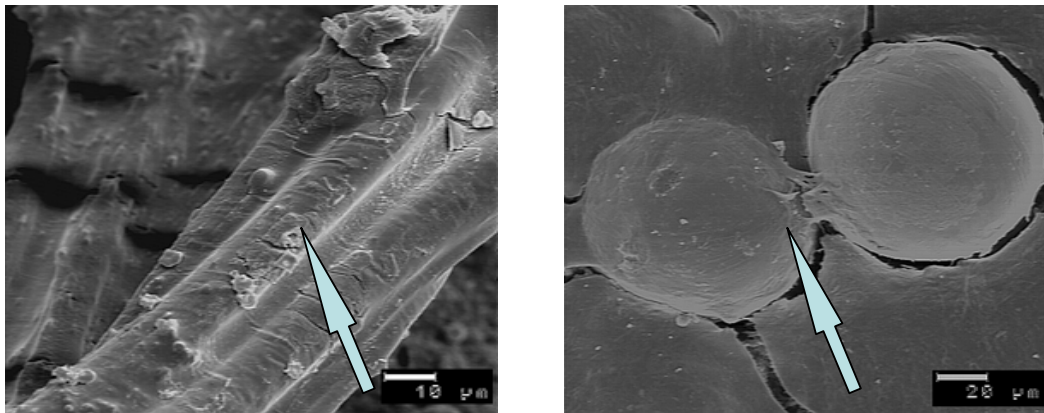


FIGURE 8 Scanning electron microscopy of the implanted LVAD's biomaterial surfaces after 7 days of implantation *in vivo*. An extensive amount of extracellular matrix and an intact, well-incorporated cellular coating (arrows) were noted on the textured polyurethane (left) and sintered titanium (right) surfaces of the implanted LVAD.

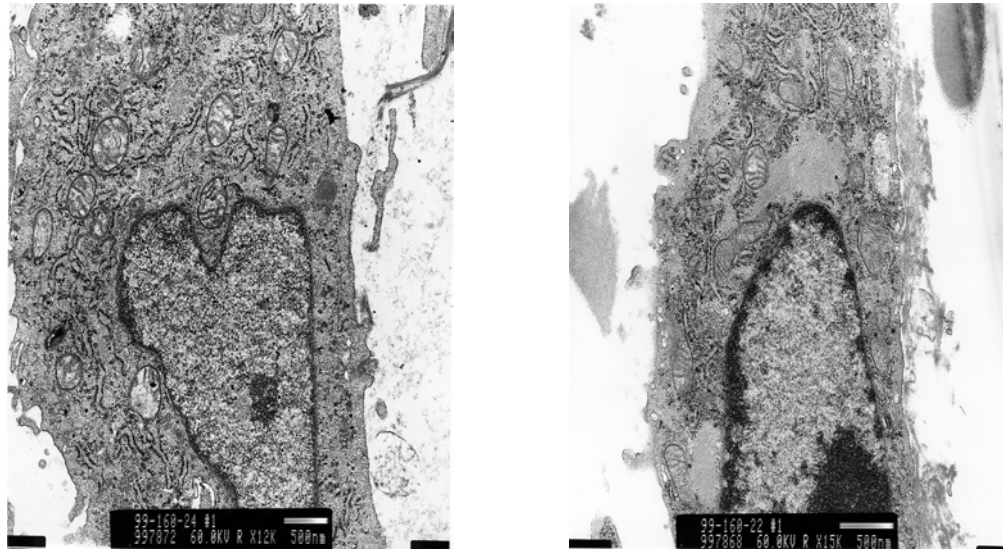


FIGURE 9 Transmission electron microscopy of the implanted LVAD's biomaterial surfaces after 7 days of implantation *in vivo*. (Left) Textured polyurethane surface; (right) sintered titanium surface. A well-established monolayer of chondrocytes was revealed. No endothelial cells were seen.

4. DISCUSSION

This study demonstrates the feasibility of using autologous chondrocytes derived from auricular elastic cartilage to line the luminal surfaces of LVAD's. Auricular elastic cartilage is an accessible source of autologous cells. Chondrocytes can be harvested from the ear under local anesthesia, and they can be isolated more efficiently than vascular smooth muscle or endothelial cells. The isolation of chondrocytes from cartilage described here is simple, fast and easy. Moreover, chondrocytes derived from auricular cartilage can adhere strongly to artificial surfaces because of their ability to manufacture collagen II, elastin and other important constituents of the extracellular matrix. Hypothetically, cardiovascular assist devices lined with auricular chondrocytes might also allow easier recruitment of circulating endothelial cells and thus improve the process known as fallout healing (11).

