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Survival and Function of Hepatocytes on a Novel Three-Dimensional Synthetic Biodegradable Polymer Scaffold With an Intrinsic Network of Channels

Stephen S. Kim, MD,*† Hirofumi Utsunomiya, MD,* John A. Koski, BA,* Benjamin M. Wu, DDS,‡ Michael J. Cima, PhD,‡ Jane Sohn,§ Kanae Mukai,§ Linda G. Griffith, PhD,§ and Joseph P. Vacanti, MD*

From the Laboratory for Transplantation and Tissue Engineering, Children's Hospital and Harvard Medical School, Boston, Massachusetts; Department of Surgery, The University of Chicago Hospitals, Chicago, Illinois†; and Department of Materials Science and Engineering‡ and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts§*

Objective

To evaluate the survival and function of hepatocytes (HCs) on a novel three-dimensional (3D) synthetic biodegradable polymer scaffold with an intrinsic network of interconnected channels under continuous flow conditions.

Summary Background Data

The authors' laboratory has investigated HC transplantation using 3D biodegradable polymers as scaffolding as an alternative approach to treatment of end-stage liver disease. Previous studies have demonstrated survival of HCs transplanted on polymer discs in peripheral tissue sites and partial correction of single enzyme liver defects. One of the major limitations has been the insufficient survival of an adequate mass of transplanted cells; this is thought to be caused by inadequate oxygen diffusion.

Methods

HCs and nonparenchymal liver cells from Lewis rats were seeded onto 3D biodegradable polymer scaffolds. Microporous 3D polymers were created using 3D printing on copolymers of polylactide–coglycolide. The cell/polymer constructs were placed in static culture or continuous flow conditions. The devices were retrieved after 2 days and examined by scanning electron microscopy and histology. Culture medium was analyzed for albumin by enzyme-linked immunosorbent assay (ELISA). Differences in culture parameters including pH, PCO_2, PO_2, glucose, lactate, and HCO_3 were examined.

Results

Scanning electron microscopy revealed successful attachment of HCs on the 3D polymer in both static and flow conditions. Histology demonstrated viable HCs in both conditions. ELISA demonstrated a significantly higher mean concentration of albumin in flow conditions than in static conditions. Culture parameter analysis revealed a significantly higher PO_2 and glucose level, and a more physiologic pH in flow conditions than in static conditions.

Conclusions

HCs cocultured with nonparenchymal cells can attach to and survive on the 3D polymer scaffolds in both static and flow conditions in the size and configuration used in this study. Flow conditions may provide a more conducive environment for HC metabolism and albumin synthesis than static conditions. The authors hypothesize that flow through directed channels will be necessary for the transfer of large masses of cells when implantation studies are initiated.

Each year 26,000 people die of end-stage liver disease in the United States, with an estimated annual cost of $9 billion.[1] Liver transplantation is currently the only established successful treatment for end-stage liver disease. The severe scarcity of donor organs, especially in the pediatric population, has become a major limitation and has stimulated investigation into selective cell transplantation.[2,3] Using the principles of tissue engineering, our laboratory has investigated hepatocyte (HC) transplantation using three-dimensional (3D) synthetic biodegradable polymer scaffolds as a novel approach to treatment of end-stage liver disease.[4,5] Previous studies performed in this laboratory

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Address reprint requests to Joseph P Vacanti, MD, Department of Surgery, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.
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have shown survival of HCs transplanted on synthetic biodegradable polymer discs in peripheral tissue sites,[6,7] improved survival of transplanted HCs with hepatotrophic stimulation using portacaval shunt and partial hepatectomy,[8,9] partial correction of single enzyme liver defects,[10] and reorganization of HCs and sinusoidal endothelium cocultured under continuous flow conditions *in vitro*.[11]

One of the major limitations has been the insufficient survival of an adequate mass of transplanted cells to correct defects in liver function permanently. We hypothesize that the limitations of oxygen and nutrient diffusion and waste exchange are critical factors for the highly metabolically active cells during the initial posttransplantation period. To address this issue, we designed and fabricated a complex 3D synthetic biodegradable polymer scaffold with an intrinsic network of interconnected branching channels using the technique of 3D printing. In this study, we evaluate the survival and function of HCs cocultured with nonparenchymal liver cells (NPCs) on the complex 3D polymer scaffolds under continuous flow conditions.

