Effects of Shear Forces and Pressure on Blood Vessel Function and Metabolism in a Perfusion Bioreactor

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Abstract

Bovine saphenous veins (BSV) were incubated in a perfusion bioreactor to study vessel wall metabolism and wall structure under tissue engineering conditions. Group 1 vessels were perfused for 4 or 8 days. The viscosity of the medium was increased to that of blood in group 2. Group 3 vessels were additionally strained with luminal pressure. Groups 1-d through 3-d were similar except that BSV were endothelium-denuded before perfusion. Groups 1-a through 3-a used native vessels at elevated flow rates. Group 3 vessels responded significantly better to noradrenaline on day 4, whereas denuded vessels showed attenuated responses ($p<0.001$). Tetrazolium dye reduction did not depend on perfusion conditions or time except for denuded vessels. pO_2 gradients across the vessels were independent of time and significantly higher in group 2 ($p<0.001$). BSV converted glucose stoichiometrically to lactate except vessels of groups 3, 1-d, and 3-d which released more lactate than glucose could supply ($p<0.001$). Group 1 vessels as well as all vessels perfused with elevated flow rates showed a loss of endothelial cells after 4 days, whereas group 2 and 3 vessels retained most of the endothelium. These data suggest that vessel metabolism was not limited by oxygen supply. Shear forces did not affect glucose metabolism but increased oxygen consumption and endothelial cell survival. Luminal pressure caused the utilization of energy sources other than glucose, as long as the endothelium was intact. Therefore, vessel metabolism needs to be monitored during tissue engineering procedures which challenge the constructs with mechanical stimuli.

Introduction

Vascular insufficiency is a common condition among the elderly especially in Western countries. In addition to percutaneous procedures like balloon angioplasty or stenting, surgical implantation of vascular grafts is still a mainstay of peripheral and coronary revascularization[1; 10; 12]. Various vessels are used for bypass procedures in the latter case, with internal mammary artery, saphenous vein, and radial artery being the most common graft sources[13]. However, a considerable number of patients lack suitable vessels as a consequence of disease, accidents, or prior removal. Small caliber vascular grafts require a functional antithrombotic surface to avoid early graft failure. Dacron or poly(tetrafluoroethene) (PTFE), which are excellent materials to create large-caliber grafts, are therefore not well suited for coronary bypass surgery[16] or for the treatment of critical lower limb ischemia[20].

Tissue-engineered blood vessels may help to alleviate the shortage in vessel replacements. They have also been suggested as hemodialysis shunts[23]. Several approaches have been developed, most of them using two-stage procedures which include an incubation of the constructs in a perfusion bioreactor prior to implantation[35; 36; 30]. Appropriate shear forces, which control vessel tone[3], and luminal pressure help to adapt grafts to physiological flow and pressure conditions. The concentrations of soluble nutrients, including oxygen, determine cell growth and function. As cell culture media lack oxygen transporters like hemoglobin, their oxygen transport capacities are comparable to that of plasma. This may limit the metabolism of tissue-engineered constructs in perfusion bioreactors[26]. Along the same lines, the main source(s) of energy may depend on the perfusion conditions. Lack or excess of substrates may limit the growth of an engineered tissue or unnecessarily increase culture medium expenses. In order to estimate nutrient and oxygen demands of vessel grafts and their changes under load,

the current study used bovine vessels as a model. Both endothelial cells[31] (EC) and smooth muscle cells[34] show higher proliferative capacities and lower degrees of differentiation in venous beds compared to arterial beds. Therefore veins lend themselves particularly well to this kind of analysis as tissue engineering procedures are likely to require less differentiated but rather prolific cells. Bovine medial saphenous veins (BSV) are adapted to approximately the same venous blood pressures as in humans, although BSV are slightly larger in diameter compared to the corresponding human vessel (approx. 5 vs. 3 mm internal diameter). Metabolic requirements may alter during cell growth, e.g. after seeding a neoendothelium, and during physiological responses to mechanical or pharmacological stimuli. While testing the former is beyond the capabilities of the adult vessel model and will be addressed in a separate study, the model is well suited to explore the effects of mechanical stimuli on key parameters of metabolism and on vessel wall structure. The influence of shear forces were investigated by increasing the viscosity of the medium to that of human blood. Luminal pressure induces smooth muscle contractions to counteract the passive distension and thus allowed to determine the metabolism under load. In order to identify a role of the endothelium in any effects of shear forces or pressure, endothelium-denuded vessels were subjected to the same perfusion conditions. Finally, some experiments were repeated at higher flow rates to approximate arterial conditions.

Materials and Methods

Harvesting of blood vessels

BSV were harvested as pedicles from the tarsal joint area immediately after sacrificing the animals at a local butcher. Samples were transferred into sterile Krebs-Henseleit buffer (KHB; NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 16.7 mM, glucose 5.5 mM, $CaCl_2$ 1.2 mM) supplemented with HEPES (25 mM), penicillin (100 U ml^{-1}), and streptomycin (100 µg ml^{-1}) and transported to the lab at 4°C within 30 min. All further prepara-

tion steps were performed in a sterile hood. The pedicles were immersed in 70% ethanol for 5 min and washed in sterile phosphate-buffered saline (PBS). The vessels were then prepared free from connective tissue and the terminal 2 cm at both ends were discarded. Two short segments were put aside for tension and dye reduction measurements. Segments of 8 cm in length were mounted in vessel chambers for the perfusion experiments after ligating side branches using silver clips (0.5 x 0.8 mm; WPI, Berlin, Germany) or surgical suture (7-0 Prolene, Ethicon, Norderstedt, Germany) as appropriate.

To investigate the role of ECs in any of the observed effects, a separate set of experiments was done with endothelium-denuded vessels. Wooden skewers were soaked in PBS and autoclaved. Skewers were inserted into the vessels and gently agitated to destroy the endothelium. Vessels were then rinsed thoroughly to remove any remaining debris. Endothelium removal was verified by histology and by evaluating the response to the endothelium-dependent vasodilator acetylcholine (ACh) in each vessel as described below.

Perfusion bioreactor system

Perfusion experiments were conducted in a set of custom-made bioreactors (FMI, Seeheim/OB, Germany; Fig. 1). Each circulation contained a medium reservoir, which was vented by a sterile filter. Due to the superiority of hollow fiber oxygenators for culture media[22] a neonatal hollow fiber oxygenator (Polystan Safe Micro, MAQUET Cardiopulmonary AG, Hirrlingen, Germany) was used to equilibrate the medium with a mixture of 20% oxygen and 5% carbon dioxide (balance nitrogen). Two peristaltic pumps (ISM444, Ismatec SA, Glattbrugg, Switzerland) fed the perfusion and superfusion compartments of the vessel chambers independently with oxygenated medium. Adjustable, air-filled compliance chambers allowed to damp down the pulsation of the pumps to the desired levels. Starling resistors between the vessel chamber and the medium reservoir allowed to apply back pressure to the

perfusion circuits. Pressure probes (DPT-6100; Codan, Forstinning, Germany) at the vessel chamber outlets were used to monitor luminal pressure. Appropriate resistor settings were determined empirically in pilot experiments. The vessel chambers contained ports to retrieve medium samples in a sterile fashion. Tygon tubing with internal diameters of 3 and 4 mm was used to connect the components, and PharMed tubing with internal diameters of 3.2 mm and 8 mm was used as pump hoses in the peristaltic pumps (all tubing from Saint-Gobain Performance Plastics, Charny, France). The medium reservoirs, the perfusion chambers, and the oxygenators were double-walled and connected to a circulating water bath. The thermostats were calibrated to maintain a temperature of $37\pm0.02°C$ inside the perfusion chamber.

The superfusion circuit was set to a flow rate of 20 ml min^{-1}, whereas the perfusion was operated at 40 ml min^{-1} in most experiments. In a subset of experiments 60 ml min^{-1} was used instead. The residence times (reactor volume/flow rate), which indicate how long it takes to replace the medium in each compartment of the perfusion chamber, were calculated as 8 s and 2.6 min for the perfusion and superfusion compartments, respectively.

M199 with Earle's salts (PAA, Pasching, Austria) supplemented with 20% FCS (PAA), penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), gentamicin (100 µg ml^{-1}; Roche, Mannheim, Germany), and amphotericin B (50ng ml^{-1}; Sigma, Deisenhofen, Germany) was used as the default medium. Media were replaced every other day. In some perfusion conditions, dextran (average molecular weight 40000, Sigma) was added at a final concentration of 12% (w/v) to increase the viscosity of the medium to that of human blood. The total medium volume per circulation was 300 ml.

