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Computer-Assisted ex vivo, Normothermic Small Bowel Perfusion

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Key Words

Small bowel perfusion · Ex vivo perfusion system · Rat model · Graft pretreatment · Norepinephrine · Krebs' solution

Abstract

Background: In the present study, a technique for computer-assisted, normothermic, oxygenated, ex vivo, recirculating small bowel perfusion was established as a tool to investigate organ pretreatment protocols and ischemia/reperfusion phenomena. A prerequisite for the desired setup was an organ chamber for ex vivo perfusion and the use of syngeneic whole blood as perfusate. ***Methods:*** The entire small bowel was harvested from Lewis rats and perfused in an organ chamber ex vivo for at least 2 h. The temperature was kept at 37 °C in a water bath. Three experimental groups were explored, characterized by different perfusion solutions. The basic perfusate consisted of syngeneic whole blood diluted with either NaCl, Krebs' solution or Krebs' solution and norepinephrine to a hematocrit of 30%. In addition, in each group *l*-glutamine was administered intraluminally. The desired perfusion pressure was 100 mm Hg which was kept constant with a computer-assisted data acquisition software, which measured on-line pressure, oxygenation, flow, temperature and pH and adjusted the pressure by changing the flow via a peristaltic pump. The viability of the preparation was tested by measuring oxygen consumption and maltose absorption, which requires intact enzymes of the mucosal brush border to break down maltose into glucose. ***Results:*** Organ perfusion in group 1 (dilution with NaCl) revealed problems such as hypersecretion into the bowel lumen, low vascular resistance and no maltose uptake. In contrast a viable organ could be demonstrated using Krebs' solution as dilution solution. The addition of norepinephrine led to an improved perfusion over the entire perfusion period. Maltose absorption was comparable to tests conducted with native small bowel. Oxygen consumption was stable during the 2-hour perfusion period. ***Conclusions:*** The ex vivo perfusion system established enables small bowel perfusion for at least 2 h. The viability of the graft could be demonstrated. The perfusion time achieved is sufficient to study leukocyte/lymphocyte interaction with the endothelium of the graft vessels. In addition, a viable small bowel, after 2 h of ex vivo perfusion, facilitates testing of pretreatment protocols for the reduction of the immunogenicity of small bowel allografts.

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Introduction

Small bowel transplantation (SBT) still lacks the success rates of liver, heart or kidney transplantation. Despite the success of new immunosuppressive agents, e.g. tacrolimus or mycophenolic acid, in other solid organ transplantation, 2-year graft survival after allogeneic SBT in adults is only 38% [1]. Increasing immunosuppression, by adding supplementary agents (e.g. antibodies) to the medication of the recipient, will increase the risk of septic complications [2]. New strategies are necessary to improve the clinical results of SBT. Among these are protocols for local immunosuppression [3] and graft pretreatment [4]. In order to test strategies for the modulation of small bowel immunogenicity, we have established a computer-assisted ex vivo small bowel perfusion model which enables a normothermic, pressure-controlled perfusion for at least 2 h. A variety of experiments carried out in the 60s and 70s showed that special additives and perfusion solutions are required to meet the need of the intestinal epithelium [5, 6]. Three different types of perfusion solutions and additives were tested in our experiments. In each case, the blood was diluted to a hematocrit of 30%. In group 1 (n = 14) NaCl, in group 2 (n = 11) Krebs' solution and in group 3 (n = 8) Krebs' solution for dilution combined with norepinephrine as an additive was used. Physiological parameters (e.g. pH, pressure, flow) were measured on-line. The viability of the intestine perfused was tested by oxygen consumption and maltose absorption.

