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Plastic Foil Technique Attenuates Inflammation in Mesenteric Intravital Microscopy

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Background. **Interpretation of intravital microscopic observations is complicated by the "inflammatory"-type response to the trauma inflicted on the tissue by the surgical preparation. The present study evaluates different experimental conditions for prolonged observations of the mesenteric microcirculation in the rat.**

Methods. **The mesentery was exteriorized through a median laparotomy and subjected to an organ bath or a modified plastic foil technique. Hemodynamic, metabolic, respiratory, and microcirculatory data were analyzed.**

Results. **In contrast to the plastic foil technique, which yielded stable baseline values over a 5-h observation period, venular velocity and wall shear rates decreased significantly in the organ bath technique, and leukocyte adhesion to the endothelium was significantly increased. Likewise, abdominal blood flow decreased significantly by 35% and base excess declined (−10.0 ± 0.4 mmol/L) in the organ bath, with reduced pCO_2 (26.4 ± 2.5 mm Hg vs 33.7 ± 1.1 mm Hg in plastic foil technique) due to respiratory pH compensation.**

Conclusions. **The plastic foil technique was found clearly superior to the organ bath technique for maintenance of stable baseline metabolic, hemodynamic, and microcirculatory conditions in mesenteric intravital microscopy.**

Key Words: **intravital microscopy; surgical preparation; microcirculation.**

INTRODUCTION

Intravital microscopy is widely employed in diverse animal models to study pathophysiological processes in the microcirculation. Yet, "inflammatory"-type responses due to the surgical insult of the tissue under observation usually limit the observation period to 2–3 h [1–6]. However, experimental protocols that bear significance for complex pathphysiological situations such as hemorrhagic shock and ischemia reperfusion injury require longer periods of stable experimental conditions.

In the organ bath technique the exteriorized segment of the intestine is immersed in Krebs–Henseleit buffer [7, 8]. Alternatively, the exteriorized mesentery can be covered with an impermeable plastic foil and superfused with buffer to minimize evaporation and tissue dehydratation [9, 10]. Surgical manipulation inevitably causes inflammatory-type responses, protein loss, and changes in blood viscosity [11–14]. Yet, it is unclear so far whether and to what extent these effects might alter basic physiological parameters and thus confound long-term observation in the respective models of intestinal microscopy [15].

In the current study we examined the stability of physiological parameters during 5-h and longer observation periods using both experimental variations. Systematic analysis of metabolic, respiratory, and macro- and microcirculatory parameters clearly demonstrates that the plastic foil technique is markedly superior to the organ bath technique for the maintenance of stable microcirculatory conditions during prolonged observation periods.

METHODS

Experimental conditions. Twenty Sprague–Dawley rats of either sex (250–400 g body wt) were maintained on standard rat chow and water *ad libitum* until the night before the experiment. Rat chow was removed 10 to 12 h before the start of the experiment to reduce

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bowel movements during intravital microscopy. After anesthesia with urethane (1.25 g/kg im, single dose) the carotid artery and jugular vein were cannulated with PE tubing for arterial blood gas (ABG) analysis and measurements of plasma protein and arterial and central venous blood pressure. For each sample 250 μL of heparinized blood was drawn. Arterial blood gases (Pa_{O_2}, s_{O_2}, p_{CO_2}, pH, BE), hemoglobin (Hb), hematocrit (Hct), lactate, potassium, and sodium were analyzed with Arterial Blood Gas Laboratory Radiometer Copenhagen 615. Pa_{O_2}, s_{O_2}, p_{CO_2}, pH, Hb, lactate, potassium, and sodium were measured; BE and Hct were calculated from measured values as previously described [16].

