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Intestinal injury and recovery after ischemia

An experimental study on restitution of the surface epithelium, intestinal permeability, and release of biomarkers from the mucosa

Thesis for the degree doctor medicinae

Trondheim, December 2007

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Ingebjørg S. Juel

LIST OF PAPERS

I. Juel IS, Solligård E, Lyng O, Strømholm T, Tvedt KE, Johnsen H, Jynge P, Sæther OD, Aadahl P, Grønbech JE. Intestinal injury after thoracic aortic cross-clamping in the pig. *J Surg Res* 2004; 117: 283-295.

II. Solligård E, Juel IS, Bakkelund K, Jynge P, Tvedt KE, Johnsen H, Aadahl P, Grønbech JE. Gut luminal microdialysis of glycerol as a marker of intestinal ischemic injury and recovery. *Crit Care Med* 2005; 33: 2278-2285.

III. Juel IS, Solligård E, Tvedt KE, Skogvoll E, Jynge P, Beisvag V, Erlandsen SE, Sandvik AK, Aadahl P, Grønbech JE. Postischemic restituted intestinal mucosa is more resistant to further ischemia than normal mucosa. *Scand J Clin Lab Invest* 2007, in press.

IV. Juel IS, Solligård E, Skogvoll E, Aadahl P, Grønbech JE. Lactate and glycerol released to the intestinal lumen reflect mucosal injury and permeability changes caused by strangulation obstruction. *Eur Surg Res* 2007, in press.

These papers will be referred to by their Roman numerals.

ABBREVIATIONS

ATP	Adenosine triphosphate
CO	Cardiac output
FD-4	Fluorescein isothiocyanate dextran 4000
F16BP	Fructose 1,6 biphosphate
HPLC	High performance liquid chromatography
HR	Heart rate
HSP	Heat shock protein
MAP	Mean aortic pressure
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
PEG-4000	^{14}C Polyethylene glycol 4000
PMP-22	Peripheral myelin protein 22
QRT-PCR	Quantitative real time – polymerase chain reaction
RNA	Ribonucleic acid
SMA	Superior mesenteric artery
SOD-2	Superoxide dismutase 2
STAT-3	Signal transducer and activator of transcription 3
TIMP-1	Tissue inhibitor of metalloproteinase 1
VCAM-1	Vascular adhesion molecule 1

INTRODUCTION

Ischemia-reperfusion injury of the intestine is associated with a high morbidity and mortality rate in both surgical and trauma patients [31,35].

Intestinal ischemia, defined as hypoperfusion of blood through the organ, may be present for example during and after aortic aneurysm surgery, development of splanchnic artery embolus, intestinal strangulation, small bowel transplantation, neonatal necrotizing enterocolitis, and collapse of the systemic circulation as in hypovolemic and septic shock [31,35,129]. Metabolically active tissues such as the intestine are particularly susceptible to ischemia [52,151]. The mitochondria, which are essential for production of ATP and thereby organ recovery, are the first organelles to be influenced by ischemia [67]. It is well established that the epithelium located at the tips of the villi are more sensitive to the effect of ischemia than cells located at the bottom of the crypts [28]. This increased sensitivity has been attributed to the location at the end of the distribution of a central arteriole which may lead to lower oxygen tension compared to the crypts [68,69]. In addition, a recent study in a rat model demonstrated that the sensitivity of an enterocyte also is dependent on its state of differentiation [63].

For more than 25 years it has been known that restoration of blood flow after ischemia paradoxically may lead to increased damage of the tissue under study [21]. This phenomenon is to a large part related to catabolism of ATP to hypoxanthine and conversion of xanthine dehydrogenase to xanthine oxidase during ischemia. Upon reperfusion the reintroduction of oxygen allows xanthine oxidase to convert hypoxanthine to xanthine and further form reactive oxygen species; O_2^- , H_2O_2, OH^-. [51,52,61].Under normal conditions O_2^- is detoxified by superoxide dismutase, but

this defence mechanism may during reperfusion be overwhelmed and available O_2^- may react with H_2O_2 leading to formation of OH^-. The hydroxyl radical is highly reactive and lead to lipid peroxidation and thereby cellular damage [64,91]. Ischemia/reperfusion injury shows the features of an inflammatory response that in the microvasculature is characterized with loose adhesion (rolling) of neutrophils primarily regulated by selectines, thereafter firm adhesion to the endothelial wall and finally emigration into the interstitial space which is largely regulated by integrins [31,53,101]. Activated polymorphonuclear leucocytes are another potential source of oxygen radicals [119,146]. They may also cause tissue damage by plugging of capillaries [7] and release of proteolytic enzymes [22]. The burst of reactive oxygen species released upon reperfusion elicits a variety of biochemical and molecular events. Under normal conditions the production of NO is much larger than that of superoxide and therefore NO effectively scavenges low intracellular levels of superoxide, reduce arteriolar tone, prevent platelet aggregation and thrombus formation, and minimize the adhesion between leukocytes and endothelium [47,148]. Upon reperfusion the balance between NO and superoxide is tipped in favour of superoxide [74,75,76] . When the relative level of NO is reduced, the formation of H_2O_2 is promoted which again can enhance activation of phospholipase A_2. The result may be accumulation of mediators such as platelet activating factor (PAF) and leukotrienes (i.e., LTB_4), which again may initiate the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). These transcription factors are probably essential for initiating expression of a large number of genes that participate in tissue injury, but also in tissue repair throughout the reperfusion period [21]. Ischemic injury to the intestinal mucosa can be divided into two types: 1) deep injury involving extensive haemorrhage and large areas of tissue necrosis, and 2) superficial

injury confined to the surface epithelium of the mucosa. The histologic changes induced by ischemia are first noted at the tips of the villi and may progress to frank transmural necrosis, depending on the duration and severity of the ischemic episode [16,149]. Repair of deep injury takes weeks and involves mitosis. Superficial injury is rapidly repaired, over hours, by epithelial restitution.