Materials and Method

Animals. The rat model was used in order to establish defined strain combinations for transplantation experiments in the future. Lewis rats served as blood donors for the ex vivo perfusion circuit and also as organ donors. The animals were anesthetized with ether followed by 0.05 mg/100 g body weight of 3.6% chloral hydrate, injected intraperitoneally. The entire small bowel was harvested on its vascular pedicle consisting of the arteria mesenterica superior and vena portae, using the technique described by Monchik and Russel [7]. The arteria mesenterica superior was cannulated with an Angiocath (Vialon-Angiocath, 20G Insyte-W, Becton Dickinson, Heidelberg, Germany). The venous outflow reached the blood reservoir directly (fig. 1).

Experimental Groups. Three experimental groups were investigated. In group 1 (n = 14) NaCl was used to dilute the blood to a hematocrit of 30%. In group 2 (n = 11) Krebs' solution and in group 3 (n = 8) Krebs' solution and continuous administration of norepinephrine as additive served for blood dilution. In each case, the hematocrit was 30% and *l*-glutamine, as a substrate for the intestinal epithelium, was instilled intraluminally.

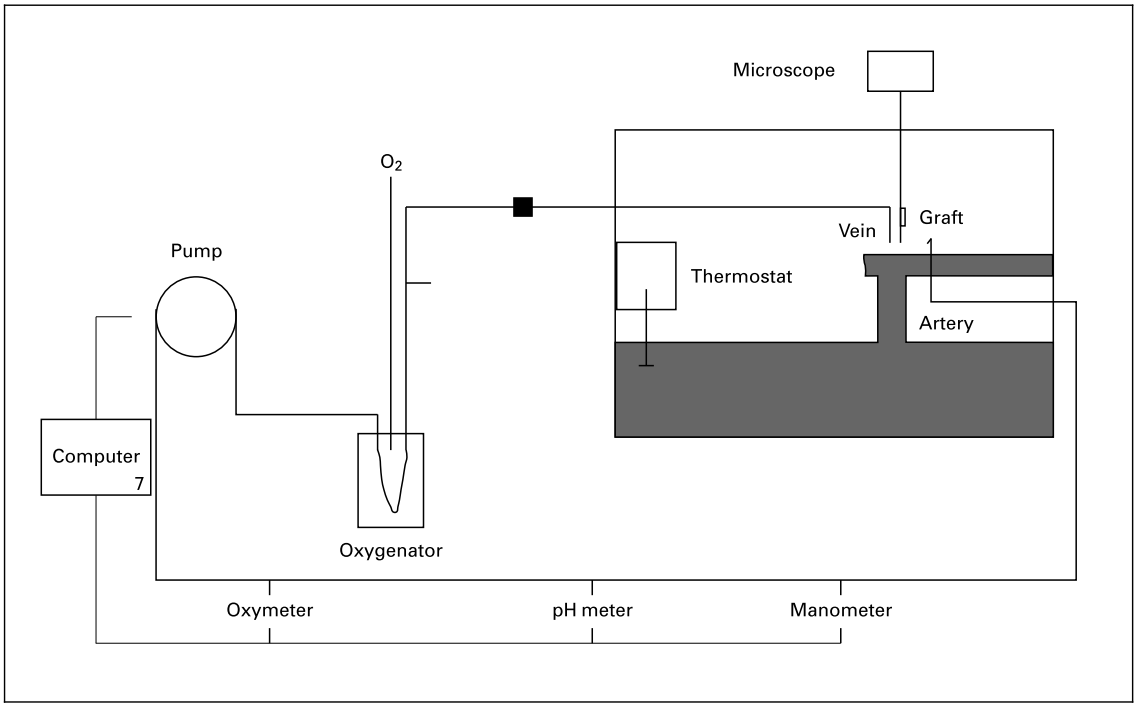


Fig. 1. Perfusion system.

Perfusion System. The perfusion system consisted of a water bath with a temperature kept continuously at 37°C by a thermostat (Haake DC5, Haake, Karlsruhe, Germany), an oxygenator, a roller pump (MCP, CA4, Ismatec, Wertheim, Germany), a pH meter (pH 540 GLP, WTW, Weilheim, Germany), a manometer (Direct BP 1, Bilaney, Düsseldorf, Germany), an oxymeter (Oxi 538, WTW), and a thermometer (INFCT, Newport Electronics, Deckenpfronn, Germany; fig. 1). The pressure during perfusion was kept at 100 mm Hg by autoregulation of the computer system via the flow rate.

