Simplified ex vivo artery culture techniques for porcine arteries

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SUMMARY

Vascular physiology may be studied in intact medium-sized arteries using an ex vivo artery culture system. By maintaining hemodynamic pressure and flow to the vascular tissue, pH, O_2, CO_2 and temperature, early changes in vascular structure and function can be measured. We present techniques which enable use of non-sterile, porcine arteries in an ex vivo system. Viabilities of vessels are demonstrated with assays for vessel contraction and relaxation and uptake of a mitochondrial stain. Our techniques represent a simplification of previously described methods and enable the use of abattoir material for a wider range of studies on vascular physiology and pathology.

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INTRODUCTION

Ex vivo artery culture represents an alternative technique for studying vascular structure and function; it is more physiological than cell culture and less costly and more easily controlled (hemodynamically and biochemically) than in vivo studies. One reported use of ex vivo artery culture techniques was by Herman et al who perfused freshly excised canine vessels for 2–24 h.[1] Pulsatile flow was generated by a disk cam and roller follower pump;[2] techniques for measuring vessel diameter with a He–Ne laser were reported in a separate study by the same group.[3] Bardy et al described an organ culture system which could maintain vessels for longer periods of time, up to 8 days.[4] Freshly excised rabbit aorta was bathed in culture medium and connected to a perfusion system which controlled pressure and steady flow independently. In order to maintain in vivo pressure and stretch, the tissue was cannulated, pressurized and then excised directly onto splints held at in vivo length. Synthesis of aortic fibronectin induced by transmural pressure and angiotensin II, and activation of extracellular signal-related kinase 1/2 were measured in vessels cultured in this system for 1–7 days.[4–6] Labadie et al[7] also described a perfusion system to maintain freshly harvested canine carotid arteries which incorporated a centrifugal pump and custom-designed, computer-controlled gate valve. Using sterile technique, arteries were isolated, cannulated, flushed with tissue culture medium, clamped and excised. Significant diameter changes were measured in canine carotid arteries perfused for 1, 24 and 48 h by an epinephrine/ acetylcholine contraction/relaxation assay.

Simpler systems can be used for experiments less than 6 or 8 h in duration. Vasomotion at different transmural pressures was studied in rat femoral and mesenteric arteries constricted with norepinephrine, maintained with steady flow for 4 h.[8] Defects in endothelial-mediated relaxation after balloon embolectomy were investigated in fetal calf carotid arteries (from an abattoir), maintained with steady flow for 1 h.[9] Arterial diameter changes induced by vasoactive substances have been measured with a He–Ne laser micrometer in order to assess endothelial damage incurred by intravascular intervention.

Each of these systems is an excellent tool for performing physiologically realistic flow studies on fresh, sterile vascular tissue with precise measurement apparatus. However, it would be advantageous if these investigations into vascular structure and function were less complex, required less expensive equipment and could be maintained for significant

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durations, up to a week. Likewise, the use of freshly excised animal tissue is prohibitively expensive for many studies. Our group has developed simple yet effective techniques for ex vivo vascular culture to study vascular biology under steady or pulsatile flow conditions for up to 7 days.

Methods

Carotid arteries were harvested directly from abattoir animals (6-month-old male and female swine) soon after exsanguination. The arteries were immediately rinsed and bathed in a phosphate-buffered saline (PBS, MediaTech 20-031-LV, Herndon, VA) solution containing 2% penicillin–streptomycin (Sigma Chemical Co. P0906, St Louis, MO), then stored on ice for transport to the laboratory. With clean but not sterile technique, side branches were tied off and vessels were inflated with air while submerged in PBS to check for leaks. Then, with sterile technique, the vessels were rinsed three to four times in sterile-filtered PBS with antibiotics. With each subsequent rinse, the vessel was bathed in the sterile solution and never bathed in the efflux solution. From this point on, only sterile solutions, tools and apparatus came into contact with the vessel.

Thus prepared, the vessel was placed in a chamber and mounted in physiologic orientation between two cannulae which pass through the chamber. These cannulae connect the vessel to the flow loop. The flow loop consists of a pumped-tubing section, a dampening reservoir for steady flow, the vessel, a gas exchange reservoir, and ports for pressure measurement, drug infusion and temperature measurement (see Fig. 1). Immediately after the vessel was mounted, the vessel chamber was filled with Dulbecco's modified Eagles medium (DMEM, Sigma D1152; approximately 100 ml) to surround the vessel, with some space reserved for gas exchange. Air was infused into the vessel under pressure to check again for leaks. To obtain physiologic stretch, the cannulae were adjusted so that the vessel was under no tension, markings were made on the vessel, and then the cannulae were separated so that the vessel was stretched 150% according to the markings. Compression fittings were tightened to secure the cannulae in this position. The chamber was then covered with a gasketed lid and clamped shut. Gas exchange was allowed through 0.2 µm sterilized airflow filters connected to ports in the chamber lid.