Intestinal barrier failure plays an important role in the development of multiple organ dysfunction syndrome by allowing translocation of bacteria or bacterial products from the intestinal lumen to blood. In addition to the surface epithelium, the intestinal barrier consists of the normal microbial flora, the mucous layer, an intact immune system, and the gut-liver axis (bile salts binding intraluminal endotoxin) [10,44]. The surface epithelium; the single layer of columnar enterocytes and goblet cells, are joined together by junctional complexes (tight junctions, adherent junctions, and desmosomes). Depending on the properties of the molecule of interest (size, configuration, charge, hydrophilic properties), transport across the epithelial lining can be paracellular or transcellular, passive or active (energy dependent) [10,48]. Under normal conditions small molecules can traverse this border, while the passage of large molecules and bacteria are prevented. Several pathophysiologic insults have been shown to compromise this cellular barrier. One mechanism of particular importance is ischemia/reperfusion injury. It has been shown that increased gut permeability and the degree of bacterial translocation correlates with the extent of intestinal mucosal injury [114,129]. Although there are conflicting data [96,117], several animal models developed to study bacterial translocation associated with intestinal ischemia show the occurrence of bacteria in mesenteric lymph nodes and blood [8,9,130,131]. Furthermore, there are several clinical studies indicating that loss of the intestinal mucosal barrier to bacteria and endotoxin contributes to the

development of systemic infections or multiple organ failure [15,34], and Ammori et al demonstrated a close correlation between the degree of endotoxinemia and intestinal permeability to a macromolecule in patients with severe pancreatitis [3]. It appears, in addition, that the translocation of bacteria and endotoxin may lead to local activation of the immune inflammatory system and the local production of cytokines and other immune inflammatory mediators. These intestinally derived mediators may then exacerbate the systemic inflammatory response and potentially lead to further increase in gut permeability [129].

Although there is a growing knowledge about mechanisms involved in or responsible for intestinal tissue injury and dysfunction caused by ischemia/reperfusion, there are still several issues that remain unclear: 1) There might be considerable species differences which could be related to much higher levels of certain enzymes (i.e., xanthine oxidase) in rodent intestinal tissue than in other animal species (i.e., pigs) or humans [11,32,73,106]. This may lead to more pronounced reperfusion injury in rodents, which by far are the most used experimental animals. 2) In most studies on ischemia/reperfusion of the intestine restitution of the surface epithelium has not been considered or reported. One reason for this may be that reperfusion injury in rat models in the early reperfusion period (i.e., 1 or 2 hours) may mask repair processes such as restitution. 3) Most studies in this field report on the effect of interventions that may modify the outcome of intestinal ischemia typically induced by occlusion of the superior mesenteric artery for defined time periods. However, there is still a need to investigate ischemia/reperfusion injury of the intestine in models that more closely mimic clinical situations. 4) Clinically applicable methods that can provide information about key manifestations of ischemia/reperfusion such as intestinal tissue injury and permeability changes are few and often unreliable.

AIM OF STUDY

The overall purpose of the present thesis was to develop experimental models and apply methods that might elucidate these issues and in particular:

- To perform a detailed morphological analysis of the intestinal mucosa after a clinically relevant period of aortic cross-clamping.

- To explore the tissue injury, epithelial repair and permeability changes caused by different duration of intestinal ischemia, and whether information obtained by the microdialysis technique reflect these changes.

- To evaluate the resistance of the postischemic restituted intestinal mucosa to further ischemia, and to identify changes in mRNA expressions that may relate to this resistance.

- To evaluate mucosal injury, repair, and permeability changes after different duration and intensity of strangulation obstruction, and whether markers of anaerobic metabolism and cellular damage released to the intestinal lumen mirror such changes.

METHODOLOGICAL CONSIDERATIONS

Experimental animals

All experimental protocols were approved by the local laboratory animal science veterinarian and the Norwegian State Commission for Animal Experimentation. Ninety-one male juvenile pigs, Norwegian Landrace/Yorkshire, were used in the present experiments. Throughout the experiments the pigs were taken care of by experienced anaesthesiologists and surgeons. The size of the animals made the ischemic interventions and instrumentations easier, and allowed repeated blood samples without causing alterations in central hemodynamics. To reduce stress, all pigs were kept in the animal laboratory for 7 days before the experiment. From the night before the experiment, they were fasted with free access to water.

Anaesthesia

All experiments were performed and terminated under general anaesthesia. Premedication was given as an intramuscular injection of azaperone and diazepam. Anaesthesia was induced with thiopental, ketamin, and atropine intravenously. Anaesthesia was maintained with fentanyl and midazolam intravenously and isoflurane gas. After total anaesthesia was confirmed, the animals were tracheotomised, intubated, and ventilated with 40 % oxygen. Tidal volume was kept at 10 mL/kg, and minute ventilation was adjusted to maintain $PaCO_2$ in the range 4.5 – 5.5 kPa. Blood volume was maintained throughout the experiment by continuous infusion (10-15 ml/kg/h) of Ringer solution.

Blood flow determination

Tissue blood flow and cardiac output were determined by the colour labelled microsphere distribution technique [71]. This method is based upon the assumption that the amount of embolized microspheres into a tissue is proportional to the blood flow to this tissue during injection of the microspheres into the left cardiac ventricle [62]. Determination of cardiac output is similarly based upon a proportional relationship to the amount of microspheres found in a reference sample [120].

$$\text{Tissue blood flow (ml/min/g)} = \frac{\text{flow in ref sample} \times \text{number of spheres in tissue sample}}{\text{number of spheres in ref sample}}$$

$$\text{Cardiac output (ml/min)} = \frac{\text{total number injected spheres} \times \text{flow in ref sample}}{\text{number of spheres in ref sample}}$$

There are several sources of error which must be considered when using this technique. Each batch must be controlled for size (range), aggregations, abberants and damage of the microspheres. Sphere size must be selected carefully to assure that all microspheres are entrapped in the peripheral microcirculation and that no significant number bypasses the organs. To avoid differences in concentrations of microspheres between different arteries mixing should be even and sufficient microspheres should be injected. Delivery of spheres into the left cardiac ventricle gives adequate mixing of spheres in the blood stream. Obtaining equal distribution of spheres to the right and left kidneys confirms this. When the number of spheres in tissue specimen is higher than 384, based on a Poisson's distribution, the measurements yield reliable statistics [19]. Altogether, this method is well validated also for the small intestine and when

appropriately applied ensures accurate measurements [36]. A disadvantage is that this method permits only a limited number of measurements.