After median laparotomy a Doppler flow transducer was placed around the abdominal aorta below the truncus iliacus and above the superior mesenteric artery (SMA) and the two branches of the left and right renal artery. Hemodynamic data (abdominal blood flow (AF), descending aorta), heart rate (HR), systolic, mean, and diastolic arterial blood pressures (SAP, MAP, DAP), and central venous pressure (CVP)) were recorded on a beat-to-beat basis using System-6 (Triton Technology, Inc., San Diego, CA). Analog data were digitized and recorded online with a computer-based system (DasyLab, National Instruments Corp., Austin, TX). The Doppler flow values presented a linear correlation to electromagnetic blood flow sensors (Skalar-Medical b.v., Delft, The Netherlands) which had two disadvantages for use in our experiments. First, the tight adaptation to the abdominal aorta results in compression of the accompanying lymphatic vessels, and second, due to reduced mechanical flexibility we observed compression of other vessels in the narrow retroperitoneal space with induction of local ischemia. An ECG in lead II was recorded during the experiment to detect morphological ECG changes [16]. Rectal temperature was kept constant at 37.5 $\pm$ 0.5°C by means of a feedback-controlled homeothermic blanket control unit (Harvard, South Natick, MA).

Animals were placed on a heating pad in the left lateral recumbent position on an adjustable Plexiglas microscope stage. A segment of the ileum was exteriorized through the abdominal incision with great care to avoid trauma to the exposed bowel and mesentery.

In Group 1 (organ bath (OB), $n = 6$) a loop of the exteriorized ileal mesentery was placed in a temperature-controlled, fluid-filled Plexiglas chamber. The mesentery was placed over a Plexiglas pedestal in the superfused chamber, and the ileum secured with fine-gauge insect pins on a cork board to stabilize the viewing field [7]. Throughout the experiment, mesentery and ileac loop were superfused with a modified Krebs–Henseleit solution [17]. Bicarbonate-buffered salt solution was equilibrated with 5% CO_2 in N_2. The pH of the Krebs–Henseleit solution and the temperature measured online in the fluid-filled plastic chamber next to the ileac loop were maintained constant at pH 7.4 $\pm$ 0.04 and 37.5°C, respectively.

In group 2 (plastic foil (PF), $n = 6$) the mesentery including the ileac loop was covered completely with oxygen-impermeable plastic foil (Folio, Germany). The previously published technique was modified by covering the complete abdominal wound with plastic foil [18]. For *in vivo* microscopic observation the mesentery was spread on a nonfluorescent glass pedestal that allowed for epi- and transillumination of a 1.5- to 2-cm^2 field. The exposed bowel segment was covered with plastic foil except for a small window of the mesentery which was continuously superfused with warm (37.5°C) bicarbonate-buffered salt solution (pH 7.4) equilibrated with 5% CO_2 in N_2. The temperature of the glass pedestal was maintained at 37°C with a constant-temperature circulator. In both groups, normal saline was infused at the rate of 0.5 mL/100 g body wt/h after fixation of the mesentery for compensation of evaporative water loss as previously recommended [19].

Animals in group 3 (no laparotomy (NL), $n = 5$) underwent only general anesthesia, with monitoring of heart rate, arterial blood pressure, CVP, and blood examinations according to the experimental protocol. ABGs and plasma protein concentration were examined without surgical trauma to the abdominal wall.

To differentiate between the effects of laparotomy plus surgical preparation of the abdominal Doppler flow probe and those of animals without laparotomy, three animals underwent the surgical procedure without extravasation of the mesentery for intravital microscopy (group 4, laparotomy, $n = 3$). Animals in groups 3 and group 4 received an infusion with normal saline at the rate of 0.1 mL/100 g body wt/h.

The duration of this procedure plus the stabilization period was 2 h. Thereafter (t_0), an observation period of 5 h followed.

For protein analysis arterial blood was drawn from the carotid artery into heparinized polypropylene syringes. Blood samples were collected in polypropylene tubes and centrifuged at 2000*g* for 15 min at 4°C. Plasma was decanted and frozen at −70°C until further analysis. Total protein analysis was performed with the biuret method [20, 21]. For measurement of albumin we used the bromcresol green-method [22].