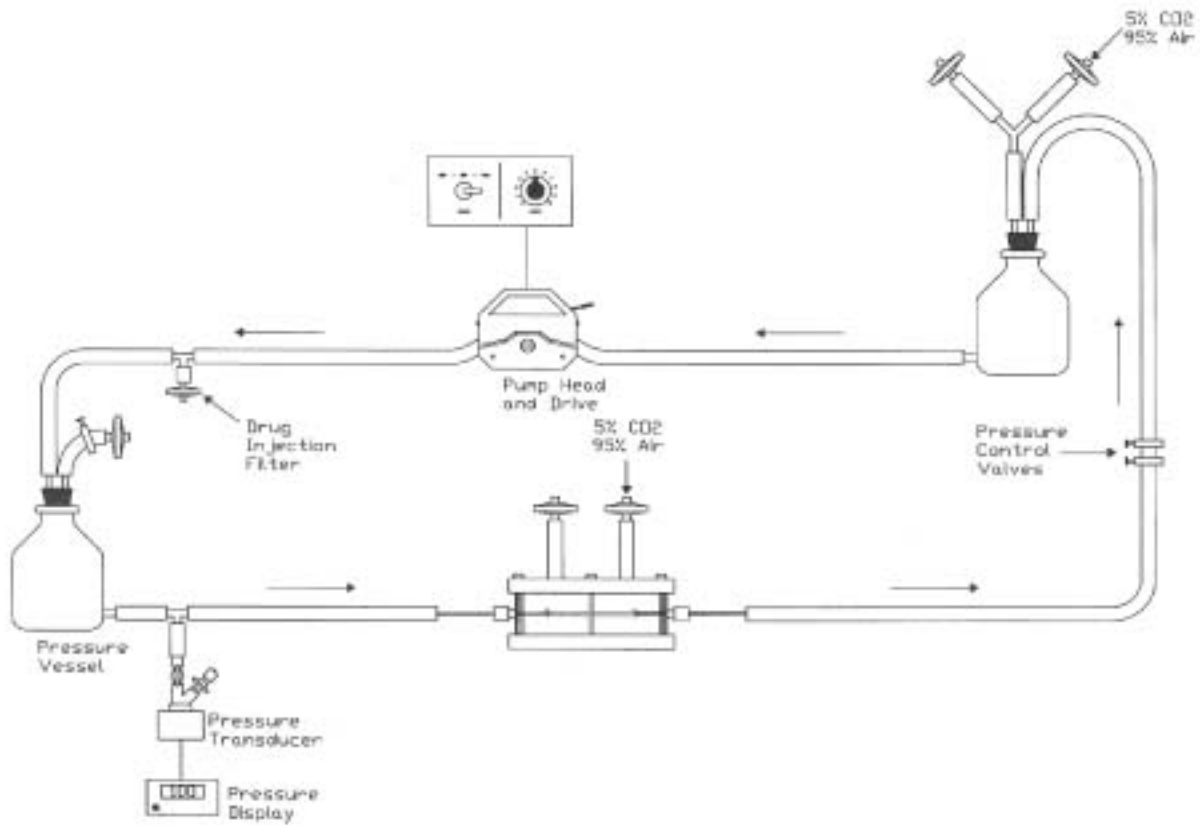

Fig. 1 Flow loop schematic. The flow loop consists of a pumped-tubing section, drug injection port, pressure vessels for dampening flow oscillations, pressure measurement port, vessel chamber which houses the vessel, sites for pressure control, and a gas exchange reservoir. The artery is mounted in physiologic orientation between two cannulae which pass through the chamber.

The autoclave-sterilized flow loop was filled with 250 ml of DMEM with 5% dextran (Sigma D7265), added to obtain a physiological fluid viscosity. Appropriate gas exchange for the flow loop media was provided through sterile airflow filters connected to the gas exchange reservoir. Physiologic pH, pO_2 and pCO_2 were maintained by infusing a 5% CO_2 – air mixture into the gas exchange regions and allowing outflow some distance away. Phenol red in the media allowed visual verification of proper pH. Temperature control was maintained by either independent closed-loop heating and control of the internal and external media, or by placing the system in a temperature-controlled incubator.