For continuous blood flow measurements ultrasound transit time flow probes were placed around the portal vein in the first study, around the portal vein and the superior mesenteric artery in the second study, around the superior mesenteric artery in the third study, and around the main mesenteric artery supplying ileum in the fourth study. The Doppler ultrasound technique is based upon measurement of movement of the red blood cells through the beam as a phase change in the received signal. The resulting Doppler frequency can be used to measure velocity if the beam/flow angle is known. The flow is proportional to the transit time. All calculations were made automatically by the flowmeter and displayed as ml/min. The Doppler ultrasound technique has been extensively used both under experimental and clinical conditions [81,89].

Morphological examination

Morphological classification of intestinal mucosal injury caused by ischemia and reperfusion has in the majority of studies been based on the classification reported by Chiu et al in 1970 [28]. Grading on a scale from 0 to 5 this classification describes varying degree of lifting of the surface epithelial cells from the surface of the villi, denudation of the villi, or disintegration of the lamina propria (grade 5). However, this classification does not take into account restitution after superficial injury of the surface epithelial lining in the gastrointestinal tract. Although Grant already in 1945 described replacement of gastric surface epithelium within 4 hours after ethanol injury [58], the importance of this phenomenon was not appreciated until 1982 when Svanes et al described morphological events after destruction of the surface epithelium in frog

stomachs mounted in Ussing chambers [128]. They showed that remaining surface epithelial cells along the sides of and at the tips of villi migrates by extending lamellipodia over the basal lamina and covered the surface of the villi within 4 hours. This process was subsequently reported after various forms of chemical injury of the mucosa in all parts of the gastrointestinal tract in vitro [5,40,95,122] and in vivo [59,77]. Much less attention has been paid to restitution in ischemia/reperfusion models. Park and Haglund reported on a high degree of regeneration of the rat small intestinal mucosa without increased mitosis at 6 hours after 45 min of warm ischemia [104]. Blikslager et al also reported on a high degree of restitution at 6 hours after ischemia in the porcine ileum [11]. In a series of in vitro studies this group has also reported on mechanisms for reassembly of tight junctions in the restituted porcine intestinal surface epithelium and thereby for functional recovery after ischemia [12,14,20,50,80,93,94]. Goblet cells may play a role in restitution [65] and epidermal growth factors enhance restitution after ischemia/reperfusion in rat models [39]. Apart from these studies there are relatively scant studies that consider the influence of restitution as a participant in recovery of intestinal barrier functions after ischemia. Preliminary histological examination of the postischemic small intestine in pigs at the start of the experiments of the present thesis work showed surface epithelial cells at the villi tips that were not normal. These cells had characteristics that closely resembled low cuboidal restituted cells described 1 to 2 hours after mucosal damage with hypertonic saline in the cat and rat stomach [37,59,60]. Ultrastructural examination of such areas showed features that were similar to those originally described in the restituting frog gastric mucosa [128], i.e. flattened elongated cells extending lamellipodia over an infolded basal lamina (paper I). At the light microscopy level changes at the villi tips, sometimes extending along the sides of the

upper parts of the villi, could be categorized using a slight modification of the description by Lacy and Ito [77], i.e. 1) normal columnar epithelium, 2) columnar epithelium with an irregular shape and basal vacuolization of the cytoplasm (in situ damage), 3) denuded basement membrane where the surface epithelium had been sloughed off, and 4) low cuboidal or restituted surface epithelial cells (paper I). Interobserver variability in the morphometric judgement performed by two investigators is presented in table 1.

Table 1

	Mean difference	SD	Range
Normal epithelium	-0.91	6.88	38 (-18,20)
Cuboid epithelium	0.27	4.14	23 (-14,9)
In situ damage	2.50	5.93	22 (-3,19)
Denuded basement membrane	-1.86	3.88	17 (-14,3)

% of surface length, n = 22

As shown in this table, there was a very good agreement between the two investigators.

Permeability

Permeability across the intestinal mucosa can be assessed in vivo by administration of a marker molecule enterally and then measure the recovery either in urine or plasma [99,112,113]. Alternatively the marker molecule may be infused intravenously and the clearance of the marker to an isolated bowel loop perfused with a buffer solution can be calculated [115]. Ex vivo, permeability can be measured by the everted gut sac method in which a segment of the intestine is everted with mucosal side out, ligated at

both ends, and placed in a bath of oxygenated solution. Permeability is then assessed by transport of the marker probe from the bath to the serosa lined lumen [23,143]. Another way of measuring permeability ex vivo is to excise a segment of the intestine, strip off the seromuscular layer and mount the mucosa in an Ussing chamber. Bidirectional transport of marker molecules between the two halves of the chamber can then be measured [29,102,103,117].

The ex vivo methods obviously have less confounding factors than in vivo methods, for example variations in blood flow, variations in urinary excretion of a marker molecule and a number of other factors [100]. In the present thesis work we still chose to use the in vivo approach because it allows more direct comparisons with the morphologic state of the mucosa (injury or repair) and with microdialysis data (glycerol and lactate).

Permeation of hydrophilic marker molecules with a cross sectional diameter of 10-30 Å across the intestinal surface epithelium most likely use the paracellular route [85,102], which to a large part is regulated by tight junctions [4,84,140]. Formation of tight junctions is an integral part of restitution of the surface epithelium. We wanted to assess permeability of marker molecules which is regulated by these paracellular bridges, and another point was to assess marker molecules with a molecular weight that approximates gramnegative endotoxins. Polyethylene glycol with a molecular weight of 4000 Da (PEG-4000) and fluoroescein isothiocyanate dextran (4000 Da, FD-4) fulfil both of these requirements. PEG-4000 has an estimated diameter of 12-16 Å [48,79], and FD-4 approximately the same [115,137], and a molecular weight of 4000 Da is about the same as endotoxins [145,147]. They are both much smaller than the size of the small pores in the microvasculature of cat ileum which has been

estimated to be 46 Å [57] and they are both stable and not metabolically degraded [24,132].