Intravital microscopy was performed with epi- and transillumination observing three to five unbranched mesenteric venules (diameter 25–40 μm; length 100–150 μm) (Zeiss Axiotech fluorescence microscope with computer-controlled scanning table; light source, AttoArc HBO 100 W). The images were recorded with a high-resolution camera (Stemmer b/w VS 450) and a videocassette recorder (S-VHS Panasonic AG-7355). Off-line analysis was performed with the Cap Image software system (Dr. Zeintl, Heidelberg, Germany, Version 6.01) on an IBM-compatible PC with a Matrox image processing card and real-time video tape digitalization [23]. The system allows measurement of various morphological parameters, such as capillary diameter, length, and density; video densitometric analysis; and measurement of leukocyte rolling and adhesion. Measurement of the erythrocyte flow velocity was performed with a frame-to-frame method using fluorescein isothiocyanate (FITC)-stained erythrocytes [24]. Centerline velocities were converted to mean blood flow velocities by dividing the centerline velocity by an empirical factor of 1.6 [25]. Wall shear rate (γ) was calculated on the basis of the Newtonian definition: $\gamma = 8$(mean velocity/venular diameter) [26]. Rolling and adherent cells were analyzed once every hour using a frame-to-frame method for an observation period of 1 min each for epi- and transillumination microscopy. Rollers and stickers were expressed as number per square millimeter calculated from the measured diameter and length of the observed microvessel assuming cylindrical geometry. For study of macromolecular leakage we injected FITC-labeled bovine albumin (50 mg/kg; Sigma Chemical Co., St Louis, MO) intravenously at the end of the experiment to avoid phototoxic side effects and scanned for a further 30 min [27]. The fluorescence intensity was recorded within a defined area of the venule under study and in the adjacent perivascular interstitium. The vascular albumin leakage index was calculated from the ratio (perivascular intensity − background intensity)/(venular intensity − background intensity) × 100. Off-line analysis of coded videorecordings was performed in a blinded manner.

All investigative procedures and the animal facilities conformed with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The protocol was approved by the regional animal care and use committee.

Experimental protocol. After anesthesia an arterial line was introduced and the first ABG and blood sample for protein analysis was drawn (t − 2 h). A central venous line was positioned and subsequently rats were placed on a temperate operating table for microscopic preparation of the abdominal aorta. A second blood sample was drawn after laparotomy and positioning of the flow cuff around the descending aorta (t − 1 h). Following exteriorization and fixation of the mesentery rats were allowed to stabilize for 30 min. The time for preparation in groups 1 and 2 was 2 h, including 30 min for stabilization after surgery and exteriorization of the mesentery. Thereafter, the experimental follow-up with observation of the mesentery microcirculation was 5 h.

During the stabilization phase, three to five unbranched mesenteric venules were studied by intravital microscopy. Leukocytes were stained by intravenous injection of rhodamine G-6 (100 μl of a

0.005% solution, Sigma Chemical Co.) [28]. Prior to the experimental phase a baseline blood sample was drawn (t_0). Further ABGs and plasma samples were collected hourly for an observation period of 5 h. Hemodynamic data were recorded continuously as described above. After 5 h we injected 50 mg/kg body wt FITC-labled bovine albumin for detection of molecular leakage, scanned a further 30 min, and concluded the experiment [10].

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed with Sigma Stat (SPSS Inc., Chicago, IL). Statistical significance of changes from baseline values within each group was tested with ANOVA for repeated measures. Differences between groups were statistically analyzed by one-way ANOVA. In the absence of a normal distribution, ANOVA for nonparametric values (Kruskal–Wallis test) with the multiple comparison method (Student–Newman–Keuls test) was used. Statistical significance was accepted at an error probability of $P < 0.05$.

RESULTS

All animals survived the total preparation and experimental time of 5–7 h. In agreement with a previous report, baseline HR, MAP, pressure rate product (PRP), and abdominal flow data did not significantly differ between groups [29].