Support for this idea has come from development of the HeartMate® LVAD. Even in early calf studies, the HeartMate®'s artificial surfaces encouraged the immediate deposition of a stable, uniform, antithrombogenic, nonhemolytic neointimal lining (12). Later, in human studies, endothelial cells were found on samples taken from the luminal surfaces of LVAD's after implantation (13). Presumably, blood-borne endothelial cells or endothelial cell precursors had been deposited on the blood-contacting surfaces, which may explain in large part the low reported incidence of thrombogenicity and clinical thromboembolic problems associated with the use of LVAD's. Another study showed endothelial cells to have

high affinity for heparinized surfaces in addition to cell surface receptors involved in adhesion to collagen (14); this suggested that a lining of autologous chondrocytes strongly adherent to artificial surfaces might provide an ideal attachment zone for endothelial cells. Yet another study showed that genetically engineered smooth muscle cells lining the luminal surface of the HeartMate® were nonthrombogenic (9) suggesting that the use of such cells might improve the hemocompatibility of artificial surfaces.

Beyond the immediate scope of our study, our findings also have some implications for the use of auricular cartilage in tissue engineering. Other investigators have successfully used a mixed-cell population of vascular cells from ovine carotid arteries to create a heart valve on a scaffold of biodegradable porous polyhydroxyalkanoate (15). Still, the ideal cell source for tissue-engineering a heart valve seem to remain a mystery (15).

In conclusion, auricular elastic cartilage is an accessible source of autologous tissue. Chondrocytes derived from such cartilage can be efficiently harvested, isolated, cultured and seeded and can adhere very strongly to artificial surfaces because of their ability to produce collagen II, elastin and other important constituents of extracellular matrix. Therefore, auricular chondrocytes are a potential source of autologous cells for lining large cardiovascular assist devices such as LVAD's and improving their long-term biocompatibility. Our successful short-term feasibility study in which auricular chondrocytes were used to line the luminal surfaces of a LVAD in a calf model warrants further study *in vivo*.

5. ABSTRACT

Background: Auricular elastic cartilage is a potential source of autologous cells for lining the luminal surfaces of left ventricular assist devices (LVAD's) to improve long-term biocompatibility. We evaluated this potential *in vitro* and *in vivo* in a calf model.

Methods: *In vitro*, auricular cartilage was harvested from the anesthetized ear of a calf, isolated, and cultured on tissue culture dishes. Primary chondrocytes were typed by immunocytochemistry, transferred into culture media, passaged twice, and seeded onto the blood-contacting luminal surfaces of four LVAD's (HeartMate®; Thermo Cardiosystems, Inc., Woburn, MA). The seeded cell linings were preconditioned under flow conditions *in vitro* to promote cell adhesion to the luminal surfaces. Seeding efficiency and cumulative cell loss under flow conditions *in vitro* were quantitated. *In vivo*, one of the four preconditioned, autologous chondrocyte-lined LVAD's was implanted into the tissue-donor calf; run for 7 days; explanted; and finally evaluated grossly, by scanning electron microscopy, and by transmission electron microscopy.

Results: Autologous chondrocytes were seeded onto the luminal surfaces of the four LVAD's. The seeding efficiency was $95.11 \pm 4.23\%$ (n = 4). Cumulative cell loss during preconditioning under flow conditions *in vitro* did not exceed 12% (n = 4). After 7 days of *in vivo* implantation, the luminal surfaces of the implanted LVAD demonstrated an intact, strongly adherent cellular lining. There was no evidence of thromboembolic events at necropsy.

Conclusions: Auricular elastic cartilage is a ready and easily accessible source of chondrocytes whose ability to produce collagen II and other important extracellular matrix constituents allows them to adhere strongly to the luminal surfaces of LVAD's. The simple method of isolating and expanding auricular chondrocytes presented here could be used to provide autologous cell linings for LVAD's and other cardiovascular devices to improve their long-term biocompatibility. Our successful short-term feasibility study in a calf model warrants further study *in vivo*.

Key words: auricular chondrocytes, tissue engineering, left ventricular assist device, elastic cartilage

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