The oxygenator was constructed by ourselves and consisted of a cylinder made of aluminum within a glass chamber. The aluminum cylinder can be heated inside with water from the water bath. Tubes consisting of a semipermeable membrane roll around the cylinder. Gas (95% O_2 and 5% CO_2) is insufflated into the glass chamber.

Hemolysis due to the perfusion circuit was measured after each experiment by analyzing free hemoglobin with a photometer. In brief, serum was taken from the perfusion circuit after 1 and 2 h of perfusion, serially diluted and the extinction was measured at 562 (A), 578 (C), 598 nm (B). Hemolysis was calculated as percentage of free hemoglobin compared with the total hemoglobin.

Computer-Assisted Control System. The hardware used was a regular personal computer (Pentium I, 133 MHz, 1.2 GB hard disc, 24 MB RAM) with a screw-terminal board for signal wiring (PCL-818L; Spectra, Leinfelden, Germany). The software consisted of Windows 95 and a data acquisition software (Notebookpro version 9.02). With the program Build-Time, one can create a block diagram of the experiment. With the so-called ICONview-Window, the settings for each block are defined and connected with the block icons. Via the connection of the block icons, a block diagram is created. By connecting input block icons with the log block icon, data can be recorded and through the display block icon they can be shown online on the computer screen either as a graph or as a numeric value. With the help of calculated block icons, data can be processed with a variety of mathematical operations. Output signals are transformed through output block icons.

Perfusion Solutions. Blood was harvested from Lewis rats via puncture of the abdominal aorta. The syringe was flushed with heparin (100 U) before puncture. No further heparin was added to the perfusion circuit. A hematocrit of 40–46% of the blood was diluted with one of the three different perfusion solutions to a hematocrit of 30%. In group 1 0.9% NaCl with 24 mmol/l $NaHCO_3$ was used, in group 2 Krebs' solution (140 mmol/l Na^+, 4 mmol/l K^+, 2.5 mmol/l Ca^{2+}, 1 mmol/l Mg^{2+}, 106 mmol/l Cl^-, 45 mmol/l lactate, 2 g/dl albumin 20%, 24 mmol/l $NaHCO_3$ 8.4%, 200 mg/dl dextrose, 7.3 mg/dl taurocholic acid, 29.6 mg/dl $MgSO_4$ and 13.6 mg/dl K_3PO_4) and in group 3 Krebs' solution and continuous infusion of norepinephrine at a rate of 4–10 μg/h. The final volume of the perfusate was 18 ml and the pH was adjusted to 7.40 by adding either $NaHCO_3$ or CO_2.

On-Line Measurements. pO_2, pH, flow, pressure and temperature were measured on-line via the computer system and recorded into an Excel file.

Clinical Chemistry. Glucose, Na^+, K^+ and osmolarity were measured using standard laboratory methods.

Oxygen Consumption. Oxygen consumption was calculated every 30 min; starting 15 min after the beginning of perfusion, by drawing blood from the perfusion system. Arterial blood was taken through a line at the oxygenator outlet, venous blood was taken from the portal outflow. Oxygen consumption was calculated using the following formulas.

Oxygen consumption of the hemoglobin-bound oxygen was calculated as follows.

$$\Theta_{Hb} = \dot{V} \cdot Hb \cdot \eta \cdot \left(\frac{Sat_a - Sat_v}{100} \right) \cdot \frac{100}{w}$$

where Θ_{Hb} = oxygen consumption, hemoglobin (ml/min/100 g), $\dot{V}$ = flow (ml/min), η = Hüfner's numbers 1.34 (ml O_2/g Hb), Sat_a = arterial oxygen saturation (%), Hb = hemoglobin (g/ml), Sat_v = venous oxygen saturation (%) and w = organ weight (g).