Arterial Blood Gas Analysis

Pa_{O_2} and Sa_{O_2}. Pa_{O_2} values of all groups did not differ at the beginning of the experimental period (group 1: 83.6 ± 3.0 mm Hg, group 2: 83.2 ± 1.6 mm Hg, group 3: 81.1 ± 1.6 mm Hg, group 4: 88.3 ± 0.2 mm Hg). Pa_{O_2} values of group 3 (no laparotomy) and group 4 (laparotomy) showed no significant difference throughout the experiment. Pa_{O_2} increased significantly in group 1 (OB) at 3 to 5 h (5 h: 103.4 ± 3.6 mm Hg) and in group 2 (PF) at 5 h (93.7 ± 3.3 mm Hg) versus all prior time points. Pa_{O_2} values of group 1 were significantly elevated from 2 to 5 h as compared with group 3. Group 1 and group 2 were significantly different only at 4 h of observation.

Pa_{CO_2}. The results of Pa_{CO_2} measurements are depicted in Fig. 1A. Data of all groups were similar at baseline without significant differences until the start of the experiment (t_0) (group 1: 38.5 ± 1.5 mm Hg, group 2: 38.8 ± 1.0 mm Hg, group 3: 42.5 ± 1.9 mm Hg, group 4: 40.6 ± 1.9 mm Hg). In group 1 (OB) Pa_{CO_2} decreased significantly beginning at 3 h until the end (26.4 ± 2.5 mm Hg). In group 2 (PF) only values at 5 h (33.7 ± 1.1 mm Hg) were significantly lower versus baseline. Group 3 (no laparotomy) and group 4 (laparotomy) presented constant values throughout the experiment. Pa_{CO_2} values of group 1 (OB) were significantly lower at 4 and 5 h versus group 2 (PF).

Arterial base excess and pH. Values of all groups at the beginning did not show any differences from pre 1 to pre 2 (Fig. 1B) (group 1: −2.6 ± 0.6 mmol/L, group 2: −1.7 ± 0.6 mmol/L, group 3: 0.0 ± 0.9 mmol/L, group 4: −1.0 ± 0.4 mmol/L). BE values of groups 1 and 2 had a similar time course, like the Pa_{CO_2} data. BE values of group 3 and group 4 were constant at all time points. Decreases in BE were significant at 2 h in group 1 and 4 h in group 2 versus baseline until the end of the experiment (5 h; group 1: −10.0 ± 0.4 mmol/L, group 2: −5.3 ± 0.6 mmol/L). Data for group 1 (OB) are significantly decreased versus group 2 (PF) from 2 h to the end at 5 h.

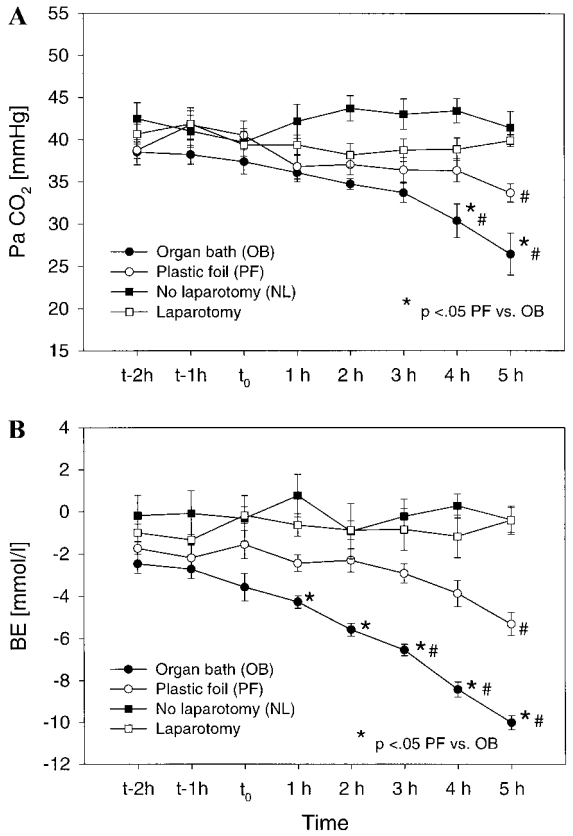

FIG. 1. Pa_{CO_2} (A) and arterial base excess (B). OB resulted in a significant decrease in Pa_{CO_2} versus PF (*). Likewise, values at 5 h in PF are significantly decreased versus t − 2 h, t − 1 h, and t_0 (#). Measurements of base excess indicated a significant drop in OB versus PF (*). Data in OB decreased significantly versus t − 2 h (#) from 3 to 5 h and in PF at 5 h.