The perfusion system was designed to provide physiological shear stress if dextran was present in the medium. Wall shear stress was estimated using the Poiseuille formula by approximating vessels with rigid tubes[27]. The internal diameter of the vessels averaged 5 mm. Viscosity val-

ues were determined at 37°C in a rotational rheometer (Haake, Karlsruhe, Germany) and amounted to 4.0 mPa s and 1.0 mPa s for medium with and without dextran, respectively. This resulted in estimated wall shear stresses of 0.22 Pa and 0.05 Pa at 40 ml min^{-1}. Venous shear stress in humans was reported to be approx. 0.1 to 0.6 Pa[27], which puts the shear stress in the presence of dextran into the physiological range as intended.

Sterility of the system was of utmost importance as even minor contaminations might influence the gas, nutrient, and metabolite readings. Standard antibacterial and antifungal agents were present in all perfusion media. In addition to monitoring the oxygen partial pressure (see below), the turbidity of the medium was checked visually, and medium samples were retrieved after terminating the experiments to be examined for bacterial or fungal growth.

Perfusion conditions

In order to assess the influence of shear forces and of pressure on the metabolic demands of vessels, three different perfusion conditions were applied to the vessel segments for four days (Table 1). In each experiment one vessel segment of an animal was used as a control (group 1) and the other segment was assigned to either group 2 or 3 to balance individual differences between animals. In addition, some of the control experiments were extended to 8 days to evaluate the linearity of substrate consumption and lactate production over more than one media exchange. Separate sets of experiments addressed two key aspects of vascular regulation. Groups 1-d, 2-d, and 3-d were designed to test the influence of the endothelium by subjecting endothelium-denuded vessels to the same perfusion conditions. The conditions in groups 1-a, 2-a, and 3-a were selected to approximate arterial flow rates. The elevated flow rates were applied after perfusing the vessels at regular flow rates for at least 2 hours.

(Table 1)

Determination of nutrients, metabolites, electrolytes, and gas partial pressures

Once per day, 0.5 ml samples of the perfusion medium were drawn through sterile ports at the inlets and outlets of the vessel chambers into syringes which were capped immediately. If an exchange of the medium was scheduled, the samples were drawn before replacing the medium. Samples were read by a blood gas analyzer (ABL 700, Radiometer, Willich, Germany) using probes for oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), pH, glucose, lactate, and several electrolytes. To facilitate comparisons of glucose consumption and lactate production data, cumulative curves of the absolute values of molar changes were computed and transformed to take into account the production of 2 mol lactate per mol glucose (stoichiometry of lactate fermentation). The glycolytic index was calculated from the ratio of the slopes according to formula (1). Differences in oxygen partial pressures between perfusion inlet and outlet were assumed to be proportional to the oxygen consumption of the specimens.

$$\text{glycolytic index} = \frac{\text{cumulative } \Delta \text{ [lactate]}}{(-2)\cdot\text{cumulative } \Delta \text{ [dextrose]}} \quad (1)$$

Organ bath experiments

Vessel responses to contracting and dilating agents were measured in an organ bath system (IOA-5300, FMI) before and after the perfusion experiments as described previously[15]. In brief, rings of 2 mm segment length were mounted in organ baths containing KHB which was gassed with 95% O_2/5% CO_2. Vessel tensions were recorded by isometric force transducers and stored by a PC data acquisition software. During an equilibration period of at least 90 min the basal tension was readjusted to 20 mN repeatedly, and again after an initial challenge with 150 mM KCl. A second KCl challenge was then used to determine the maximum contraction induced by depolarization.

After recording a cumulative norepinephrine (NE, Aventis, Frankfurt/Main, Germany) dose-response curve to determine the maximum contraction, the rings were allowed to return to the baseline. A second NE dose-response curve was used to constrict the vessels to 80% of the maximum tension, and acetylcholine (ACh, Alexis, Lausen, Switzerland) dose-response curves were then recorded to assess endothelium-dependent relaxation. A single excess dose of sodium nitroprusside (SNP, 500 µM, Alexis), a spontaneous nitric oxide donor, was added at the end of each experiment to verify endothelium-independent relaxation.

Determination of tetrazolium dye reduction

Reduction of tetrazolium salts to coloured formazans by cellular enzymes causes an increase of absorbance which is proportional to the intracellular redox state and thus to the number of viable cells[2]. Tetrazolium dye reduction on the luminal surface of vessels as a measure of tissue viability was assessed as described previously[15]. In brief, the vessels were opened longitudinally and mounted with the luminal side facing up in custom-made holders which exposed individual wells of 0.30 cm^2 surface area. The conversion of the chromogenic substrate 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was then determined in these wells according to the manufacturer's instructions (Promega, Madison, WI, USA).

Histology

Short segments were removed from the vessels before and after the perfusion experiments and fixed in phosphate-buffered formaldehyde (4%). Specimens were embedded in paraffin and thin sections were prepared and mounted on slides. The general wall structure was visualized by hematoxylin and eosin (H&E) staining using standard methods. Apoptosis was assessed by terminal dUTP nick end labeling (TUNEL) using the kit manufacturer's protocol (Millipore, Schwalbach, Germany). Von Willebrand Factor (vWF) and E-selectin were labelled with poly-

clonal rabbit anti-human von Willebrand Factor (Dako, Glostrup, Denmark) and monoclonal mouse anti-human E-Selectin (Millipore) antibodies, respectively. Bound primary antibodies were visualized by biotinylated anti-rabbit and anti-mouse IgGs (Vector Laboratories, Burlingame, CA, USA) as appropriate, and the Vectastain Elite ABC kit (Vector Laboratories) using diaminobenzidine (Sigma) as substrate.

Statistical analysis

Numerical data are presented as mean±standard deviation. n refers to the number of animals. Four to eight rings were analyzed per vessel sample in organ bath experiments. Three to five wells per sample were used for dye reduction assays. Single-dose data with two different treatments were compared by paired t-tests. Multiple treatments were compared by ANOVA, followed by a Holm-Sidak test. Dose-response curves were compared by two-way repeated measurements ANOVA, followed by a Holm-Sidak test. Differences were assumed to be significant if the error probability p was less than 0.05.

Results

General performance of the perfusion system

Pilot studies were conducted to obtain perfusion flow rates which were as close as possible to arterial flow rates without causing irreversible vein distension. The empirically determined settings of the starling resistors were applied in groups 3, 3-d, and 3-a and resulted in luminal pressures of 20.5±3.3 mm Hg (2.7±0.4kPa) above the corresponding controls. Flow rates of 60 ml min^{-1} did not cause distension in the absence of luminal pressure, whereas partial distension was detected if such pressure was applied. Flow rates of 80 ml min^{-1} or higher caused irreversible vessel distension if additional luminal pressure was applied. Therefore a flow rate of 60 ml min^{-1} was used in the subset of experiments using increased perfusion flow rates (groups

1-a, 2-a, and 3-a), even if this was less than physiological flow rates of arteries of similar sizes.

The pulse wave form in the presence of additional luminal pressure resembled venous wave-forms in amplitude (approx. 7 mm Hg) but not in shape as the latter was determined by the design of the peristaltic pump head. In the absence of additional luminal pressure, pulsation was negligible.

Impact of endothelium removal on vessel function

In order to evaluate whether mechanically denuded vessels are suitable controls to investigate the involvement of endothelium in transmitting shear stress or pressure-related signals, the impact of denuding on several functional parameters was determined. Mechanical denudation did not significantly alter the response of these vessels to KCl (43.74±23.07 vs. 44.22±16.83, p=0.981, paired t-test). Half maximal effective concentrations (EC_{50}) of NE were also not affected by denudation [log(EC_{50}): -6.76±0.56 vs. -6.55±0.35, p=0.453, paired t-test]. Tetrazolium dye reduction indicated similar reductive capacities, although denuded vessels tended to show a lower reductive capacity (0.71±0.30 vs. 0.43±0.15, p=0.108, paired t-test). The organ bath tracings in Fig.4 demonstrate that denuded vessels no longer relaxed to intermediate concentrations of the endothelium-dependent vasodilator ACh. However, they retained the endothelium-independent constricting response at higher concentrations, which can also be seen in the native controls. These results indicate that the chosen method of denudation selectively abolished endothelial function while leaving both responses to vasoconstrictors and reductive metabolism intact.

Influence of perfusion on contractile responses and on tetrazolium dye reduction

KCl induced contractions were not affected by perfusion except in groups 1', 1-d, and 2-d, which were attenuated compared to the controls on day 0 (p=0.009, ANOVA, Fig. 2), indicat-

ing that a long duration of the perfusion under standard conditions, as well as the perfusion of denuded vessels in the absence of luminal pressure for 4 days is detrimental to contractile function.