MATERIALS AND METHODS

Animals

Adult male inbred Lewis rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 150 to 300 g were used in all experiments. Animals were housed in the Animal Research Facility of Children's Hospital, Boston, Massachusetts, in accordance with NIH guidelines for the care of laboratory animals. Animals were maintained in a temperature-regulated environment (21°C) on a 12-hour light/dark cycle, housed in cages with soft bedding and a microisolator cover, and given access to rat chow (Prolab 3000 Rat Diet; PMI Feeds, St. Louis, MO) and tap water *ad libitum*.

Polymer Fabrication

Microporous 3D synthetic biodegradable polymers were fabricated from copolymers of polylactide–coglycolide (PLGA, 85L:15G, 100,000 MW; Ethicon, Somerville, NJ) using the Three-Dimensional Printing (3DP) fabrication technique. 3DP involves selectively directing a solvent onto polymer powder packed with sodium chloride particles (45 to 150 μm) to build complex 3D structures as a series of very thin two-dimensional slices[12–14] (Fig. 1A). The polymer scaffolds were warm gas-sterilized with ethylene oxide (H.W. Anderson Products, Chapel Hill, NC), and the salt crystals were leeched out with distilled water over 48 hours to yield highly porous (60%) devices with micropores 45 to 150 μm in diameter.

Flow Bioreactor System

The Cellmax Quad flow perfusion system (Cellco, Germantown, MD), modified to house the cell/polymer constructs, was used to produce the continuous flow condition. The culture medium was pumped at a flow rate of 1.4 ml/min from a 100-ml reservoir through the oxygenation tubing and the cell/polymer construct housing unit, and recirculated back to the reservoir. The flow rate needed for cell survival was estimated based on cell mass and reported values for nutrient consumption. The entire perfusion unit was maintained at 37°C with 10% CO_2 supplementation.

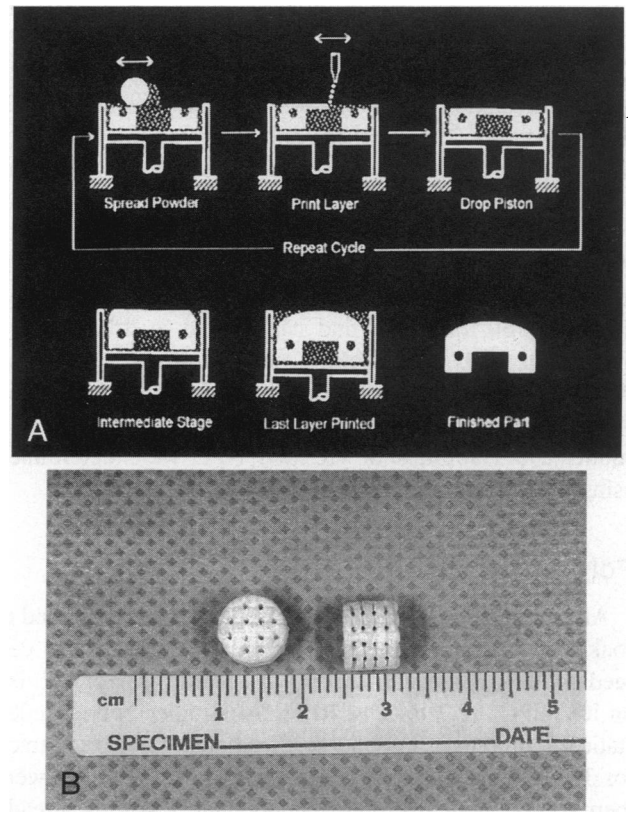

Figure 1. The 3DP technique. (A) The 3DP sequence of operation. (B) 3D biodegradable polymer scaffold, demonstrating the interconnected longitudinal and radial channels.