No significant changes in pH could be calculated between groups and for repeated measurements. The pH was compensated at all time points.

Hematocrit. The decrease in hematocrit during repetitive blood sampling for ABG and protein analysis is depicted in Fig. 2A. There were no statistically significant differences at the beginning of the experiment (group 1: 52.2 ± 1.7%, group 2: 53.2 ± 0.4%, group 3: 49.8 ± 0.4%, group 4: 47.8 ± 0.7%). Groups 2, 3, and 4 showed a significant decrease in Hct values from baseline to the end of the experiment. Hct of group 1 decreased until 1 h and then remained stable. Hct values from 3 to 5 h differed significantly between group 1 (OB) and group 2 (PF).

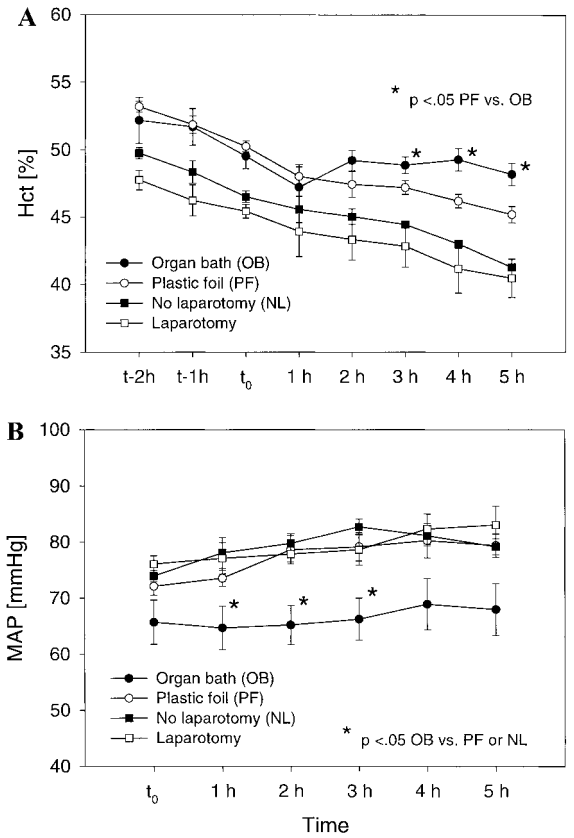

FIG. 2. Hematocrit (A) and mean arterial pressure (B). Hct decreased significantly in all groups. Yet, the Hct of OB was significantly elevated after 2 h versus PF (*) presumably due to fluid evaporation. Values of MAP at 1, 2, and 3 h are significantly lower in OB than in PF or NL (*).

Hemodynamic Measurements

Heart rate, mean arterial blood pressure, and pressure rate product. HR and MAP (Fig. 2B) did not show any significant difference at the beginning of the experiment (HR: group 1: 360 ± 21 beats/min, group 2: 350 ± 10 beats/min, group 3: 362 ± 11 beats/min, group 4: 353 ± 14 beats/min; MAP: group 1: 66 ± 4 mm Hg, group 2: 72 ± 2 mm Hg, group 3: 74 ± 1 mm Hg, group 4: 76 ± 1 mm Hg). PRP of all groups is presented in Fig. 3B and showed no significant differences between groups.