Analysis of the NE dose-response curves showed that group 3 vessels responded stronger and group 1' vessels responded weaker to NE compared to the day 0 controls (p<0.001 ANOVA, Fig. 3A). Denuded vessels also contracted weaker to NE on day 4 compared to day 0 (p<0.001, ANOVA, Fig. 3B). The post-test identified a stronger response of group 3-d vessels compared to 1-d on day 4 at the highest NE doses, indicating a small but significant benefit of luminal pressure even in the absence of endothelium. NE dose response curves of vessels perfused at higher flow rates did not differ significantly between days 0 and 4 (p=0.580, ANOVA). EC_{50} values (Table 2) were not affected significantly by any of the treatments (groups 1,2,3,1': p=0.208; groups 1-d, 2-d, 3-d: p=0.098; groups 1-a, 2-a, 3-a: p=0.316; ANOVA).

(Table 2)

Endothelial function before and after perfusion was assessed by determining the response to the endothelium-dependent vasodilator ACh. As shown in Fig. 4, fresh vessels relaxed at ACh concentrations up to 1E-6 M, whereas 1E-5 M elicited a constriction. The relaxing action was selectively suppressed by removing the endothelium. In contrast, perfused vessels did not show any response to ACh independent of the perfusion conditions. Fresh and perfused vessels relaxed almost completely after administration of 500 μM SNP in all groups (Fig. 4).

Tetrazolium dye reduction data indicated an influence of denudation on reductive capacity on day 4 in groups 1-d and 2-d compared to native vessels on day 0 (p<0.001, ANOVA, Fig 5), whereas the process of denudation itself did not reduce reductive capacity significantly on day 0. Denuded vessels also showed lower tetrazolium dye reduction compared to the corresponding vessels from groups 1, 2, and 3 on day 4. Vessels of groups 1, 2, 3 as well as of groups 1-a,

2-a, and 3-a did not alter significantly compared to native vessels on day 0 with respect to tetrazolium dye reduction, whereas vessels of group 1' had a lower reductive capacity on day 8.

Changes of gas partial pressures

pO_2 values measured at the vessel chamber inlet were independent of time and perfusion conditions and averaged 147.4 ± 2.4 mm Hg. Oxygen gradients between inlet and outlet differed significantly between groups 1, 2, and 3 (Fig. 6, $p < 0.001$, ANOVA). Delta pO_2 was independent of the incubation time except for a difference between days 1 and 4 in group 1. In group 1', delta pO_2 significantly changed over time ($p = 0.036$, ANOVA) although the post-test failed to identify individual days. pCO_2 did not change over time ($p = 0.821$), indicating a constant oxygenator performance. There were no significant differences in terms of oxygen gradient or pCO_2 between groups 1-d, 2-d, and 3-d as well as between groups 1-a, 2-a, 3-a.

Changes of lactate, glucose, and pH

Glucose was steadily consumed, and lactate produced over time (Fig. 7 and 8). To test the hypothesis of a glycolytic conversion of glucose to lactate, the absolute changes of glucose and lactate concentrations (assuming a stoichiometric yield of 2 mol lactate per mol of glucose) were compared by ANOVA. Data obtained from groups 1, 2, 1', 2-d, 1-a, 2-a, and 3-a confirmed the hypothesis of lactate fermentation. This indicates that luminal pressure as well as denudation altered nutrient usage in BSV. In these groups (3, 1-d, 3-d) the amounts of glucose used were not sufficient to make up for the amounts of lactate produced. The glycolytic indexes computed from these data are summarized in Table 3.

(Table 3)

pH values remained within the physiologic range (7.35 <pH<7.45) during the entire duration of the experiments in all groups. There were no significant changes over time.

Histological Analysis

Evaluation of H&E-stained thin sections did not reveal changes in the gross structure of the tunica media and of the tunica externa, whereas the tunica intima showed a loss of ECs without apparent changes of basement membranes after 4 days in group 1 (Fig. 9). In the presence of dextran (groups 2 and 3), ECs were still detectable after 4 days, although the density of the cells in the sections appeared to be lower compared to fresh controls. Applying luminal pressure (group 3) did not further enhance survival of the endothelium. After 8 days (group 1') the subendothelial layer showed signs of disintegration in addition to the complete loss of ECs. TUNEL staining did not reveal any signs of apoptosis up to 4 days under all perfusion conditions, whereas all layers of the vessel wall showed apoptotic cells after 8 days (group 1').

Vessels of groups 1 through 3 were also analyzed for histological markers of endothelial function (Fig. 10). VWF, a glycoprotein stored in and secreted by ECs, was detectable before and after perfusion both in ECs and close to basal membrane in groups 2 and 3, whereas group 1 showed staining only of the basal membrane due to the loss of most endothelial cells. E-selectin, a shear-stress inducible adhesion molecule, stained sparsely in fresh BSV, but was readily detectable after perfusion in groups 2 and 3, indicating a shear-stress induced response in the presence of dextran.

Denuding removed the endothelium entirely without compromising the basement membranes (Fig. 11). Freshly denuded vessel did not show any signs of apoptosis, as expected. While the gross structure of the vessels did not change during perfusion, apoptosis was noticeable at varying degrees. The luminal side was affected under all perfusion conditions, whereas vessels perfused in the absence of dextran showed apoptotic nuclei throughout the vessel wall.

Perfusion at elevated flow rates caused a loss of endothelium within 4 days (Fig. 12), which was least prominent in vessels incubated with luminal pressure (group 3-a). There were only

few apoptotic cells which were all located close to the luminal face of the vessel wall, suggesting a gradual loss over time when compared to the results seen in mechanically denuded vessels (cf. Fig. 11). Again, the overall structure of the tunica externa and tunica media was not affected.

Discussion

This study has compared the influence of shear forces, luminal pressure, and flow rates on bovine saphenous vein function and metabolism in a perfusion bioreactor. The main objective was to study the oxygen and glucose consumption of vascular tissue under conditions similar to the ones used in vascular tissue engineering protocols, in order to improve procedures for building vessel grafts usable in coronary or peripheral reconstructions.

Oxygen supply is a critical parameter for blood vessel cultivation as hypoxia or hyperoxia causes a variety of abnormalities[18]. On the other hand, hypoxia is considered a strong signal in wound healing and may thus promote in vitro tissue growth[19]. For vascular tissues, pO_2 values of 150 mm Hg (20.0 kPa) were reported to be sufficient[14]. The perfusion system used in this study delivered medium to the vessels with a pO_2 of 147.4±2.4 mm Hg. A literature search performed at the beginning of the project did not reveal specific information about the oxygen consumption of native vascular tissues in perfusion models. However, a variety of data are available from fragments in microrespirators or from strips in organ baths[28]. Resting bovine mesenteric veins, the only bovine veins included in this work, were reported to consume 6.9E-8 mol g^{-1} min^{-1}. According to Henry's law, the solubility of oxygen in aqueous media at 37°C is 2E-7 mol ml^{-1}. The theoretical upper limit of oxygen supply at a total flow rate of 60 ml min^{-1} of medium (40 ml min^{-1} perfusion + 20 ml min^{-1} superfusion) is approx. 1.2E-5 mol min^{-1}. The mass of the vessel segments used in this study was close to 1 g, which according to the above-

mentioned data can be expected to consume approx. 6.9E-8 mol min^{-1}. Therefore it seems unlikely that resting vessels deplete oxygen to critical levels, which was confirmed by the pO_2 values at the vessel outlets which did not drop below 129 mm Hg (17.2 kPa) in all groups.

The general purpose culture medium used in this study (M199) contained glucose as the major energy source at a concentration of 1 g l^{-1} (5.6 mmol l^{-1}). The analysis of glucose consumption and of lactate production in this study (Figs. 7 and 8) clearly indicates a glycolytic metabolism in groups 1, 1', and 2, i.e. one mol glucose is converted stoichiometrically to two mol lactate with a yield of 2 mol ATP, compared to a theoretical yield of 38 mol ATP if glucose was converted to CO_2 using aerobic respiration. This agrees with earlier findings that hardly any carbon from glucose is released as CO_2 by blood vessels[24]. Of the basic groups, only group 3 differed from this pattern in that significantly more lactate was produced compared to the glucose consumption. Using a mass of approx. 1 g per vessel segment and a culture medium volume of 300 ml, this results in lactate formation rates ranging from 0.25 to 0.29 µmol g^{-1} min^{-1} which agrees well with published data[28]. Aerobic glycolysis in blood vessels under static[4] and laminar flow[7] conditions has been observed earlier. In a recent study of silk-based tissue-engineered vessel grafts, glycolytic metabolism was found only after approx. 7 days of perfusion, whereas static controls showed a glycolytic index above unity which slowly increased over time[37]. It is interesting to note that in our study neither flow with subphysiological shear forces in groups 1 and 1' nor flow with physiological shear forces in group 2 influenced glucose metabolism compared to the data under static conditions reported in the literature. However, application of circumferential strain by increasing luminal pressure caused significant changes in metabolism. Obviously the vessels still did not employ oxidative phosphorylation as this would have reduced the glucose formed/lactate produced ratio. Instead, even more lactate was produced per glucose, indicating that the vessels utilized alternative energy sources

instead. Several alternative energy sources are available in serum-supplemented culture medium, e.g. L-glutamine, L-leucine, L-valine, and fatty acids, all of which can be utilized for energy production by vascular smooth muscle cells[4; 9; 28]. The hypothesis of <u>Zhang et al.</u> that endogenous glycogen acts as an additional energy source[37] must also be considered, although it is questionable whether the small amount available in the vessels is sufficient to fuel the metabolism for several days. A more thorough analysis of the spent medium will have to show which of these sources are utilized.