The arterial oxygen pressure was kept at 200 mm Hg, therefore the physically dissolved oxygen as part of the oxygen consumption had to be calculated.

$$\Theta_{Ph} = \dot{V} \cdot \alpha \cdot (p_a - p_v) \cdot \frac{100}{w}$$

where Θ_{Ph} = oxygen consumption, physically (ml/min/100 g), α = coefficient for oxygen in blood at 37°C: $2.813 \cdot 10^{-5}$ (ml O_2/l·mm Hg), p_a = arterial oxygen pressure (mm Hg) and p_v = venous oxygen pressure (mm Hg).

Final oxygen consumption:

$$\Theta_G = \dot{V} \cdot \left[Hb \cdot \eta \cdot \left(\frac{Sat_a - Sat_v}{100} \right) + \alpha \cdot (p_a - p_v) \right] \cdot \frac{100}{w}$$

where Θ_G = oxygen consumption $\Theta_{Hb} + \Theta_{Ph}$ (ml/min/100 g).

Maltose Absorption. 105 min after the start of the ex vivo perfusion, maltose (0.5 mg/g body weight of the organ donor) was dissolved in 2.5 ml saline and instilled into the bowel. Blood was taken before maltose instillation and 15 and 30 min thereafter. The glucose concentration was measured using the hexokinase method. As a positive conctrol, maltose was instilled into the small bowel of an anesthetized Lewis rat (n = 4) in a concentration of 0.5 mg/g body weight. The glucose concentration was measured before and 15 and 30 min after maltose administration. As a negative control, the same maltose test procedure was carried out after 45 min of warm ischemia (Arteria mesenterica clamped in an anesthetized Lewis rat, n = 4).

Statistics. All results are calculated as means ± SEM. Comparisons between the different groups were performed with one-way analysis of variance and the pairwise multiple comparison procedure (Student-Newman-Keuls method). Differences were considered statistically significant if $p < 0.05$.

Results

In group 1 (perfusion solution diluted with NaCl), a marked vasodilatation during the first 20–25 min was registered. The blood pressure fell below the desired perfusion pressure of 100 mm Hg and could not be compensated by an increased flow rate (fig. 2). About 10–15 min after the start of perfusion, a hypersecretion into the bowel lumen and hypermotility started, which finally led to the distension of the intestine. After 1 h, hyperemia and an increase in the vascular resistance developed and the flow rate fell below 1.5 ml/min. After instillation of maltose into the bowel lumen, after 105 min of ex vivo perfusion, no increase in the glucose concentration within the