Abdominal blood flow (descending aorta). Abdominal blood flow measured in the descending aorta is depicted in Fig. 3A as a percentage of baseline value (t_0). No significant differences between groups were observed at baseline (group 1: 42 ± 3.8 mL/min; group 2: 50 ± 3.4 mL/min; group 4: 46.6 ± 5.3 mL/min). Data in group 1 significantly decreased at 3, 4, and 5 h (−35.6 ± 6.5%) versus baseline. The time courses of groups 2 and 4 showed no significant changes. At 5 h the decrease in AF in group 2 was −7.9 ± 8.9%. Data for groups 1 and 2 significantly differed at 4 and 5 h.

Total protein and albumin concentrations and vascular albumin leakage. Measurement of total protein showed a significant decrease during the observation period (Table 1). Data for all three groups did not differ significantly at the beginning of the surgical preparation (pre 1). TP values decreased significantly until t_0 and 5 h in all groups and showed a significant difference between groups 1 and group 3 at 5 h. Albumin data in group 1 showed a significant reduction over time parallel to the decrease in TP.

Densitometric data on FITC-labeled albumin did not show any significant increase or differences between both groups at 5 h of experimental time.

Rolling and adherent leukocytes. Rhodamine-labeled leukocytes were counted using epiillumination rather than transillumination because detection of the cells was more accurate with fluorescence after determination of the intra- and interobserver variability of both methods. Cell counts were normalized to the endothelial surface assuming cylindrical geometry (i.e., one leukocyte in a standard venule 30 μm in diameter and 100 μm in length corresponds to 106 cells/mm^2). The numbers of rolling and adherent leukocytes of all observed venules did not differ significantly between

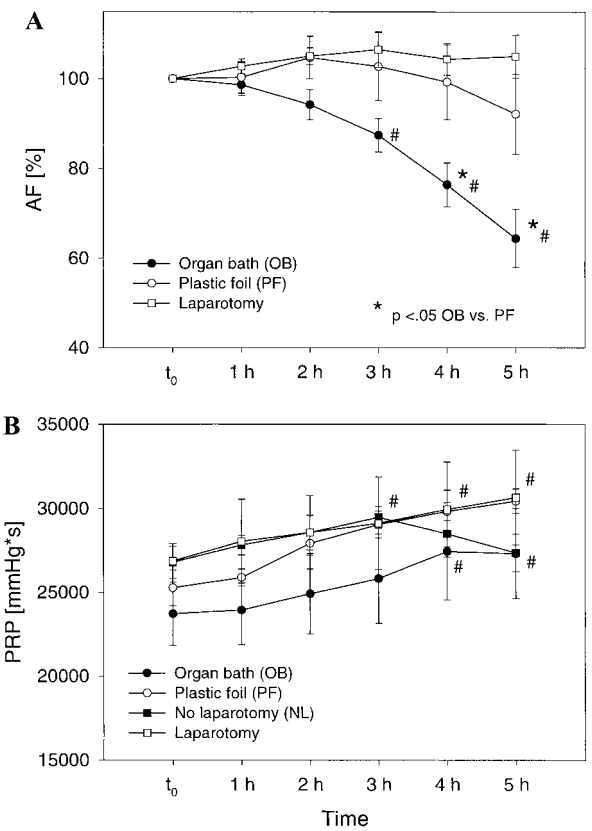

FIG. 3. Abdominal flow as percentage of baseline value (A) and pressure rate product (B). Abdominal flow of OB was significantly different than that of PF (*) and showed a significant decrease during the experiment versus t_0 and 1 h (#). PRP increased significantly during the experiment in OB (#) and PF (#) versus t_0 and 1 h.