Our approach to assess the influence of endothelium on vessel function and metabolism during perfusion was twofold. First, it was necessary to demonstrate that ECs maintain key synthetic properties during perfusion, as this is a prerequisite for any assumed role in regulation. VWF is expressed constitutively in intact ECs[6] and is found both inside the cells as well as in the subendothelium. This makes vWF suitable as a specific marker for endothelium. Histological analysis of fresh vessels and of vessels of groups 1 through 3 demonstrated that vWF was expressed in ECs until day 4 of perfusion. A decrease of vWF expression was noticeable after 8 days (group 1'). E-selectin is an adhesion molecule and is considered a specific EC surface marker. Expression of E-selectin has been shown to be upregulated by shear stress[8]. Our histological data agree with this hypothesis (Fig. 10). Fresh vessels displayed only a moderate expression of E-selectin, whereas vessels of groups 2 and 3 stained strongly for the adhesion molecule. Vessels of the control group 1, which were perfused using lower shear forces, displayed no staining due to the loss of endothelial cells. These results indicate that ECs not only maintain synthetic activities, but also respond to mechanical stimuli in our perfusion system. Next, we mechanically removed ECs of some vessels (groups 1-d through 3-d) to exclude any regulatory influence of these cells. In the absence of endothelium, all perfusion conditions caused an increased use of energy sources other than glucose, although the overall lactate production

was not different from groups 1 through 3 (Fig. 8). However, care must be taken when speculating about a role of endothelium in regulating the vessel wall metabolism from these experiments, as there were strong signs of apoptosis in the vessel wall which are also likely to affect metabolism. On the other hand, the experiments conducted with increased flow rates caused a loss of ECs on day 4 while there were only few apoptotic cells. Under these conditions, luminal pressure was not able to induce a shift of the metabolism towards alternative energy sources. With all due care, ECs thus may play a role in regulating energy metabolism in response to luminal pressure.

Lactate production was not associated with a significant drop of pH in our experiments, mainly because the medium was replaced every other day. To produce vessel grafts economically, the medium composition may have to be optimized to reflect the particular requirements of vessels in terms of energy sources. In all groups the glucose consumption and lactate production appeared to be nonlinear in the initial 24 h. This may be due to a slow recovery from the stress inflicted on the animal before and during slaughter, or from the trauma of vessel resection, or it may be a consequence of ischemia/reoxygenation damage. It is interesting to note that this nonlinear glucose consumption was most prominent in mechanically denuded vessels, which may imply endothelial mechanisms to control energy metabolism in the neighboring parts of the vessel wall.

Contractile functions of the vessels remained intact in our perfusion system for at least 4 days. Contractions induced by depolarization with KCl were identical in groups 1, 2, and 3 before and after perfusion (Fig. 2). Mechanical denudation caused a loss of contractile force on day 4 in the absence of dextran, indicating a protective effect of shear forces. There were no differences between NE dose-response curves in group 1 before and after perfusion, whereas vessels of group 3 responded stronger to NE after perfusion compared to fresh vessels (Fig. 3). Stress

during animal transport and slaughter is likely to increase catecholamine levels which act as stress hormones. Prolonged exposure to these hormones causes a desensitization[29] which may still be effective when the fresh vessels are investigated. Perfusion with sufficient shear forces and luminal pressure appears to be more efficient in restoring and maintaining vessel function. Mechanically denuded vessels responded weaker to NE after 4 days. This can be explained by the obvious signs of apoptosis in the vessel wall in these groups. When using elevated flow rates, luminal pressure no longer shows a beneficial effect for vessel function. This may again be explained by the loss of endothelium in these groups, which indicates that the endothelium is mandatory for the salutary effects of luminal pressure.

The amount of tetrazolium dye reduction indicates the metabolic state of cells and tissues[21]. In our perfusion system, metabolic states did not change within 4 days, unless the vessels were mechanically denuded (groups 1-d and 2-d, Fig. 5). These data are in line with KCl-induced constrictions and with histological evidence of apoptosis, so attenuated responses to KCl can be explained with a lack of mitochondrial activity. Endothelium-dependent relaxation was abolished after perfusion regardless of the perfusion conditions, as seen by the absence of a reaction towards ACh (Fig. 4). On the other hand, endothelium-independent relaxation as shown by the response to SNP was unaffected. This indicates a rather specific loss of contractile function, in contrast to histological evidence of other endothelial functions, and without a general loss of smooth muscle function. Obviously, neither increased shear forces nor application of luminal pressure as used in groups 2 and 3 were able to prevent this loss of function. It would require further analysis of ECs after perfusion to determine the changes in the levels of expression of relevant enzymes in order to better describe this partially functional state of ECs. This, however, was beyond the scope of the current study. At this time we can only speculate that our perfusion system lacked certain aspects of the human circulation, e.g. true arterial flow

with an appropriate pulse waveform or axial stretch, both of which might help to retain the fully functional state of ECs.

Vessels perfused for 8 days showed a significant decrease of KCl-induced and of NE-induced vasoconstriction. Interestingly, this was not accompanied by a loss of tetrazolium dye reduction, so the loss of contractile function cannot simply be attributed to a reduced energy metabolism. This is further supported by the glucose consumption and lactate production which appeared to be linear until day 8.

Histological analysis revealed a loss of ECs within 4 days of incubation in group 1. In contrast, vessels of groups 2 and 3 retained ECs although their density appeared lower (Fig. 9). Dextran, which was present in these groups, has previously been shown to increase the survival of ECs exposed to shear stress[33]. Clerin et al. [5] have investigated the decay of native endothelium during long-term perfusion experiments using porcine arteries as a model. This study concluded that subphysiological flow rates were necessary to retain the endothelium at least partially. In all other conditions, a loss of endothelium and a progredient loss of smooth muscle starting from the luminal side was observed. The results of the present study indicate a similar process, which can apparently not be prevented by the flow conditions which were applied (Fig. 12). We have also confirmed (M. Hoenicka, unpublished observations) the finding of many studies, reviewed in[16], that freshly seeded ECs do not deteriorate under similar conditions. As the bovine vessels in our study were without a proper oxygenation for at least 30 min, an ischemia/reperfusion damage is a reasonable explanation for the loss of ECs in native vessels. The data obtained with mechanically denuded vessels support the hypothesis that an intact endothelium is mandatory to maintain vessel wall integrity in perfusion experiments.

This study also attempted to address the question whether increased flow rates affect vessel function and metabolism. This is analogous to the changes in flow and pressure which human

veins encounter when used as grafts in the arterial system. This is also analogous to many vascular tissue engineering protocols which try to adapt tissue engineered constructs to arterial flow conditions by gradually applying elevated flow rates and pressure. However, our pilot experiments had shown that BSV do not withstand true arterial conditions, as they show signs of irreversible distension. True arterial flow rates would have required sufficient pressure in the superfusion circulation, or even external stenting of the vessels, to counteract the flow-induced vessel distension. Also, the vessels lost their endothelium regardless of the perfusion conditions even with the moderate "arterial" flow rates used in this study (Fig. 12). This is in contrast to published studies using arterial vessels in similar perfusion experiments[32] which demonstrated a preservation of endothelium under arterial flow conditions. Experiments with porcine saphenous veins using similar flow rates as in the present study had shown a flow-rate-dependent beneficial effect of perfusion versus static culture in terms of EC coverage, although in all cases coverage was reported to be less than in native vessels[11]. It would be interesting to see whether BSV could be slowly adapted to conditions closer to arterial flow by using the "ramped pressure" or "controlled shear stress" approaches described in this study. In any case, our results indicate that there are no signs of a completely different energy metabolism under elevated flow rates. Actually, there was no usage of energy sources other than glucose in response to pressure, which may be due to the apparent loss of endothelium in group 3-a compared to group 3.

Some other limitations of this study need to be addressed in future studies. First, the consumption of other energy sources should be monitored by analyzing the spent medium to further identify the major sources of energy under load. Next, the incubation times of 4 days and 8 days are arguably too short to provide data comparable to tissue-engineering protocols which often take several weeks to complete. Axial stress, which has been shown to affect vascular

function and remodelling ex vivo [17; 25], could not be modulated with the setup used in this study. Finally, only equivalent experiments using seeded scaffolds to test the oxygen and nutrient consumption of growing cells will provide more specific information in order to optimize vascular tissue engineering processes.