Permeability, from the intestinal lumen to the blood, was assessed by ^{14}C PEG-4000 (paper I, II, IV). An isolated small intestinal loop of 30 cm was filled with PEG-4000. Samples from the solution instilled into the intestinal loop, from venous blood, and urine were counted in a β–scintillation counter. The net count rate in each sample was assumed to be proportional to the concentration of PEG-4000. PEG-4000 is cleared only by urinary excretion. The coefficient of variability regarding laboratory handling (sampling, handling on site, transport, etc) was for the plasma samples 3.5 % (60 measurements), and for the urine samples 1.4 % (32 measurements). For the β-counting the co-efficient of variability for the plasma and urine was 3 % and 1 %, respectively.

To assess permeability from the blood to the intestinal lumen (paper III, IV) a bowel loop of 20 cm, ligated at each end, was perfused with Ringer acetate. FD-4 was injected intravenously and clearance to the intestinal perfusate was measured with a fluorescence spectrophotometer. A potential source of error using this technique could be dye quenching caused by blood staining of the perfusate, particular in paper IV. To assess this possibility a set of in vitro experiments in which increasing amounts of blood were added to standard samples with concentrations of FD-4 in the actual range of the present experiments. Staining with blood to an extent much more than ever observed in our experiments did not influence the calculated concentrations of FD-4.

Microdialysis

In medical practise most sampling from the body is achieved by drawing blood. The microdialysis technique, on the other hand, gives you a “sneak preview” of what goes

on in the tissue – before any chemical events are reflected in changes of systemic blood levels. The basic principle is to mimic the function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue with a physiological liquid [124,138]. The microdialysis catheter consists of an inner tube and an outer tube with a semi-permeable membrane (length 20 mm, diameter < 1mm). The catheter is placed in the interstitial space of the tissue of interest. The inner tube is continuously perfused with a perfusion fluid. During the return through the outer tube an exchange of compounds between the tube and the interstitial space takes place over the semi-permeable membrane (diffusion along concentration gradients). This dialysate is then collected and its contents are analysed. The completeness of exchange or recovery depends on the length of the catheter, membrane characteristics (pore size), properties of the compound of interest, and the perfusion rate. Former comparable studies, own pilot studies and measurements of in vitro recovery were the basis for selection of suitable catheter and perfusion rate [paper II, 78,83,118]. In our experiments microdialysis catheters were placed in the intestinal wall and lumen, into the peritoneal cavity, and into the subclavian artery for sampling of lactate, pyruvate, glucose, and glycerol. Although relatively non-invasive, the insertion of a catheter will cause damage to a small number of cells. Therefore we used an equilibration of $\geq$ 75 min before baseline measurements.

High energy phosphates

During hypoxia the ATP synthesizing capacity of the mitochondria is inhibited and ATP will be depleted. The ability of the mitochondria to re-synthesize ATP upon re-oxygenation predicts the cellular viability [92] and has been used as a predictor of recovery in many ischemia-reperfusion models [110,116,127,142,143,144]. The

content of ATP, ADP, and AMP in the intestinal wall was measured by high performance liquid chromatography (HPLC). Intestinal specimens (whole wall) were immediately frozen in liquid nitrogen and thereafter freeze-dried. The specimens were later homogenized and extracts were made prior to the analysis of contents of high energy phosphates by the HPLC [121]. The HPLC technique is particularly sensitive and has gained general acceptance in most analytical laboratories.

Myeloperoxidase

Myeloperoxidase is an enzyme found in neutrophils and, in much smaller quantities, in monocytes and macrophages [88]. Myeloperoxidase activity has been shown to be directly proportional to the number of neutrophils in the intestinal mucosa and submucosa [72]. Intestinal tissue samples were after some preparation added a detergent that releases myeloperoxidase from the primary granules of the neutrophil. Myeloperoxidase activity was measured spectrophotometrically. The determination of myeloperoxidase activity has been shown to be a reliable and simple biochemical assay to quantitate neutrophil infiltration.

Microarray

DNA microarray can be used for gene expression studies [17,18,26]. The DNA microarray is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon forming an array for the purpose of detecting expression levels for thousands of genes simultaneously. The affixed DNA segments (probes), each representing a specific gene, are placed in known locations on a single microarray. In the microarray system used here, extracted mRNA from the biological sample is reverse transcribed to cDNA and labelled with a fluorescent reporter

molecule. In this two-colour microarray system, two samples to be compared are labelled with one fluorophore each and hybridized simultaneously to the microarray. The same cDNA species from each of the two samples then competitively bind to their probe on the array. After stringency washes, the array is scanned in a dual laser system which in each of the thousands of spots induces emission from both fluorophores if any cDNA has bound. The relative intensity of fluorescence between the two determines how much of each specific mRNA was present in the two original samples. This advanced technique and the following analysis require a close collaboration between specialized laboratory personnel and bioinformaticians. It is important to remember that this method reveals mRNA abundance, and thus gives only an indirect assessment of protein concentration.

Quantitative, real-time RT-PCR

The Reverse-transcription polymerase chain reaction (RT-PCR) amplifies specific segments of mRNA with an exponential kinetics and can reveal extremely small amounts of specific mRNA in a sample. Further technological development has allowed quantitation of mRNA by a process called Quantitative real-time RT-PCR (QRT-PCR).

PCR is based on the use of thermostable DNA polymerase to copy a DNA template in repeated cycles of replication. In RT-PCR mRNA is first reverse-transcribed to cDNA using an oligonucleotide primer and reverse transcriptase enzyme. Thereafter, a thermostable DNA polymerase is guided to the cDNA by a set of oligonucleotide primers that are complementary to the flanking regions of the sequence to be amplified. The PCR reaction amplifies the DNA sequence in question through repeated cycles of denaturation, annealing and polymerization [2].