In steady flow, the shear stress in the vessel is determined by the flow rate and viscosity of the medium (200 ml/min and 4 cP, respectively) and the diameter of the vessel (typically 4–6 mm) according to the formula:

$$\tau = \frac{32\,\mu Q}{\pi D^3},$$

where τ is the wall shear stress, μ is the fluid viscosity, Q is the volumetric flow rate, and D is the vessel or cannulae inner diameter.[13] An appropriate length of rigid metal tubing precedes the vessel in the flow loop to ensure the flow is fully developed for physiologically relevant flow rates. This length can be calculated according to:

$$L = \frac{0.06\,\rho Q}{\pi\mu}$$

where ρ is fluid density. For pulsatile flow, the dampening reservoir was removed and a T-connector with an extra 60 cm of tubing was added. The oscillation amplitude was adjusted by shortening this elastic section with a hemostat clamp. With a Masterflex roller pump (Cole Parmer model 7553-80, Vernon Hills, IL; pump head model 7518-10), pulse frequency is dependent on the pumping speed (1–100 rpm).

Transmural pressure can be varied independently of the shear and flow parameters. Typically, the vessel chamber medium was kept at atmospheric pressure while the luminal pressure was varied with an adjustable tubing clamp. Increasing resistance in the flow loop with a load-independent pump (such as the Masterflex for pressures in the physiologic range) will increase the loop pressure. Pressure can be easily measured with an in-line pressure sensor (Harvard Apparatus model 60-3002, South Natick, MA; range 1–1000 mmHg).

Contraction and relaxation assay

Arterial vasoconstriction in response to norepinephrine (NE, Sigma A9512), and vasodilation in response to a subsequent dose of Carbachol, a direct-acting parasympathomimetic structurally similar to acetylcholine (Sigma C4382) were measured. Since the relaxation pathway requires an intact endothelium and both contraction and relaxation require viable, contractile smooth muscle cells, the viability of each cell type can be tested.[10] Low-concentration solutions (10^{-5} mM in dH_2O, final concentration) were infused into the flow loop at point 1 (see Fig. 1) and allowed to mix before reaching the vessel. Vessel diameter changes were recorded with a video camera placed directly on top of the chamber lid and recorded on a VCR. The data were analyzed with NIH Image 1.60 on an Apple PowerPC 7100/80AV with a video card. The resolution was computed to be 0.066 mm/pixel in the vertical direction.

Mitochondrial staining assay

Methylthiazol tetrazolium (MTT, Sigma M5655) binds to the mitochondria of living cells and stains the cells dark blue.[11] To ascertain the presence of viable cells after artery culture, vessels were incubated at 37°C in a 0.5 mg/ml solution of MTT in PBS for 1 h. Vessels were then photographed with a Zeiss 4000C stereomicroscope.

Results

Vessels harvested from 6-month-old male and female swine were 6–8 cm long unstretched and ranged from 3 to 5 mm in diameter. Typically, 1/5 of the vessels harvested had leaks which could not be tied off. Vessels rarely developed leaks with prolonged artery culture.

Vasoconstriction induced by NE and subsequent relaxation by Carbachol can be observed in cultured arteries for up to 7 days. An example of the diameter changes in response to NE and Carbachol is shown in Figure 2. NE was injected at time $t=0$; the arrow indicates Carbachol injection. After 15 min, the vessel relaxed to 90% of its initial diameter, which is within the range of control vessels cultured for 6 h. Evidence of active mitochondria by MTT staining can also be seen in vessels cultured up to 7 days. Dark staining indicates live cells with active mitochondria, whereas no staining indicates non-living cells. A representative pair of vessels is shown in Figure 3; the vessel on the right was cultured for 7 days, and the vessel on the left was kept in PBS at 4°C for over 3 weeks.

The drug-delivery port is amenable to any pharmacological agent with which the investigator is

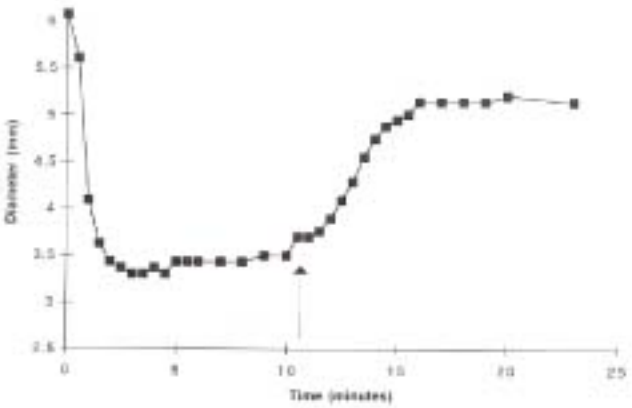

Fig. 2 Representative contraction/relaxation assay results at 48 h. Vessel diameter is plotted versus time; norepinephrine injected at time $t=0$. The arrow indicates Carbachol injection. Data were recorded for 25 min.