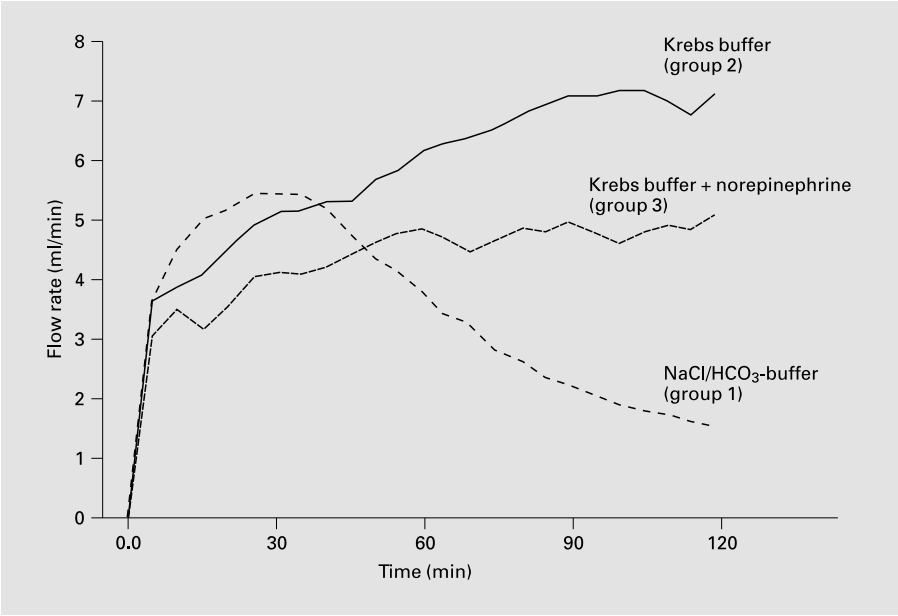


Fig. 2. Comparison of the flow rates of all three groups. Flow rates are given as means from all experiments performed in each group. They were measured every 2 min (error bars were deleted for better illustration). ANOVA and pairwise multiple comparison procedures (Student-Newman-Keuls test) were performed: $p > 0.05$ (all groups, 30 min), $p < 0.05$ (group 2 vs. group 1, 60 min) and $p < 0.05$ (group 1 vs. group 2, group 1 vs. group 3, and group 2 vs. group 3, 90 and 120 min).

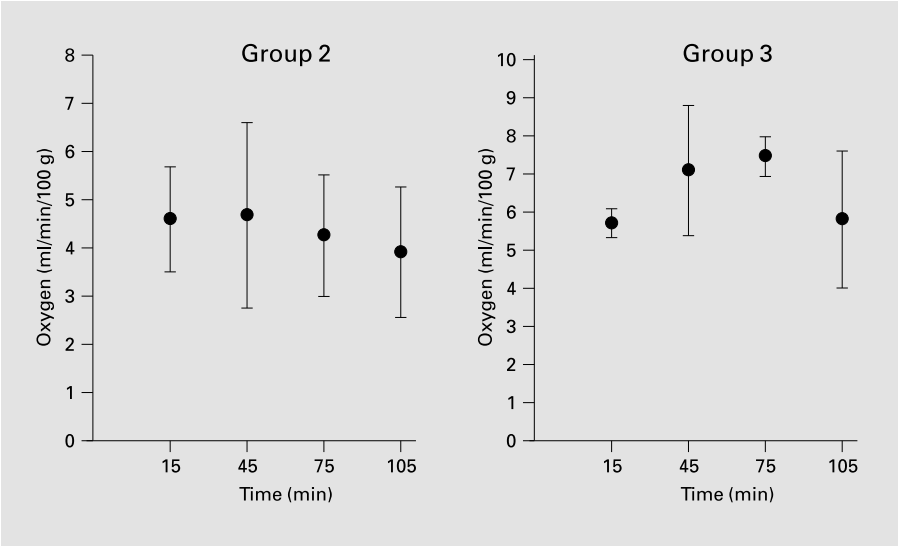
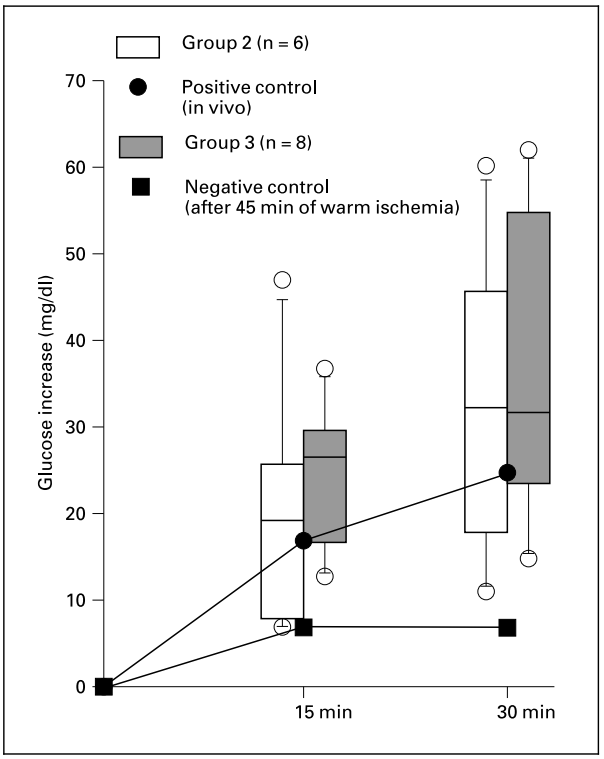