Quantitation of gene expression can be done using real-time RT-PCR [49]. All real-time PCR systems rely upon the detection and quantitation of a fluorescent reporter, in the present work SYBR Green, which releases a signal that increases in direct proportion to the amount of PCR product in the reaction. Real-time PCR measures accumulation of a product via the fluorophore during the exponential stages of the PCR, and the exponential increase of the product is used to determine the threshold cycle, C_T, i.e. the number of PCR cycles at which a significant exponential increase in fluorescence is detected, and which is directly correlated with the number of copies of DNA template present in the reaction.

Statistics

In paper I and II parametric procedures were applied. The within subjects factor (time) and the between group factor (ischemia) were evaluated by two-way analysis of variance (ANOVA) for repeated measurements. Group comparison at specific time points determined in advance was done with t-tests for independent samples.
Based on reviewers comments and subsequent discussions among the researchers, we reconsidered the assumption of data as normally distributed with equal variance. Because of the rather small sample sizes, nonparametric statistical methods, which make fewer assumptions about the shape of the distribution, were suggested as alternatives. In addition, the variance of our data was not constant for the sake of comparison as required when using the ANOVA. We therefore slightly altered our approach, and non-parametric procedures were employed throughout in paper III and IV: Friedman's test for two-way classification, Wilcoxons' signed rank test for pairwise comparisons, Kruskal-Wallis and Mann-Whitney's rank sum test for independent samples. A potential drawback of nonparametric procedures is that some

power may be lost relative to using a parametric procedure if normality and constant variance is indeed present.

Regarding the morphometric outcome (i.e. distribution of epithelial categories) in papers III and IV, we chose to report the mean proportion cell categories. As no readily available parametric procedure would yield confidence intervals, these were obtained through non-parametric bootstrap [6].

SUMMARY OF PAPERS

In **Paper I** we performed a detailed morphological analysis of the intestinal mucosa after a clinically relevant period of aortic cross-clamping and compared the outcome with changes in permeability and restoration of ATP. The thoracic aorta was cross-clamped for 1 hour followed by reperfusion for 2 hours. Ischemia caused epithelial shedding and denudation of the basement membrane at the intestinal villi tips. During the reperfusion period the denuded area was gradually covered with low cuboidal and squamous-shaped cells extending lamellipodia over the basal lamina i.e., restitution. Restoration of ATP during reperfusion correlated inversely to the extent of denuded basement membrane. Increased intestinal permeability during reperfusion was correlated to the area of denuded basement membrane.

In **Paper II** we wanted to evaluate microdialysis as a method to assess different degrees of intestinal damage and recovery during ischemia and reperfusion. The superior mesenteric artery was cross-clamped for 1 or 2 hours followed by 4 hours reperfusion. Depletion of intestinal tissue ATP, permeability changes across the mucosa, or lactate in the intestinal lumen, wall or peritoneal cavity did not mirror the more extensive injury caused by 2 hours as compared to 1 hour of ischemia. In contrast, glycerol as measured in the intestinal lumen, wall, and peritoneal cavity showed a good correlation with structural injury caused by 1 or 2 hours of ischemia. Also during the ensuing 4 hours recovery period glycerol in the gut lumen and wall, but not in the peritoneal cavity, correlated fairly well with restitution of the surface epithelium.

In **Paper III** we wanted to evaluate the resistance of the postischemic restituted intestinal mucosa to a further ischemic insult. The superior mesenteric artery was cross-clamped for 1 hour, reperfused for 4 hours, cross-clamped once again for 1 hour, and finally reperfused for 3 hours. The first ischemic insult caused more extensive damage of the intestinal mucosa than the identical second ischemic insult. The increase of intestinal permeability after the first ischemic insult was completely abolished after the second insult. ATP decreased more during the first than during the second period of ischemia. We therefore concluded that the postischemic restituted intestinal mucosa is more resistant to further ischemia than the normal mucosa. Microarray and subsequent QRT-PCR analysis revealed increased gene expression for proteins throughout the recovery period that are associated with protection against ischemia/reperfusion injury, and proteins that may be involved in reassembly of tight junctions and cell migration. Therefore, these data provide rational working hypothesis for mechanisms behind the increased resistance of the postischemic restituted mucosa.

In **Paper IV** we wanted to find out whether microdialysis of lactate and glycerol reflect mucosal injury and permeability changes after strangulation obstruction of the small intestine. Strangulation obstruction was induced by gradually and partially or totally tightening a rubber band around a small bowel loop and its mesentery until its venous pressure was close to the diastolic aortic pressure (partial strangulation), or further until complete cessation of arterial blood flow in the feeding artery (total strangulation). Then a reperfusion period of 4 hours followed. Mucosal injury and hyperpermeability of the mucosa correlated to the much more pronounced release of

glycerol to the intestinal lumen seen after total- as compared to partial strangulation. Microdialysis catheters placed into the intestinal lumen detected that lactate increased to a similar plateau level after both partial and total strangulation. During the reperfusion period lactate remained elevated after 2 hours partial and total strangulation, but declined to baseline level after 1 hour partial strangulation. We concluded that the microdialysis technique is particularly suited to assess structural and functional changes of the mucosa after strangulation obstruction.

DISCUSSION

Intestinal ischemia caused injury that on the light microscopy level was confined to the surface epithelium, predominantly at the villi tips. This was evident in all of the experimental models used in the present thesis, and is in accordance with the classic study by Chiu et al [28]. Aortic cross-clamping for 1 hour (paper I) produced considerably less extensive injury than clamping of the superior mesenteric artery (SMA) for a similar time period (paper II and III). A likely explanation for this observation is that aortic cross-clamping did not cause complete ischemia of visceral organs. The greatly reduced, but still present portal blood flow during aortic clamping may support this view. In paper IV was partial strangulation of the intestine for 1 hour associated with a reduction of intestinal wall blood flow to only about 20 % of normal level. Again, injury in terms of denuded basement membrane was greatly reduced as compared to experiments with complete ischemia. One explanation for these observations may be redistribution of blood flow towards the mucosal layer during low flow states [43,54,82] as actually demonstrated in paper IV.