Cell Isolation

Male Lewis rats were used as cell donors. HCs were isolated using a modification of the two-step collagenase perfusion procedure,[15,16] and NPCs were isolated by differential centrifugation, as previously described.[17,18] Briefly, the animals were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Mundelein, IL) inhalational anesthesia and the abdomen was prepared in sterile fashion. A midline abdominal incision was made and the infrahepatic inferior vena cava was cannulated with a 16-gauge angiocatheter (Critikon, Tampa, FL). The portal vein was incised to allow retrograde efflux and the suprahepatic inferior vena cava was ligated. The perfusion was performed at a flow rate of 29 ml/min initially with a calcium-free buffer solution for 5 to 6 minutes, then with a 0.05% type D collagenase solution (Boerhinger-Mannheim, Indianapolis, IN) at

37°C. The liver was excised after adequate digestion of the extracellular matrix and mechanically agitated in William's E medium (Sigma, St. Louis, MO) with supplements to produce a single cell suspension. The suspension was filtered through a 300-μm mesh and separated into two fractions by centrifugation at 50g for 2 minutes at 4°C. The pellet containing the viable HC fraction was resuspended in William's E medium and further purified by an isodensity Percoll centrifugation. The supernatant from the first centrifugation containing the viable NPC fraction was filtered through a 60-μm mesh and centrifuged at 150g for 10 minutes at 4°C. The resulting pellet was resuspended in William's E medium. HCs and NPCs were differentiated based on their morphologic and cell size differences. Cell counts and viabilities of HC and NPC were determined using the trypan blue exclusion test.

Polymer Seeding

After salt leeching, the polymer scaffolds were allowed to soak in culture medium for 30 minutes on ice before cell seeding. The entire cell seeding procedure was performed on ice. NPCs (2.7 to 3 $\times$ 10^7 cells/polymer) were seeded statically onto each polymer scaffold and allowed to attach for 30 minutes. HCs (5.3 to 7.8 $\times$ 10^7 cells/polymer) were then seeded statically onto the polymer scaffolds already coated with NPCs. The HC suspension was directed into the longitudinal and radial channels during seeding. The cell/polymer constructs were then incubated (10% CO_2/humidified air) in either static or continuous flow conditions at 37°C. These constructs were harvested after 2 days in culture.

Culture Media

William's E medium supplemented with 1 g sodium pyruvate (Sigma) and 1% glutamine–penicillin–streptomycin (Gibco BRL, Gaithersburg, MD) was used during the cell isolation process. HCs and NPCs were cocultured in Dulbecco's modified eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum (Sigma), 1% glutamine–penicillin–streptomycin, 10^{-6} mol/ml dexamethasone (Sigma), 1 μg/ml insulin (Sigma), and 10 ng/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA).

Study Design

For experiment I, NPCs (2.7 to 3 $\times$ 10^7 cells/polymer) and HCs (5.3 to 7.2 $\times$ 10^7 cells/polymer) were seeded statically onto each polymer scaffold and placed in either static (n = 5) or flow (n = 5) conditions. After 2 days, the medium was sampled and analyzed for differences in culture parameters.

For experiment II, NPCs (2.8 to 3 $\times$ 10^7 cells/polymer) and HCs (5.9 to 7.8 $\times$ 10^7 cells/polymer) were seeded onto each polymer scaffold and placed in either static (n = 5) or flow (n = 8) conditions. The cell/polymer constructs were harvested after 2 days in culture and prepared for subsequent examination. Culture medium was sampled for determination of albumin concentration.

Specimen Analysis

For experiment I, the media from both static and flow conditions were sampled after 2 days under culture conditions. Culture parameters including pH, PCO_2, PO_2, glucose, lactate, and HCO_3 were measured using the Nova Stat Profile Plus 9 Analyzer (Nova Biomedical, Waltham, MA) and compared using the Student's t test. $p < 0.05$ was considered statistically significant.

For experiment II, the cell/polymer constructs were harvested after 2 days in culture. For examination under scanning electron microscopy (Hitachi Instruments, San Jose, CA), the devices were fixed in 10% formalin, dehydrated, and critical-point dried. Specimens were sputter-coated with gold and analyzed by scanning electron microscopy. For histology, the devices were fixed in 10% formalin, dehydrated, and embedded in JB-4 plastic (Polysciences, Warrington, PA). Three-micron sections were prepared and stained with hematoxylin and eosin. Culture media were analyzed for albumin by enzyme-linked immunosorbent assay (ELISA). Mean albumin concentrations between the two conditions were compared using the Student's t test. $p < 0.05$ was considered statistically significant.

RESULTS

Polymer Scaffold Fabrication

The polymer scaffolds were fabricated in the shape of a cylinder 8 mm in diameter and 7 mm high. They contained 12 interconnected longitudinal channels (800 μm in diameter) running through the length of the scaffold and 24 interconnected radial channels (800 μm in diameter) at various lengths of the devices (Fig. 1B). After salt leeching with distilled water over 48 hours, the polymer scaffolds attained a microporosity of 60% with micropores 45 to 150 μm in diameter.