Fig. 3. Oxygen consumption (groups 2, n = 11 and 3, n = 8).

perfusate could be detected. Also, no oxygen consumption was registered. 2 h after the start of ex vivo perfusion, the intestine showed no signs of viability. In group 2, the flow rate increased steadily till the end of the experiment at 2 h (fig. 2), whereas blood pressure remained stable at 100 mm Hg. The increase in the flow rate was due to low vascular resistance. If the experiment was continued for more than 3 h, flow rate increased above 15 ml/min (data not shown). During the entire perfusion procedure, oxygen consumption was determined every 30 min. At every time point, oxygen uptake was higher than 5 ml O_2/min/100 g, demonstrating a viable organ (fig. 3). At the end of the perfusion procedure, a maltose absorption test was performed. The increase in glucose concentration measured within the perfusate 15 and 30 min after instillation of maltose into the bowel lumen did not differ from measurements of rats in vivo (fig. 4, positive control), again indicating viability of the intestine. In group 3, norepinephrine was added to the Krebs' solution initially, and the concentration was kept at the basic value by continuous infusion. The effect of norepinephrine was mainly a marked reduction in hypersecretion and absence of low vascular resistance at the end of the perfusion procedure (fig. 2). The flow remained within the desired range of 4–



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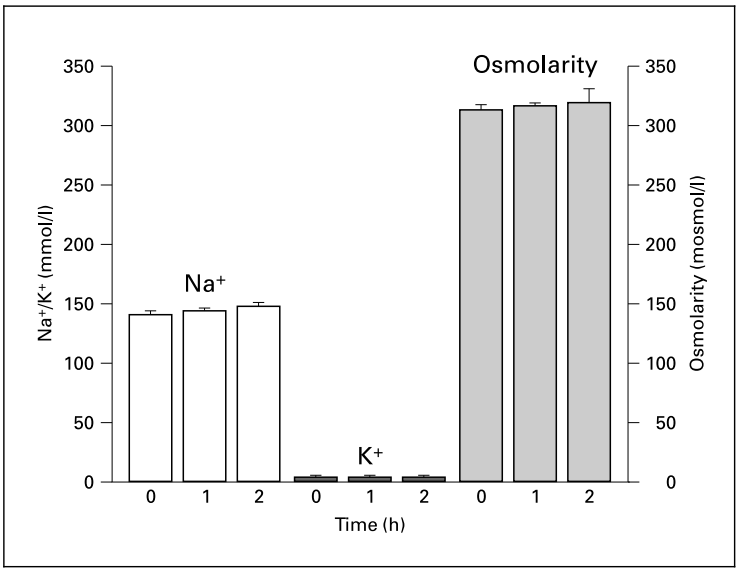

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Fig. 4. Maltose absorption (groups 2 and 3).
Fig. 5. Na^+, K^+, osmolarity at 0, 1 and 2 h ($p > 0.05$), all three groups.

6 ml/min over the entire period (2 h). Oxygen consumption and maltose absorption revealed a viable organ (fig. 3, 4).

In comparison, the addition of norepinephrine to the Krebs' solution improved ex vivo perfusion significantly (fig. 2). In all three groups, Na^+, K^+ and osmolarity did not change significantly during perfusion (fig. 5). pH was kept at 7.4 in all groups and the temperature remained stable at 37.6°C in all experiments. pO_2 was 180–200 mm Hg in each case and was adapted by changing the O_2 insufflation to the oxygenator. Glucose consumption was equal in groups 2 and 3, but there was no glucose uptake in group 1 (fig. 6). During the 1st and 2nd h of ex vivo perfusion, a significant amount of glucose was consumed by the small bowel in groups 2 and 3 compared with group 1. Glucose consumption in group 1 did not differ from perfusion of blood through the perfusion system without an organ graft (control; glucose consumption by the erythrocytes of the perfusate, fig. 6), thus demonstrating viable organs in groups 2 and 3, but not in group 1.