That aortic cross-clamping for 1 hour caused limited denudation of the basement membrane of the intestinal villi tips may be one reason for the fact that this was the only experimental model in which we were able to demonstrate increased mucosal injury during the early reperfusion period. In paper II and III clamping of the SMA probably produced too extensive injury to enable demonstration of additional injury, and in paper IV histologic assessment was performed only at 4 hours of reperfusion. The view put forward here is consistent with the study by Park et al [105] who showed that intestinal reperfusion injury in a rat model was only detectable if the initial ischemic injury was not too small or too extensive. Another contributing factor

for reperfusion injury in paper I may be that upon release of the aortic clamp there is much more tissue subjected to reperfusion and probably a more pronounced burst of reactive oxygen metabolites than in the other experimental models. In addition to these considerations there are data showing that intestinal level of enzymes (i.e. xanthine oxidase) instrumental for formation of reactive oxygen metabolites, is much higher in rats than in juvenile pigs [11,32,73,106]. Of note in this context is that even in the adult human intestine the level of xanthine dehydrogenase /xanthine oxidase is only about 10 % of that found in the adult rat [11]. This may indicate that the pig intestine is better suited than the rat intestine for evaluation of ischemia/reperfusion injury relevant for human disease.

Taken together the findings and issues discussed above emphasize that there is a need to evaluate the pathophysiological relevance of ischemia/reperfusion for human disease both in animal species which are closer to humans, and in models that are more relevant to that encountered in clinical practise.

That intestinal ischemia leads to increased permeability as a consequence of disruption of the intestinal barrier is known for decades [45,55,56,57,107]. This was confirmed in all of the present studies (I-IV). The severity and extent of injury of the surface epithelium was reflected in the degree of hyperpermeability. Support for this contention was obtained by the relationship between the extent of denuded basement membrane and excretion of PEG-4000 in urine demonstrated in paper I (Fig 6), and the fact that limited injury caused by partial strangulation in contrast to total strangulation was associated with less permeability of the marker molecules both in the lumen to blood direction and vice versa. This does not mean that increased permeability in the present studies merely is a function of lost surface epithelial cells and thereby denuded basement membrane. There are ample evidence to show that

improper function of tight junctions between adjacent surface epithelial cells is of fundamental importance for diffusion of marker molecules via the paracellular route, particularly for probes of the size as that used in the present study [4,48,140]. It is therefore conceivable that part of increased transport of marker molecules was due to dysfunctioning intercellular junctions between cells that by light microscopy appeared more or less normal. These considerations could account for the slower normalization or decrease of permeability than covering of denuded basement membrane with restituted epithelium noted in all studies in the present thesis. For example, 1 hour of SMA clamping in paper II was at 4 hours of reperfusion associated with about 90 % covering with cuboidal cells but still a substantial hyperpermeability (lumen to blood) at that time. In paper III there was virtually complete covering with cuboidal cells and a large portion of cylindrical epithelium at 6 hours of reperfusion and again permeability (blood to lumen) was clearly higher than baseline. A similar observation was made in animals with 2 hours partial strangulation at 4 hours of reperfusion in paper IV. All of these observations suggest that there are other factors than restitution per se that in addition are responsible for normalization of barrier function, and a major factor may indeed be formation of tight junctions between restituted cells or repair of such bridges between cells still in situ. Strong support for this view was recently obtained in Ussing chamber studies showing that restitution after acute injury of the intestinal surface epithelium was ineffective for recovery of transepithelial electrical resistance if paracellular closure was inhibited with indomethacin [13,14,50].

An important issue in the present thesis was to identify substances or biomarkers that may reflect tissue injury and which in addition can be measured and monitored in a practical way clinically. Glycerol has been shown to be a marker of disrupted cell

membranes in several tissues [38,87,98,109,139]. In accordance with other recent observations [125,126,139] it was shown in paper II that glycerol as measured with the microdialysis technique both in the intestinal wall and lumen reflected fairly well the time period of complete intestinal ischemia. However, a key observation, not shown before, was the close correlation between the degree of surface epithelial injury and release of glycerol to the intestinal lumen (paper II fig 6). In addition, that study also indicated that release of glycerol to the intestinal lumen at least to some extent can be used to assess restitution of the surface epithelium after ischemic damage of the intestine. The reason for this contention is that glycerol in animals with 1 hour of intestinal ischemia decreased to a level not different from baseline at 4 hours of reperfusion corresponding to an almost complete covering of the villi tips with cuboidal cells. In contrast, animals with 2 hours of intestinal ischemia showed persistent elevation of glycerol at that time and a substantial portion of denuded basement membrane (paper II fig 2 and 5). In paper IV it was shown that partial strangulation of the small bowel causing relatively limited injury of the surface epithelium was associated with only a small and short-lived elevation of glycerol. Total strangulation, on the other hand, causing extensive injury, elicited a large sustained elevation of glycerol throughout the 4 hours reperfusion period, and again there was a considerable portion of non-restituted surface area at 4 hours after release of total strangulation.

The findings referred to above suggest that release of glycerol to the intestinal lumen is a sign of substantial injury of the mucosa. Whether or not a sustained elevation of glycerol in the intestinal lumen after release of strangulation obstruction of the bowel in a clinical setting translates into increased risk of perforation or other local or general complications should be further investigated.

Formation of lactate in the intestinal mucosa in response to ischemia and reperfusion is another marker that could be measured and monitored in the intestinal lumen with the microdialysis technique [111,125,126,134,135,136,139]. Unlike glycerol, lactate increased rapidly to a plateau level that was similar in animals with 1 or 2 hours of clamping of the SMA (paper II) or total or partial strangulation with different duration (paper IV). In paper II the time pattern of lactate release to the intestinal lumen during the reperfusion period was much alike that of lumen to blood permeability of PEG-4000 (paper II, fig 1A and fig 2C). In paper IV the release of lactate to the lumen was sustained throughout the entire reperfusion period in animals with 2 hours partial and total strangulation, whereas 1 hour of partial strangulation was associated with a decline of lactate to baseline level at the end of the reperfusion period. Again permeability changes showed a similar pattern as that of lactate. Apart from being a marker of anaerobic metabolism in a dysfunctioning mucosa, the findings referred to above may indicate that lactate also can be regarded as a marker of permeability changes across the intestinal mucosa.