Bioreactor Flow Rate

The flow rate needed for cell survival was estimated based on reported values for nutrient consumption and cell mass. Under standard culture conditions, oxygen becomes the limiting nutrient. The volumetric oxygen consumption rate (QO_2) is approximately 2 to 6 $\times$ 10^{-5} mmol/cm^3 cell mass-second for metabolically active cells.[19] The polymer scaffold contains <20% by volume cells after cell seeding, and the scaffold void volume (V) is 0.11 cm^3. We specified that the oxygen concentration should not decrease >50% from the inflow to the outflow of the cell/polymer housing

unit. The flow rate (F) needed to maintain the specified oxygen concentration after cell seeding is thus estimated by: $F = (0.2 \times V \times QO_2)/(0.5 \times CO_2$ inflow), for a given value of inflow oxygen concentration (CO_2 inflow). The oxygen concentration in the 10% CO_2/humidified air-saturated culture medium at 37 °C is 1.5×10^{-4} mmol/ml. Therefore, the flow rate needed for survival of the initial cell mass is estimated to be at most 1.1 ml/min. The flow rate in our perfusion system was set at 1.4 ml/min to ensure adequate oxygen delivery.

Cell Isolation

All cell isolations yielded 3 to 6 $\times 10^8$ HCs and 1.6 to 1.8 $\times 10^8$ NPCs per isolation, with >80% viability for HCs and >95% viability for NPCs.

Specimen Analysis

For experiment I, the culture parameters, including pH, PCO_2, PO_2, glucose, lactate, and HCO_3 for static and flow conditions, were measured after 2 days under culture conditions. The analysis revealed a significantly higher PO_2 in the flow conditions (166.4 ± 5.9 mmHg) than in static conditions (149.1 ± 3.8 mmHg) ($p < 0.05$), a significantly higher level of glucose in the flow conditions (432.4 ± 12 mg/dl) than in static conditions (412 ± 16 mg/dl) ($p < 0.05$), and a more physiologic pH in the flow conditions (7.42 ± 0.03) than in static conditions (7.33 ± 0.08) ($p < 0.05$) (Fig. 2). Although statistically significant differences were not attained for PCO_2 and lactate levels, there was a clear trend toward a lower PCO_2 in the flow conditions (43.6 ± 5.6 mmHg) than in static conditions (54 ± 3.4 mmHg) and a higher lactate level in the flow conditions (2.6 ± 0.5 mmol/l) than in static conditions (1.9 ± 0.1 mmol/l). There was no significant difference in HCO_3 levels between the flow (28.6 ± 4 mmol/l) and static (28.9 ± 5 mmol/l) conditions.

For experiment II, scanning electron microscopy revealed successful attachment of large numbers of HCs on the surfaces of the 3D polymer scaffolds in both static and flow conditions after 2 days in culture (Fig. 3). Histologic sections demonstrated viable HCs attached to the outer surfaces of the polymer scaffolds and along the surfaces of the longitudinal and radial channels in both culture conditions (Fig. 4). The HCs were noted to have infiltrated approximately 200 μm into the bulk of the polymer scaffolds through the micropores. ELISA demonstrated a significantly higher mean concentration of albumin in the flow conditions (1799.94 ± 892.38 ng/ml) than in static conditions (653.50 ± 351.95 ng/ml) ($p < 0.05$) (Fig. 5).