Conclusion

The results of the present study indicate that metabolism should be monitored closely in all vascular tissue engineering projects as it reflects the perfusion conditions under which the vessel graft is developed. Vessel wall metabolism is apparently not limited by the oxygen supply provided by aqueous media at 20% oxygen which permits application of partial pressures which best stimulate growth of varous vascular cell types. As glucose is apparently not the only energy source of vessels under load, tissue culture media may have to be adapted to supply sufficient amounts of alternative substrates and to decrease lactate production in order to avoid costly media changes during the production of tissue-engineered vessel grafts. The results also suggest beneficial effects of increased shear forces in maintaining vessel integrity as well as a mandatory role of the endothelium in transmitting salutary effects of luminal pressure.

Acknowledgements

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Table 1. Perfusion conditions

	1	1'	2	3	1-d	2-d	3-d	1-a	2-a	3-a
n	16	8	5	8	5	5	5	4	4	4
dextran	-	-	+	+	-	+	+	-	+	+
pressure	-	-	-	+	-	-	+	-	-	+
duration	4d	8d	4d	4d	4d	4d	4d	4d	4d	4d
denuded	-	-	-	-	+	+	+	-	-	-
flow rate (ml min^{-1})	40	40	40	40	40	40	40	60	60	60

Table 2. NE log(EC$_{50}$) values

group	log(EC$_{50}$)
control day 0	-6.48±0.5
group 1 day 4	-6.36±0.16
group 2 day 4	-6.77±0.32
group 3 day 4	-6.67±0.32
group 1' day 8	-6.57±0.23
group 1-d day 4	-6.13±0.18
group 2-d day 4	-6.02±0.03
group 3-d day 4	-6.25±0.23
group 1-a day 4	-6.56±0.44
group 2-a day 4	-6.79±0.52
group 3-a day 4	-6.45±0.10

Table 3. Glycolytic indexes

group	value	significantly different from unity (two way ANOVA)
1	1.16	n.s.
2	1.01	n.s.
3	1.58	p<0.001
1'	1.01	n.s.
1-d	1.94	p<0.001
2-d	1.54	n.s.
3-d	1.82	p<0.001
1-a	1.17	n.s.
2-a	1.08	n.s.
3-a	1.02	n.s.

Figures
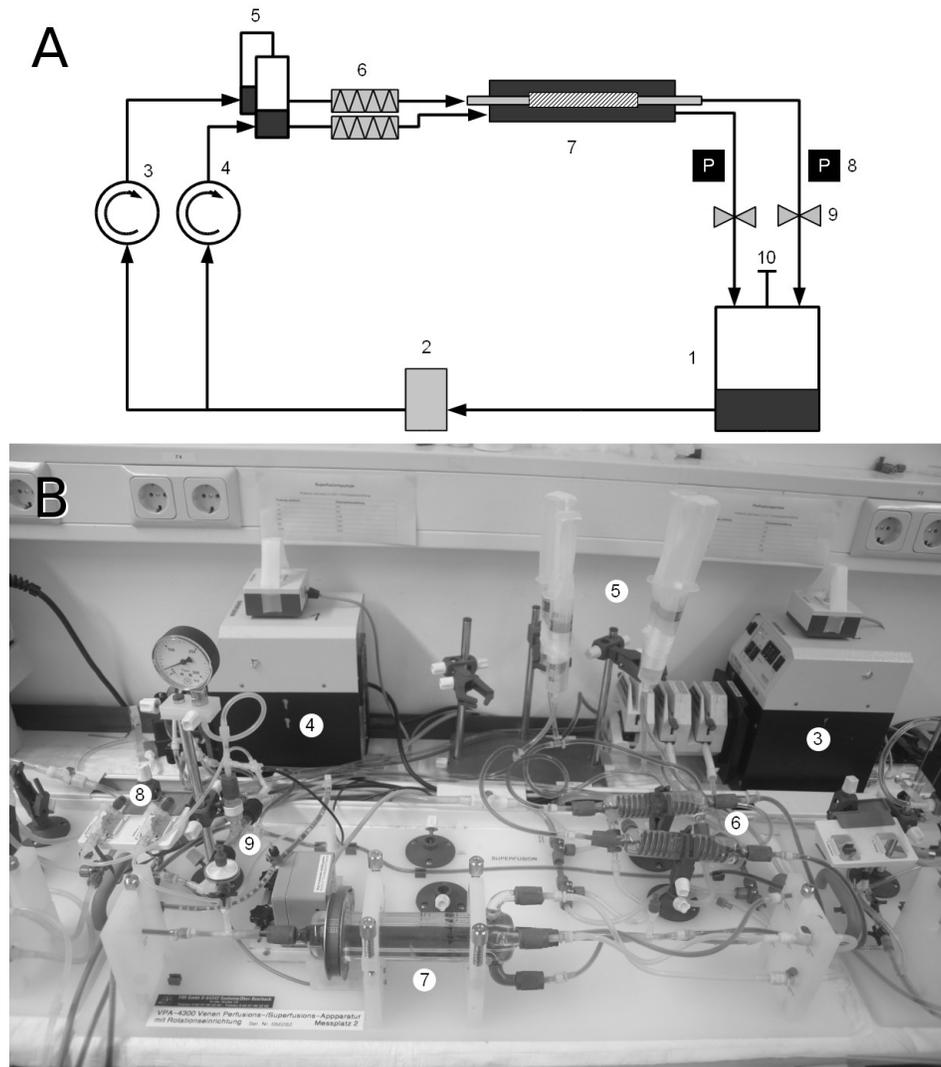
Figure 1

Schematic representation (A) and overview image (B) of the perfusion bioreactors used in this study. 1, medium reservoir; 2, hollow fibre oxygenator; 3, peristaltic pump (perfusion); 4, peristaltic pump (superfusion); 5, compliance chambers; 6, vessel chamber with vessel (hatched); 7, pressure transducers; 8, Starling resistors; 9, sterile vent. (B) shows the shared pumps (3 and 4) and one of three circulations. Medium reservoirs and oxygenators (1 and 2) are mounted on a separate cart and are not visible in image B.
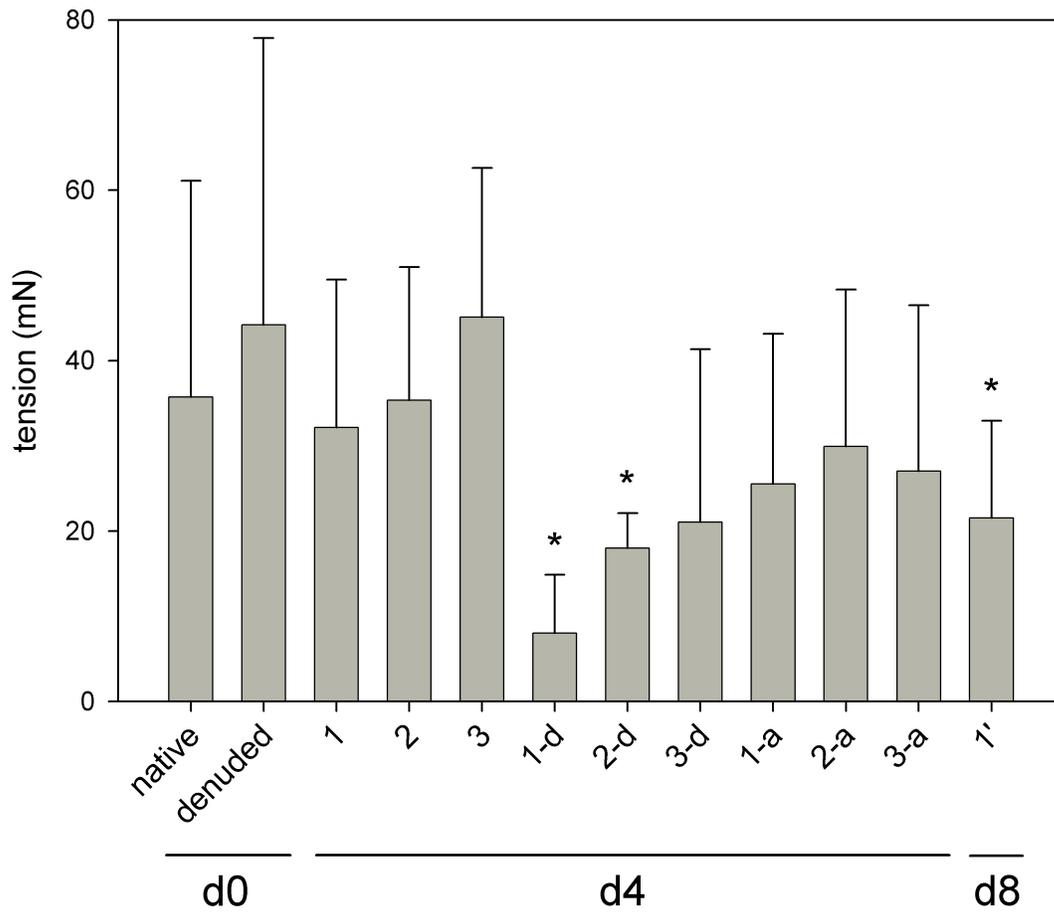
Figure 2