Taken together the observations and issues discussed above indicate that simultaneous measurements of lactate and glycerol provide complementary information about the state of the intestinal mucosa after ischemic injury.

A part of the ischemia/reperfusion pathophysiology of the intestine that has received particular attention during the last decade is preconditioning [1,30,33,41,42,90,108, 123,133,141,150]. This adaptive response elicited by a few minutes of ischemia may obviously have a potential to be used in order to improve outcome after intestinal transplantation and perhaps also certain planned vascular procedures. Surprisingly, though, we were not able to identify any study addressing the influence of a prolonged period of initial intestinal ischemia on the outcome after a subsequent

repeated ischemic insult a few hours later. Given the near complete restitution of the surface epithelium detected at 4 hours after 1 hour of ischemia in paper II, we used this experimental model and inflicted a second 1 hour period of ischemia at that time. The results in paper III indicated less structural injury, no increase in intestinal permeability, and less consumption of ATP. These findings are of interest particularly in a clinical perspective because 1 hour of complete or near complete intestinal ischemia is a time frame that is relevant for example during surgery involving (high) clamping of the aorta, or before effective resuscitation after hypovolemic shock.

It was beyond the scope of that study to explore in depth the mechanisms that were responsible for the enhanced resistance of the postischemic restituted mucosa. However, the microarray and QRT-PCR techniques identifying and quantifying a change in mRNA expressions during the course of the experiments, illustrate the power of these techniques in order to formulate rational working hypothesis. A major challenge related to the microarray technique is handling of the extremely large amount of data. After filtering of the data based upon the degree of up- or downregulation by using standard criteria, there is at present no other practical approach than to select genes for further analysis based upon for example time points of particular interest in an experimental protocol, or whether the gene products are known to influence the phenomenon under study. Among the differentially expressed genes reported in paper III, superoxide dismuthase 2 [51,52,86,108] and heat shock proteins [25,27,46,70], are in particular likely candidates to be involved in the mechanisms for the increased resistance of the postischemic restituted mucosa. Another finding of interest is the upregulation of mRNA for Connexin 43 because accumulating and recent studies suggest that this protein plays an important role for tight junctions and paracellular permeability [66,97].

IMPLICATIONS

The knowledge about intestinal ischemia and reperfusion injury and repair is extended. In particular we have shown that:

- Intestinal mucosal repair after aortic cross-clamping correlates to change in intestinal permeability and restoration of ATP.

- Glycerol as measured by microdialysis reflects the extent and severity of intestinal damage and the ensuing recovery after ischemia of the small intestine.

- The postischemic restituted intestinal mucosa is more resistant to further ischemia than the normal mucosa.

- Microdialysis of lactate and glycerol is suitable for assessment of functional and structural changes of the intestinal mucosa after strangulation obstruction.

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Erratum

Paper I: The morphometric analysis was carried out using a magnification of x 100 (i.e., with an ocular of x 10 and an objective of x 10).

Paper II: see erratum in Crit Care Med 2007;53:333

ERRATUM

In the article by Solligård and Juel et al., published in the October issue of *Critical Care Medicine*, the numbers on the Y-axis of Figure 1B on page 2280 should appear as follows:

.12
.10
.08
.06
.04
.02
0

The authors regret the errors

Paper I

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Intestinal Injury after Thoracic Aortic Cross-Clamping in the Pig

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Background. **The mucosal surface epithelium is an essential part of the functional intestinal barrier, but its structural response to ischemia/reperfusion is only partly characterized. The purpose of this study was to provide a detailed morphological evaluation of intestinal surface epithelium after aortic cross-clamping.**

Material and methods. **Pigs were subjected to thoracic aortic cross-clamping for 60 min and subsequent reperfusion for 120 min. Tissue blood flow and high-energy phosphates were measured with microspheres and HPLC, respectively. Urinary excretion of ^{14}C polyethylene glycol (MW 4000 Da) (PEG-4000), loaded into an intestinal loop, provided an index of intestinal permeability.**

Results. **Jejunal blood flow was restored at 10 min after aortic declamping. Denudation of the basement membrane of the intestinal villi tips, as a consequence of epithelial shedding, increased markedly during the initial 60 min of reperfusion ($P = 0.002$). During the following 45 min, the denuded basement membrane was partly covered with low cuboidal and squamous-shaped cells extending lamellipodia over a wavy basement membrane. Restoration of ATP at 60 min after aortic declamping correlated inversely to the extent of denuded basement membrane ($r = 0.75$, $P = 0.032$). Permeability of PEG-4000 increased markedly after aortic declamping and was linearly correlated to the area of denuded basement membrane ($r = 0.87$, $P = 0.01$).**

Conclusions. **Reperfusion for 2 h after aortic cross-clamping is associated with initial aggravation of ischemia-induced injury in the porcine jejunum, but thereafter with restitution of the surface epithelium. Restoration of ATP may be important to avoid intestinal injury after ischemia. Increased permeability of a macromolecule in response to reperfusion is closely correlated to injury of the surface epithelium.**

Key Words: **intestinal ischemia; surface epithelium; restitution; ATP; permeability; reperfusion injury; aortic surgery; aortic cross-clamping.**

INTRODUCTION

Open repair of aortic aneurysms, requiring aortic cross-clamping, is associated with profound hemodynamic changes [1]. These hemodynamic changes, notable both during and after aortic cross-clamping, may in turn cause a variety of complications from nearly all organ systems. Complications caused by ischemia and altered microcirculation of the gastrointestinal tract are a major threat to such patients [2]. The pathophysiology of this type of complications is often thought to be related to reperfusion injury of the gut mucosa. Although reperfusion injury has been well characterized in rodents and cats [3–6], it is noteworthy that its relative importance appears to be less pronounced or even lacking in other species (i.e., pigs) [7, 8]. One reason for such discrepancies may be related to large variations in xanthine oxidase activity in the intestinal mucosa among various species, humans included [7, 8].