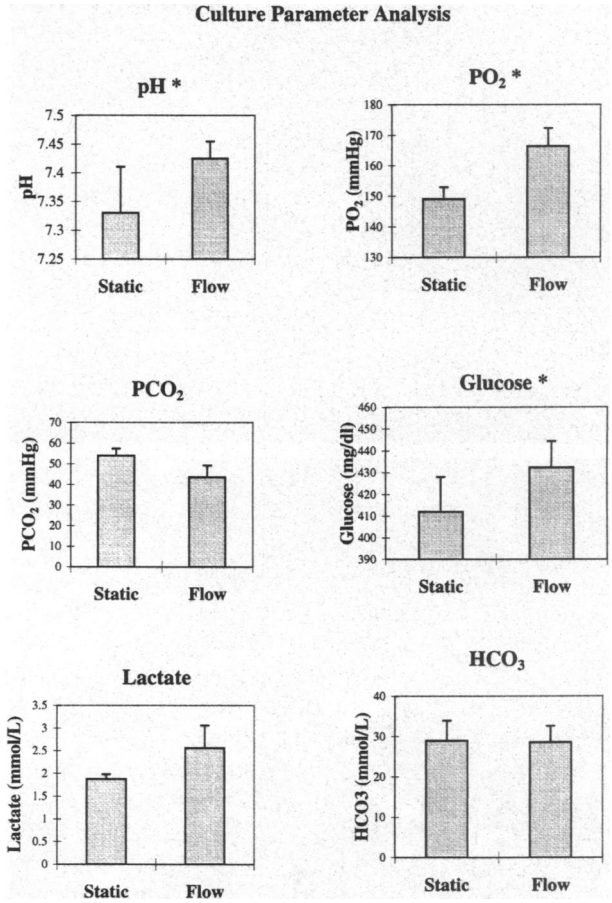

Figure 2. Comparison of culture parameters between flow and static conditions. * $p < 0.05$ between the flow and static conditions.

DISCUSSION

Our laboratory has investigated HC transplantation using 3D synthetic biodegradable polymer scaffolds as a novel approach to treatment of end-stage liver disease. One of the major limitations has been the insufficient survival of a large number of transplanted HCs on polymer discs. We have hypothesized that the limiting factor may be the inadequate diffusion of oxygen and nutrients to the cells on the polymer discs initially after implantation until adequate neovascularization had occurred. To address this issue, we fabricated a highly complex 3D synthetic biodegradable polymer scaffold with an intrinsic network of interconnected branching channels. In this study, we have demonstrated the capacity of HCs to attach to these devices in high densities, to survive after attaching to these polymers in static and flow conditions, and to synthesize albumin.

Previous studies performed in this laboratory have demonstrated survival of HCs transplanted on thin polymer discs in peripheral tissue sites such as the omentum, mesentery, and subcutaneous tissue, and the partial correction of single enzyme liver defects. The main obstacle has been the survival of only a small number of the transplanted cells, mainly as clusters adjacent to blood vessels. Several groups have shown HC spheroid formation on tissue culture dishes and have reported possible beneficial effects of the cell–cell interactions on the viability of HCs *in vitro*.[20–22] The formation of the spheroids has been suggested to mimic the