Hemolysis occurred during all perfusion experiments. Due to extensive measurements of hemolysis after 2 h, with and without a small bowel graft inserted into the perfusion circuit, we found that the main reason for hemolysis was due to impaired erythrocytes at the connection sites of the perfusion tubes. By improving this part of the perfusion system, after 2 h of ex vivo small bowel perfusion, free hemoglobin was reduced to 1.6% of the total hemoglobin.

Discussion

Isolated ex vivo gut perfusion offers a variety of experimental possibilities to study either ischemia/reperfusion phenomena [8], or the potential role of local immunosuppression [3], as well as the ability of organ pretreatment to reduce immunogenicity [9]. A large number of experiments to study intestinal physiology was carried out in the 1960s to early 1980s. Most of the experimental protocols used large animals such as dogs and cats [10–12]. When the rat model was used, either in situ perfusion [13], xenogeneic blood cells as perfusate [3, 14], oxygenated buffer solution as perfusate [15], or non-recirculating preparations [16] were used. The goal of our study was to establish a perfusion model for intestinal perfusion that meets the following requirements: (1) ex vivo perfusion in an organ chamber, (2) recirculation of the perfusate, (3) normothermic perfusion, (4) syngeneic blood, including leukocytes and lymphocytes as perfusate and (5) rat model.

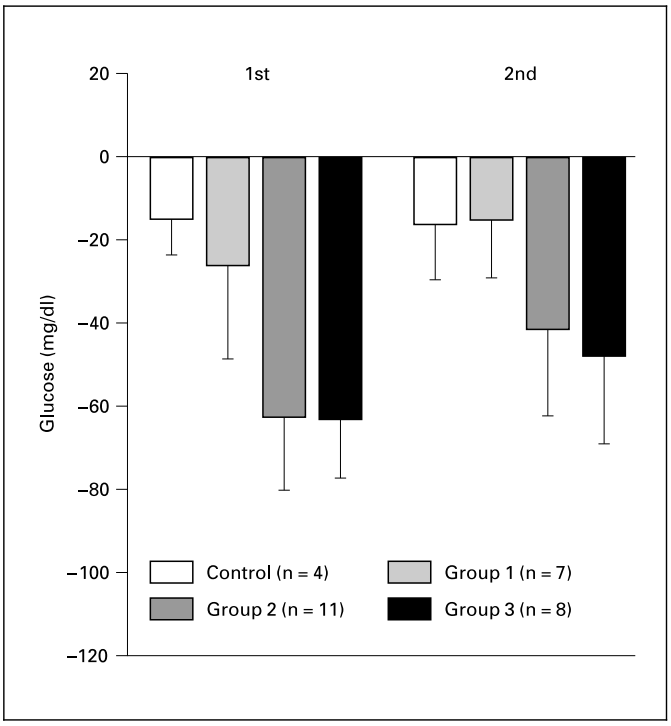

Fig. 6. Glucose consumption (all three groups).

Despite the introduction of tacrolimus in the field of small bowel transplantation, only little progress concerning graft survival has been made throughout the last years [1, 17]. The 3-year graft survival improved from 29% in 1996 to 38% at 3 years in 1997. These data are superior to those of the cyclosporine era (11% at 3 years) but still far behind the success rate of kidney or liver transplantation.

We therefore assume that further improvement will only be achieved by applying new concepts in the field of small bowel transplantation. A possibility is the pretreatment of the graft to reduce its immunogenicity. Several modalities are feasible: pretreatment with immunosuppressive agents (e.g. cyclosporine A and 15-deoxyspergualin) [3], pretreatment with monoclonal antibodies to prevent lymphocyte-endothelial cell interaction [18] or pretreatment with viral vectors for the expression of biologically active molecules directly in the graft, to prevent rejection [19], pretreatment to prevent ischemia-reperfusion injury [20] and pretreatment to change the function of endothelial cells [21].