TABLE 1

Total Protein and Albumin Concentration during Surgical Preparation and Experimental Time[a]

Time point	Total protein [g/L], group 1 (OB)	Albumin [g/L] and [% total protein], group 1	Total protein [g/L], group 2 (PF)	Total protein [g/L], group 3 (NL)
Pre 1	49.9 ± 1.5	24.5 ± 1.5 = 49%	49.1 ± 1.5	50.3 ± 0.6
Baseline (t_0)	43.4 ± 1.3*	18.1 ± 1.2 = 42%*	41.7 ± 1.0*	46.4 ± 0.9*
5 h	38.0 ± 1.0*	12.7 ± 1.0 = 34%*	40.2 ± 0.6*	41.9 ± 1.1*

[a] Total protein and albumin at t_0 and 5 h decreased significantly in all groups (* $P < 0.05$). Differences between groups were not significant except for 5-h values between OB and NL. Values are means ± SEM.

the groups at the beginning of the experiment (Fig. 4A). Rolling leukocytes did not increase significantly throughout the observation time of 5 h in both groups. However, leukocyte adhesion increased significantly from 1 to 5 h in groups 1 and 2 versus baseline values. The number of adherent leukocytes in the plastic foil group reached a plateau after 1 h of observation, whereas stickers in the organ bath group increased significantly from 3 to 5 h of observation versus baseline and 1 h. At 3, 4, and 5 h, the number of adherent leukocytes differed significantly between the groups. Systemic leukocyte counts did not change significantly.

Velocity and shear rate. Venular diameters did not differ significantly between group 1 (29.5 ± 0.9 μm) and group 2 (30.7 ± 1.4 μm). Thus, wall shear rate (γ) was virtually dependent on microvascular velocity. Data for centerline velocity and shear rate are depicted in Fig. 4B and were not significantly different between groups at baseline. Velocity and shear rate in group 1 were significantly decreased at 4 and 5 h versus baseline, 1 h, and 2 h. Data in group 2 were stable throughout the whole experiment. Differences between groups 1 and 2 were significant at 5 h of observation.

DISCUSSION

The principal finding of this study is that the plastic foil technique for intravital microscopy of the mesentery yields a preparation that remains hemodynamically stable for up to 5 h with little evidence of inflammation. In contrast, the organ bath technique is characterized by a significant increase in leukocyte adhesion as a manifestation of local inflammation (Fig. 4A). At the same time leukocyte adhesion was closely correlated with the continuous decline in aortic blood flow and with microcirculatory venular velocity (Figs. 3A, 4A, and 4B) [15, 30].

Procedures for examining leukocyte–endothelium interaction in the mesentery harbor changes in artifacts arising from surgery. The plastic foil technique can attenuate these effects, although they cannot completely eliminate them.

Most investigators who use the organ bath technique immerse the mesentery in a modified buffered salt solution (Krebs–Henseleit). The pH is equilibrated with 5% CO_2 in N_2. Methods for controlling gas tensions in tissues have been published but control of gas tensions in the bathing medium is rather difficult [31–33]. In our experiments, pH and temperature were