Maximum contraction of the vessels induced by depolarization with 150 mM KCl before and

after perfusion. * significantly different from native control on day 0 (p=0.009, ANOVA).
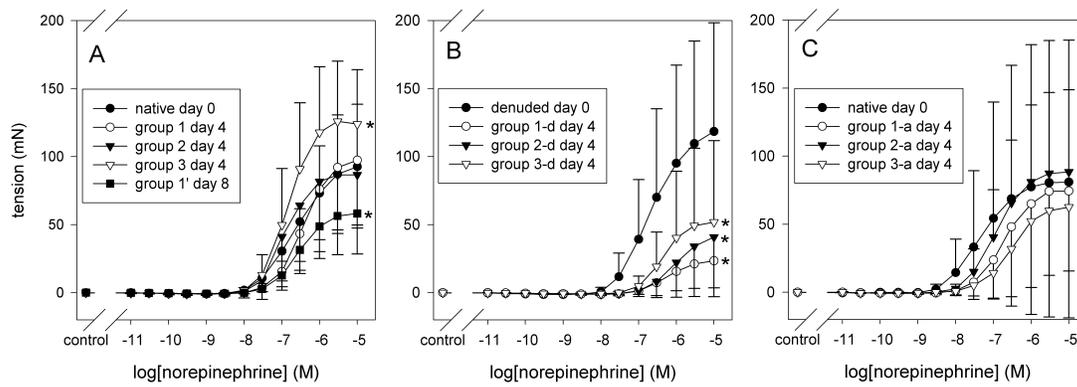
Figure 3

Norepinephrine dose-response curves of fresh and perfused vessels. (A) Comparison of groups 1, 2, 3, 1'. *, significantly different from day 0 control (p<0.001, ANOVA). (B) Comparison of groups using denuded vessels. *, significantly different from denuded control on day 0 (p<0.001, ANOVA). (C) Comparison of groups using increased luminal flow rates. There were no significant differences (p=0.580, ANOVA).
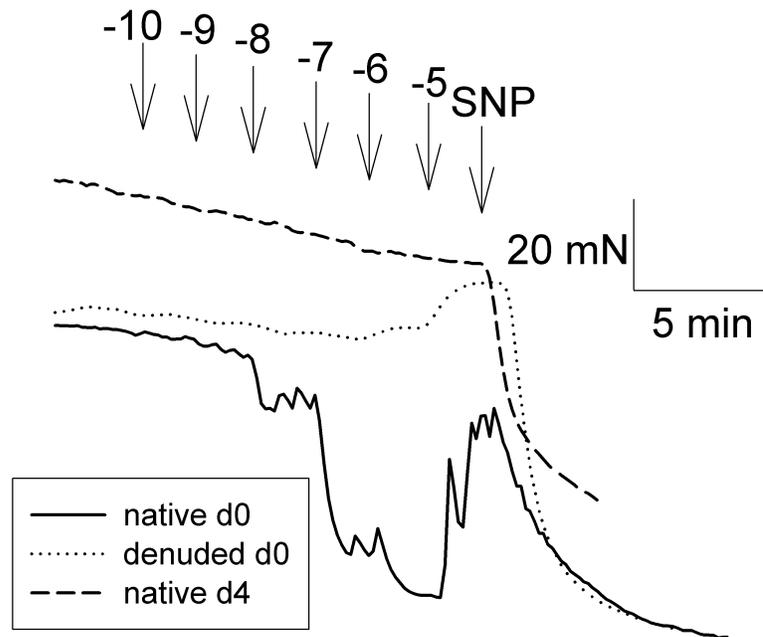
Figure 4

Organ bath tracings of acetylcholine dose-response curves of native vessels on day 0 (solid line), denuded vessels on day 0 (dotted line), and of perfused vessels on day 4 (dashed line). The response of perfused vessels to ACh was independent of the perfusion conditions, therefore the group 1 vessel shown here is representative for all other groups. Arrows with numbers indicate the logarithm of the concentration of acetylcholine. Endothelium-independent relaxation was assessed by a single dose of SNP (5E-4 M).
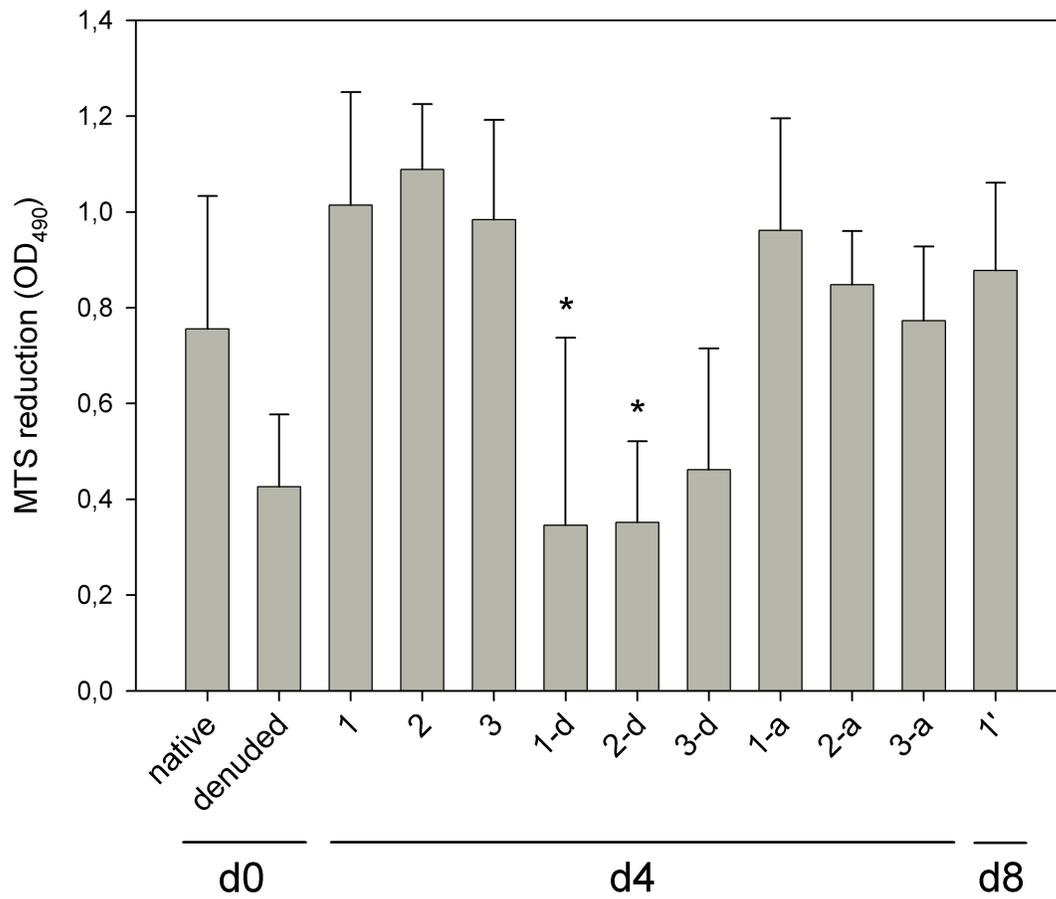
Figure 5