concerned. Given the low circulating volume of fluid (250 ml), high solute concentrations are possible. Biochemical agents could also be administered adventitially, through the vessel chamber medium. In addition to imposing hemodynamic perturbations on the vessel (i.e. altering pressure and flow rate), various mechanical stimuli may be investigated, such as stretch, catheterization and clamp injuries. Enzymatic assays, or more structural assays such as scanning electron microscopy, may be used to study the vascular biological response to changes in hemodynamic conditions.[12]

Conclusions

Vascular tissue obtained at low cost from a local abattoir was maintained in a simple artery culture system for up to 7 days with no substantial loss of viability. Two assays which can quantify the functioning of smooth muscle cells, endothelial cells, and cellular mitochondria in cultured vessels were described and demonstrated on 2-day and 7-day cultured vessels, respectively. Our techniques represent a simplification of existing methods with more widely available tissue. With such a system, early changes in vascular structure and function can be measured in response to altered hemodynamics, biochemical and pharmacological changes or mechanical perturbations. Simplified ex vivo artery culture may provide an important model for investigating vascular physiology and pathology.

Acknowledgements

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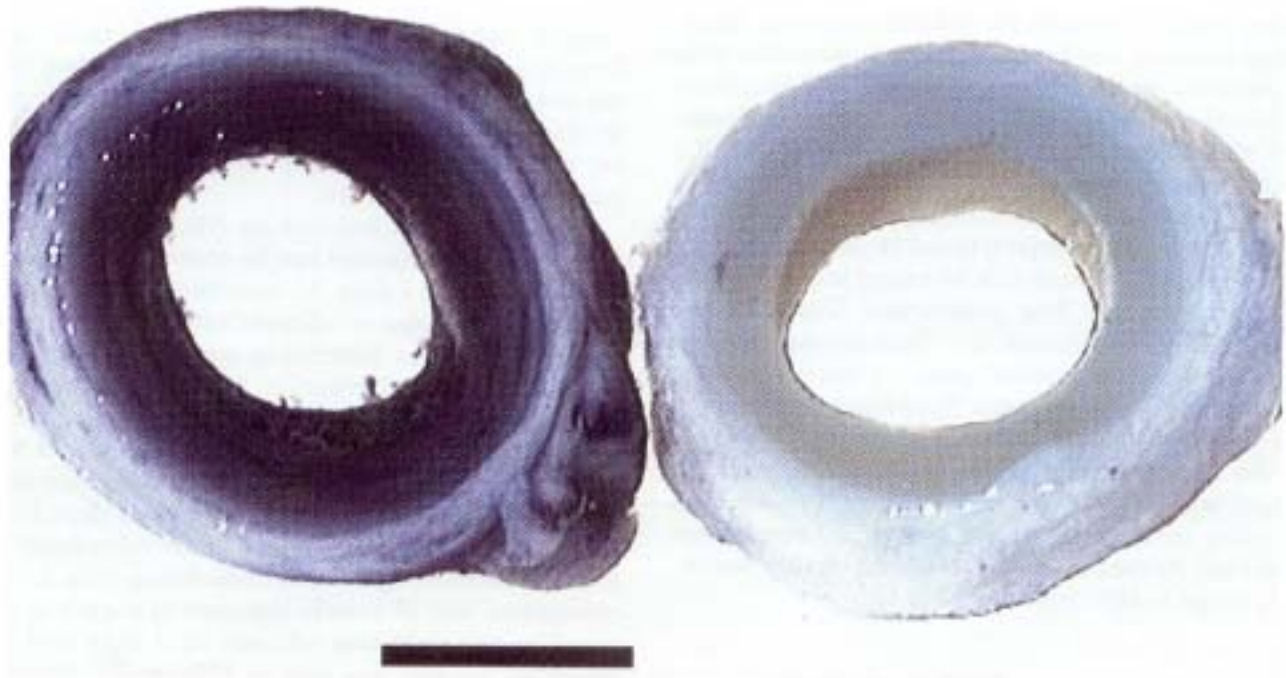

Fig. 3 MTT assay results for cultured vessel (left) at 7 days. Dark staining indicates live cells with active mitochondria. Left figure shows vessel cultured with pulsatile flow for 7 days. Right figure shows non-living vessel kept at 4°C for over 3 weeks and warmed just prior to assay. Scale bar is 2 mm.

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