Our model of ex vivo normothermic small-intestine perfusion fulfills all of the prerequisites to study these new concepts. We use an ex vivo model which mimics the clinical situation, where a pretreatment of the donor, from an ethical point of view, is not feasible. We use a recirculating perfusion chamber in order to achieve sufficient contact between the graft and biologically active substances during a 2-hour perfusion period. We have established a normothermic perfusion circuit to enable biological reactions to take place. Our perfusion solution consisted of whole blood, which allows the investigation of leukocyte/lymphocyte-endothelial cell interaction by means of intravital microscopy [22]. Finally, we have chosen the rat model, which gives the opportunity to test pretreatment protocols in a defined transplantation setup.

Windmueller et al. [6] described the problems involved in small bowel perfusion.

(1) A hypersecretion into the bowel lumen that starts only a few minutes after initiation of the ex vivo perfusion circuit: this finally leads to a distention of the bowel with decreased microvascular perfusion. We have observed the same problem in group 1, where the perfusate was diluted with NaCl.

(2) The hypermotility of the bowel, which was ascribed to insufficient oxygenation [23], may in part be due to intrinsic innervation: in our experiments, except for group 1, hypermotility did not occur.

(3) Hyperemia at the beginning and low vascular resistance at the end of the perfusion [6]: in their experiments, Windmueller et al. [6] could solve these problems by adding norepinephrine and dexamethasone to the perfusate. Some of the side effects (e.g. low vascular resistance) could be avoided by adding atropine. Before we started the experiments in groups 2 and 3 we added atropine to the protocol in group 1, but we were not successful in preventing hypersecretion and low resistance (data not shown). Dexamethasone as an additive to our perfusate was avoided, because our model is supposed to be an experimental model for the study of the interaction between leukocytes-lymphocytes and endothelial cells, a phenomenon which is abrogated by the addition of glucocorticoids. In contrast to experiments described so far, we have therefore used syngeneic whole blood, diluted to a hematocrit of 30% with Krebs' solution. Preparations with washed xenogeneic erythrocytes [5], buffer solutions alone [8], or nonrecirculating perfusion systems [16] are easier to perform, but do not enable to study pretreatment protocols or ischemia/reperfusion phenomena.

The major problems of ex vivo intestinal perfusion, e.g. hypersecretion, hypermotility and hyperemia, are at least in part due to the denervated small bowel preparation. Despite the existence of an autoregulation of intestinal perfusion [24], loss of the effect of sympathetic postganglionic vasoconstrictor fibers from the splanchnic nerves leads to the above-described phenomena [25].

Norepinephrinestimulates predominantly α-adrenoreceptors and leads to constriction of the isolated perfused mesenteric vasculature [26]. Thus, the addition of norepinephrine to the Krebs' solution was, in our experiments, sufficient to prevent the major problems described for ex vivo small bowel perfusion.

In ex vivo organ perfusion experiments, it is very important to test the viability of the perfused organ. The best way to test the viability of a perfused organ is, of course, syngeneic transplantation after perfusion. However, this requires an extension of the experiments with additional difficulties (e.g. microsurgery). Usually oxygen consumption and glucose absorption serve as viability parameters in small-intestinal preparations [5]. Oxygen consumption in groups 2 and 3 showed sufficient uptake to prove organ viability. The values measured are in good agreement to previously published data [8, 13]. Maltose absorption was used as an absorption test. It requires intact enzymes of the mucosal brush border to beak down maltose into fructose and glucose. An intact intestinal brush border also indicates small bowel viability [16]. Glucose levels measured 15 and 30 min after intraluminal administration of maltose agree with the results of Billiar et al. [27] in small bowel isografts.

In summary, we have shown that ex vivo, normothermic, computer-assisted small bowel perfusion using whole blood supplemented with Krebs' solution and continuous administration of norepinephrine as an additive enables to study pretreatment protocols and ischemia/reperfusion phenomena.

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