Tetrazolium dye reduction on the luminal vessel surface before and after perfusion. *, significantly different from native control vessels on day 0 (p<0.001, ANOVA).
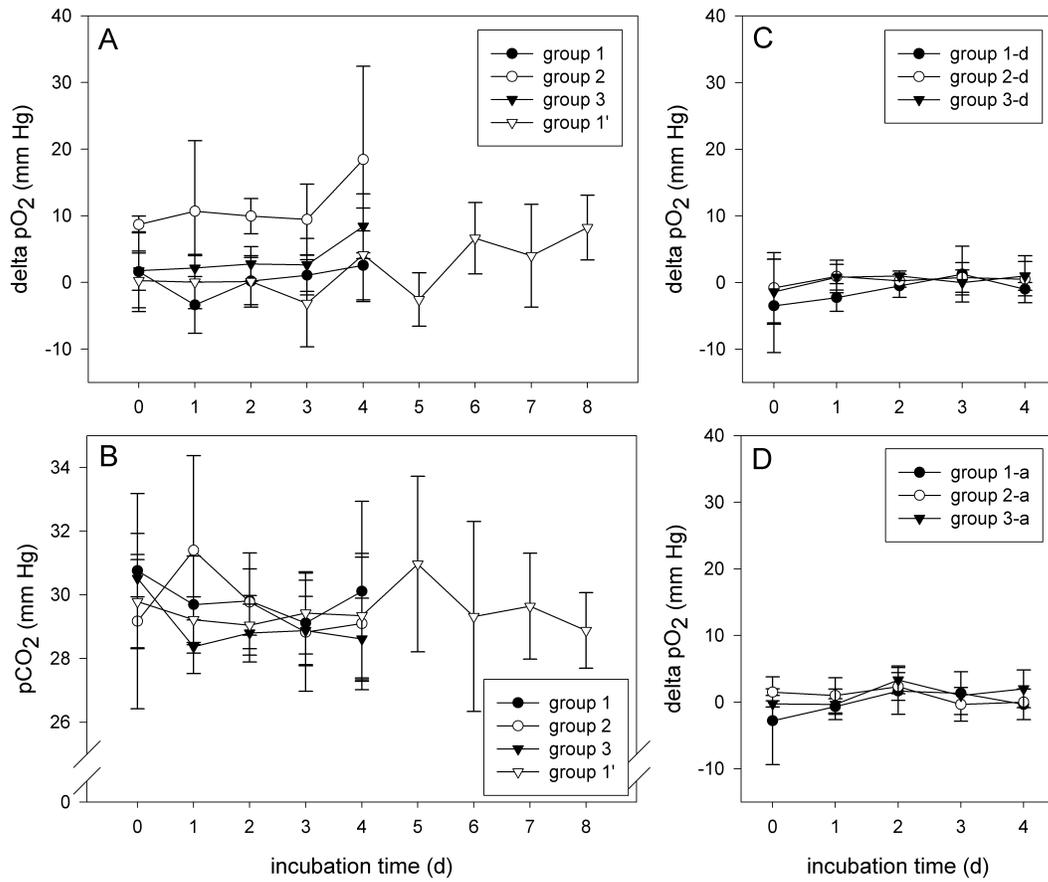
Figure 6

Time courses of oxygen gradients between vessel inlet and outlet (delta pO_2) and carbon dioxide partial pressures (pCO_2). (A) Oxygen gradients in groups 1, 2, 3, and 1'. (B) Carbon dioxide partial pressures in groups 1, 2, 3, and 1'. (C) Oxygen gradients in groups using denuded vessels. (D) Oxygen gradients in groups using increased luminal flow rates. See text for statistical evaluation.
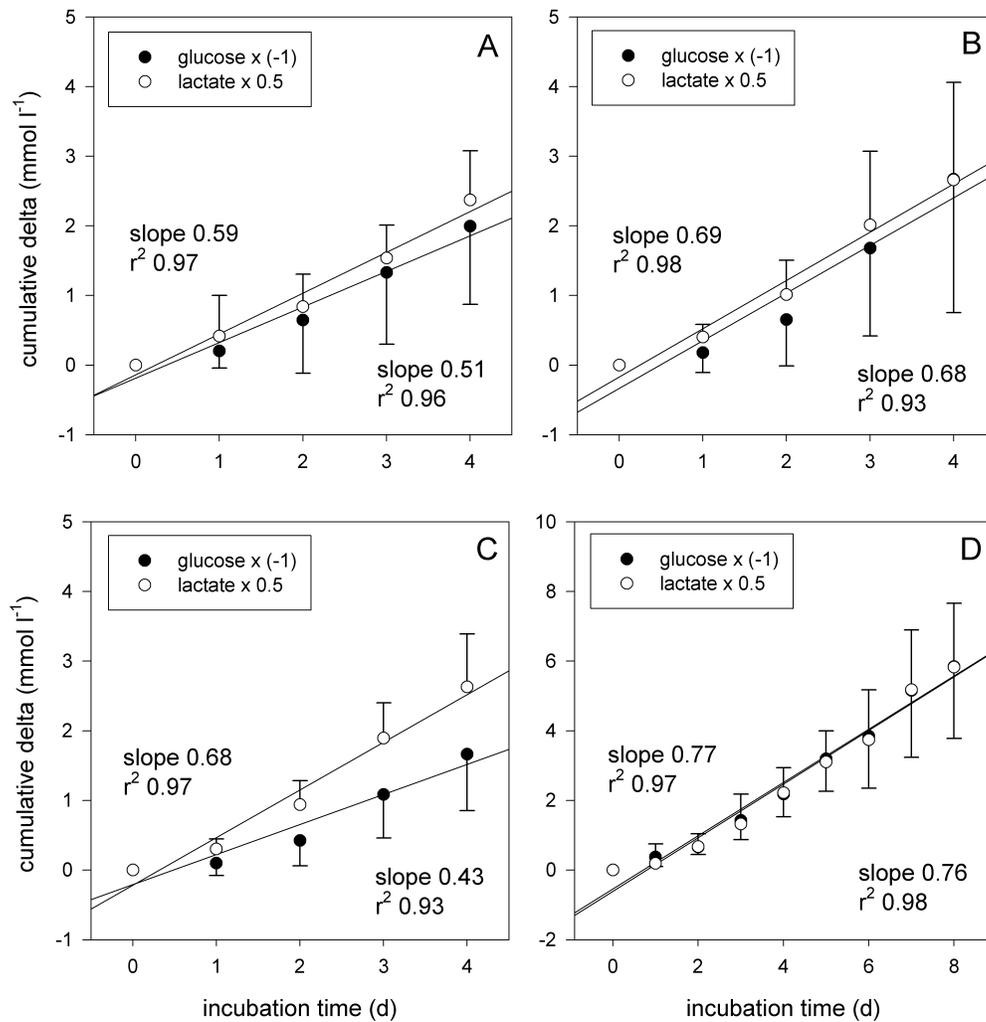
Figure 7

Cumulative absolute changes of glucose and lactate concentrations. The values represent the changes compared to the initial concentrations in the medium on day 0, corrected for the media changes every other day. To facilitate comparison and statistical analysis, glucose values were multiplied by -1, and lactose values by 0.5 to correct for glycolytic stoichiometry (two mol lactate produced per mol glucose consumed). (A) group 1; (B) group 2; (C) group 3; (D) group 1'. The curves in panel C are significantly different (ANOVA, p=0.001).
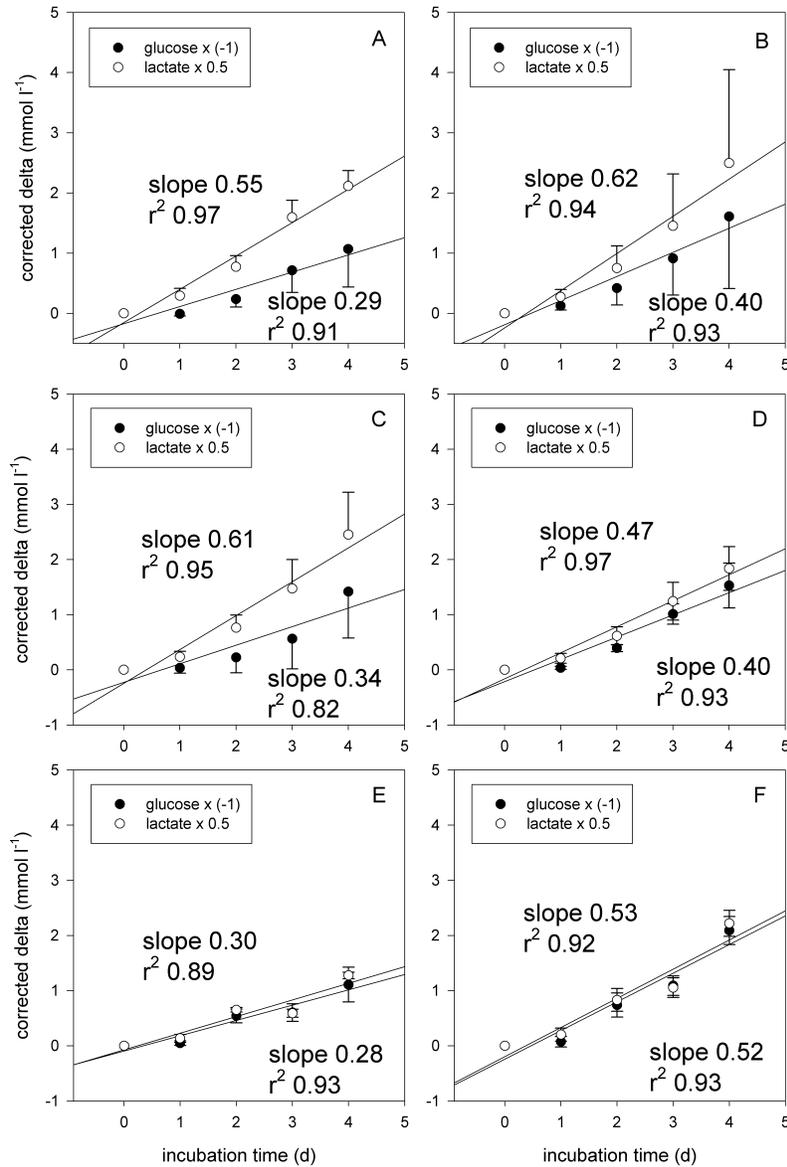
Figure 8