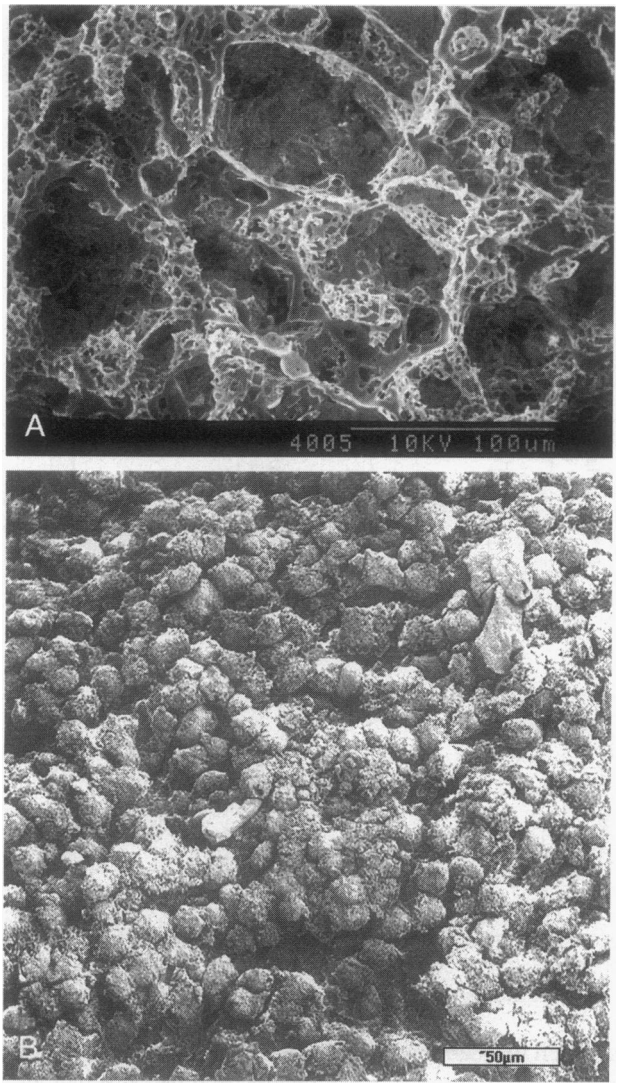

Figure 3. Scanning electron micrographs. (A) 3D biodegradable polymer scaffold after salt leeching before cell seeding, depicting the microporosity (original magnification ×400). (B) HCs densely attached to the polymer scaffold after 2 days under flow conditions (original magnification ×300).

cellular architecture found in native liver tissue. It has been reported that the maximum size of the spheroids formed is about 150 to 175 μm in diameter,[20] and any larger structures would require an intrinsic capillary network to support nutrient and waste exchange.[23]

The 3DP fabrication technique, which we used, allows the creation of polymer scaffolds with a highly complex microarchitecture and macroarchitecture. The larger size and the high porosity of these devices produce a much larger surface area for cell attachments than the previously studied polymer discs. The intricate network of channels and micropores provides a structural template to guide cellular organization, enhance neovascularization, and increase the capacity for oxygen and nutrient delivery and waste removal. Conditioning the cells in a flow system *in vitro* may allow cells to remodel along the channels of the

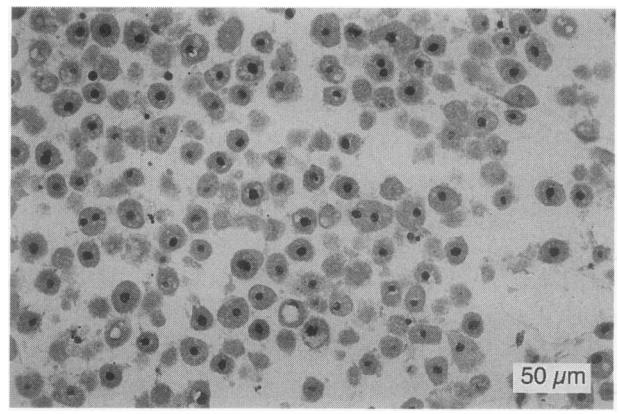

Figure 4. Histologic photomicrograph demonstrating viable HCs after 2 days under flow conditions (hematoxylin and eosin; original magnification ×300).

scaffolds and provide cues for HC and NPC reorganization. In addition, continuous flow conditions may provide more efficient O_2 delivery and CO_2 removal, a more physiologic pH, and an environment more favorable for cell metabolism and function.

In conclusion, in this preliminary study, we have demonstrated that HCs were able to attach and survive on the complex 3D synthetic biodegradable polymer scaffolds in large numbers, and that the flow conditions may provide an environment more conducive for HC metabolism and albumin synthesis. Future investigations in this laboratory will be directed toward extended *in vitro* evaluations, improved implantation methods, and subsequent *in vivo* demonstrations of HC survival and function.

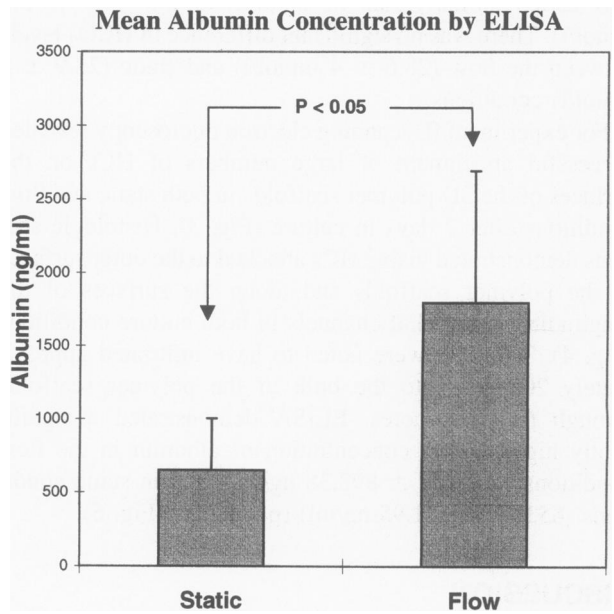

Figure 5. Comparison of mean albumin concentrations measured by ELISA between flow and static conditions.

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