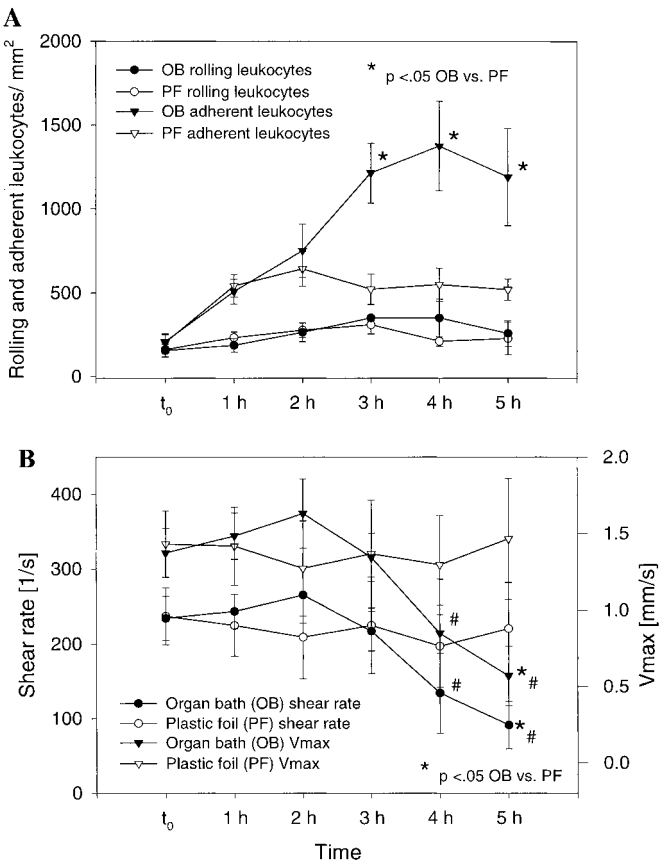

FIG. 4. Rolling and adherent leukocytes (A) and shear rate and velocity (B) during the experimental observation time of 5 h. Data for rolling leukocytes did not show any significant changes. Adherent leukocytes increased significantly in OB over time versus t_0 and 1 h and versus PF (*). Velocity and shear rates in OB were significantly decreased at 4 and 5 h versus t_0, 1 h, and 2 h (#) and versus PF (*). In contrast, velocity and shear rates remained unchanged in PF.

measured online in the superfused segment and organ bath buffer solution and found to be constant within physiological ranges. Likewise, local conditions in the immersed bowel loop itself are very difficult to control. This pertains especially to temperature gradients on the upper surface of the loop, which might be altered through evaporation. This problem is circumvented by the impermeable plastic foil, which keeps the ileal loop in its *milieu interne*.

Proteins are an important compound in peritoneal fluid [34] and protein loss during abdominal surgery is a well-known phenomenon [11, 35]. Indeed, we found a 15% decrease in total plasma protein during the first 2 h of our experiments, continuously falling to 80% of baseline levels at the end of the experiment. Albumin values presented a decrease parallel to total plasma protein, which is in accord with clinical experience [36]. Washout of proteins from the mesentery into the tissue bath has been difficult to measure accurately. Unexpectedly, direct leakage of albumin from the extended intestinal segment seemed not to be the major route of protein loss, because plasma decrease was similar in the plastic foil group and was even seen in the no laparotomy group (Table 1). Examinations with FITC–BSA did not reveal any vascular leakage from mesenteric venules after 2 h preparation and 5 h follow-up, and edema formation was not observed. Thus, the decrease in total protein cannot explain the observed differences in hemodynamic data between the experimental groups (i.e., abdominal flow, Fig. 3A). Further experiments will have to address the exact mechanisms involved in the loss of plasma protein during surgery and/or anesthesia [27, 36].

As recommended by others, volume loss was compensated by intravenous infusion of normal saline at 0.5 mL/100 g/h [9]. As a consequence of the dilution effect we found a significant drop in hematocrit in all groups (Fig. 2A), which was further aggravated by the repetitive blood draws. The plateau at 2 h in the organ bath group could be due to volume loss by evaporation. This notion is further supported by the observation that mean arterial pressure was lower in the organ bath animals (Fig. 2B) and that aortic flow decreased at 3 h (Fig. 3A). Surprisingly, the pressure rate product was not a reliable parameter of hemodynamic control (Fig. 3B). Covering the bowel with an impermeable plastic foil seemed to effectively prevent these adverse hemodynamic effects.

There was a marked decrease in base excess in the tissue bath, possibly indicating reduced peripheral perfusion secondary to the decrease in abdominal blood flow (Fig. 1B). Arterial pH was compensated over the duration of the experiment, and compensatory hyperventilation due to metabolic acidosis improved areas of underventilation, thus explaining the observed moderate increase in Pa_{O_2}, which was always within physiological ranges. As an effect of surgery an attenuated base excess decrease could be seen in the plastic foil group in contrast to the control group with or without laparotomy, which presented stable metabolic parameters (Fig. 1B).

SUMMARY

These findings emphasize the advantages of the plastic foil technique for use in intravital microscopy of the mesentery for long-term experiments up to 5 h of observation. Keeping the bowel loop in impermeable plastic foil affords better organ protection, reduces fluid evaporation, and yields better hemodynamic, microcirculatory, metabolic, and respiratory stability for prolonged intravital microscopy.

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