Cumulative absolute changes of glucose and lactate concentrations in groups using denuded veins or increased luminal flow rate. See Fig. 7 for data presentation. (A) group 1-d; (B) group 2-d; (C) group 3-d; (D) group 1-a; (E) group 2-a; (F) group 3-a. The curves in panels A and C are significantly different (ANOVA, $p<0.001$ and $p=0.002$, respectively).
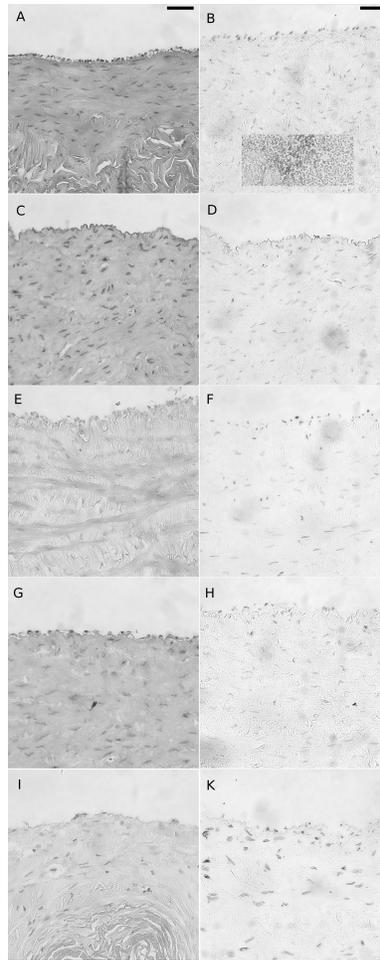
Figure 9

Histological analysis of vessel sections (groups 1, 2, 3, 1'). Images show HE-stained (left column) and TUNEL (right column) stained sections of fresh BSV (A,B), BSV perfused under standard conditions for 4 days (group 1) (C,D), BSV perfused in the presence of 12% dextran for 4 days (group 2) (E,F), BSV perfused in the presence of 12% dextran and luminal pressure for 4 days (group 3) (G,H), and BSV perfused under standard conditions for 8 days (group 1') (I,K). The lumen is on top in all images. The insert in panel (B) shows a human tonsil section as an apoptosis positive control. Bars indicate 50 μm.
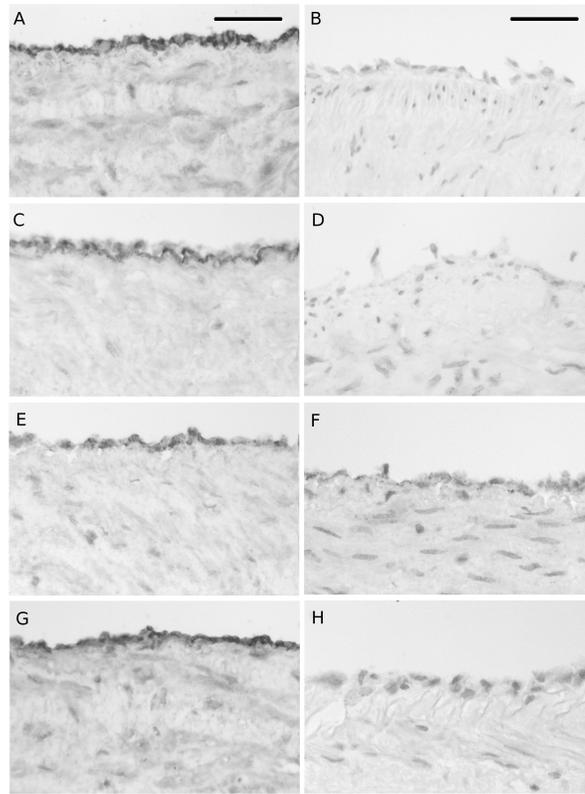
Figure 10

Immunostaining of vessel sections of groups 1 through 3. Images show sections stained for

vWF (left panel) and for E-selectin (right panel) of fresh BSV (A,B), BSV perfused under

standard conditions for 4 days (group 1) (C,D), BSV perfused in the presence of 12% dextran

for 4 days (group 2) (E,F), BSV perfused in the presence of 12% dextran and luminal pressure

for 4 days (group 3) (G,H). The lumen is on top in all images. Bars indicate 50 µm.
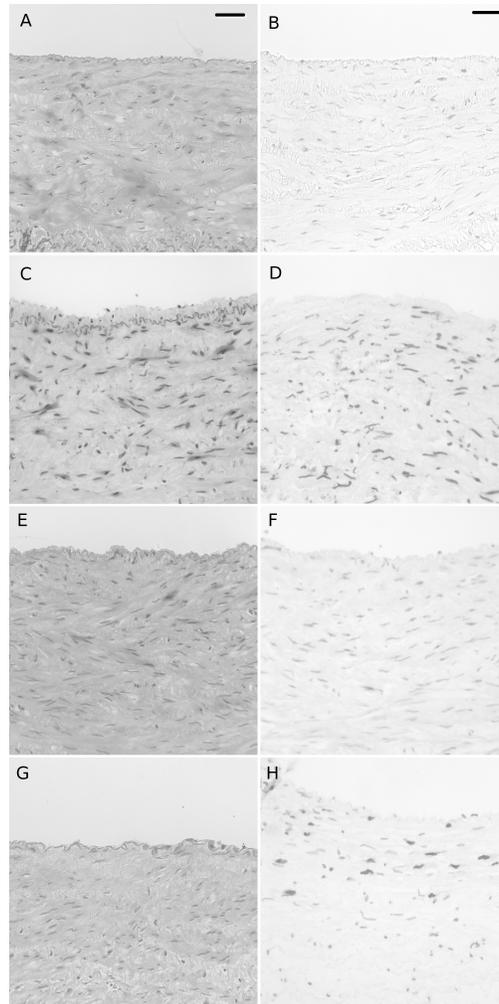
Figure 11

Histological analysis of vessel sections (denuded groups). The left column shows HE-stained sections, the right column shows TUNEL stained sections of (A,B) denuded BSV, denuded BSV perfused under standard conditions for 4 days (group 1-d) (C,D), denuded BSV perfused in the presence of 12% dextran for 4 days (group 2-d) (E,F), and BSV perfused in the presence of 12% dextran and luminal pressure for 4 days (group 3-d) (G,H). The lumen is on top in all images. Bars indicate 50 µm.
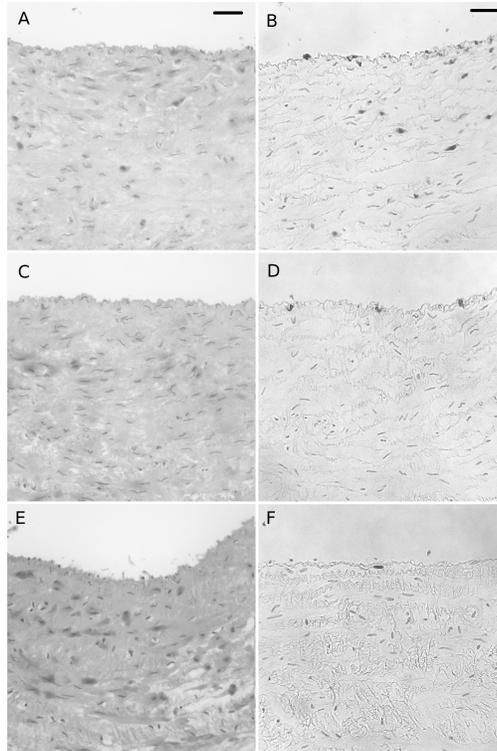
Figure 12

Histological analysis of vessel sections (increased flow rate groups). The left column shows HE-stained sections, the right column shows TUNEL stained sections of BSV perfused at 60 ml min^{-1} for 4 days (group 1-a) (A,B), BSV perfused at 60 ml min^{-1} in the presence of 12% dextran for 4 days (group 2-a) (C,D), and BSV perfused at 60 ml min^{-1} in the presence of 12% dextran and luminal pressure for 4 days (group 3-a) (E,F). The lumen is on top in all images. Bars indicate 50 μm.