Almost all information related to reperfusion injury of the gut has been obtained by studies examining segments of the intestine after clamping of the superior mesenteric artery or vessels close to the intestinal wall. The advantage of such models includes reduced influence of systemic factors that by themselves may alter the phenomenon under study. On the other hand, it is

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also pertinent to assess such changes in models mimicking clinical situations. The purpose of the present study was to perform a detailed morphological analysis of the intestinal mucosa after a clinically relevant period of aortic cross-clamping in a pig model. In particular the reperfusion period was of interest because structural damage might be worsened or signs of repair might be detected. Morphological data were correlated to a marker of neutrophil granulocyte accumulation in the intestinal wall, content of high-energy phosphates, and a marker of intestinal permeability.

MATERIALS AND METHODS

The experimental protocol for this study was approved by the Norwegian State Commission for Animal Experimentation.

Surgical Preparation

Fourteen juvenile male pigs (Norwegian Landrace/Yorkshire) (18–26 kg) were used. The pigs were kept on standard laboratory chow and a 12:12-h light-dark cycle. The animals were deprived for food, but not water, for 24 h before the experiment. Premedication was given as an intramuscular injection of azaperone (400 mg) (Stresnil; Janssen-Cilag, Vienna, Austria) and diazepam (10 mg) (Stesolid; Dumex-Alpharma, Copenhagen, Denmark). Anesthesia was induced with thiopental (175 mg) (Pentothal; Abbot Scandinavia AB, Solna, Sweden), ketamine HCl (250 mg) (Ketalar; Parke-Davis, Solna, Sweden), and atropin (1 mg) (Atropin; Nycomed Pharma AS, Oslo, Norway) administered via an auricular vein. Anesthesia was maintained with isoflurane (Isofluran; Rhodia, Bristol, UK) 1.0% during surgery and 0.5% throughout the remaining part of the experiment. Additional fentanyl (75 μg/kg/h) (Fentanyl, Alpharma AS, Oslo, Norway) and midazolam (0.5 mg/kg/h) (Dormicum; Roche, Basel, Switzerland) dissolved in Ringer-acetate solution (Fresnius Kabi, Halden, Norway) was administered intravenously via a Terumo infusion pump (Terufusion Syringe Pump, model STC-521, Vindmed AS, Lysaker, Norway). The pigs were tracheotomized and then ventilated with a Servo ventilator (Servo ventilator 900B, Siemens-Elema, Sweden) at a rate of 20 breaths/min and 40% oxygen in the inspiration air. The pigs were kept in a supine position throughout the experiment, and a heating blanket maintained normothermia. A double-lumen catheter was introduced into the right femoral vein for blood tests and infusions. The animals received Ringer acetate, 10–15 ml/kg/h, throughout the experiment. A triple lumen catheter (Certofix Trio SB 720, Braun Melsungen AG, Melsungen, Germany) was introduced into the right subclavian artery for monitoring arterial blood pressure and heart rate (Tram-rac 4A/Tram-scope 12C, Marquette Electronics, USA), sampling of arterial blood gases (ABL 330, Radiometer, Copenhagen, Denmark), and withdrawal of reference samples for tissue blood flow measurements (see later). Another catheter (O.D. 1.3 mm Portex Intravenous Cannula, Portex Ltd., Hythe, Kent, UK) was inserted, via the right carotid artery, into the left cardiac ventricle. A correct position of the catheter was assured by obtaining a typical pressure trace with low diastolic pressures. A left thoracotomy was performed and the descending aorta was prepared for cross-clamping distal and close to the left subclavian artery. A midline laparotomy was performed. A Foley catheter (Bard Ltd., West Sussex, UK) was placed in the urinary bladder, secured with a purse string suture, for monitoring urine output and for urine samples. The portal vein was dissected free and a Doppler ultrasound probe (I.D. 5 mm) was placed around the vessel. About 100 cm distal to the ligament of Treitz, four separated jejunal loops of about 20 cm were labeled and prepared with loose ligatures at each end. The loops were removed during the experiment according to the protocol. A fifth loop of 30 cm was also prepared and later filled with a probe for assessment of permeability across the intestinal wall (see later). After completion of surgery, both the thoracotomy and the laparotomy were closed with towel clamps. The pigs were allowed to stabilize for 60 min before the start of the experiment.

Blood Flow

Portal blood flow was measured with a precalibrated 5-mm ultrasonic transit-time flow probe positioned without constriction on the portal vein and connected to a flow calculator with continuous online display of flow (Transonic Systems Inc., Ithaca, NY, USA).

Tissue blood flow and cardiac output were determined by means of the distribution of microspheres, 15.5 ± 0.45 μm in diameter, labeled with three different colors (blue, yellow, or eosin) (Dye-Trak Microspheres; Triton Technology, San Diego, CA, USA). Three measurements were performed in each animal, with the color sequence randomized. The spheres were suspended in saline (containing 0.05% Tween 80 and Thimerosal), sonicated the same day as the experiment, and mechanically shaken immediately before injection. Microspheres (3 ml, about 9 mill spheres) were injected into the left cardiac ventricle for a total of 30 s. A reference blood sample was drawn from the right subclavian artery by a constant-rate pump (Cole-Parmer Instrument Co., 74900 serial, IL, USA) set at 4 ml/min, starting 15 s before sphere injection and terminating 75 s after its completion. Collected blood samples were later weighed to determine the exact extraction rate. After completion of the experiments, tissue samples were taken from the jejunal wall, pancreas, kidneys, and stomach. All tissue samples were weighed and 4 M KOH (Merck, Darmstadt, Germany) were added to dissolve the tissue and release the microspheres. After digestion for 2 days during shaking at 60°C, the samples were filtrated through a 10-μm filter (Mitex membrane filter, Millipore, Bedford, MA, USA) using a vacuum pump (Ameda Egnell-Universal, Switzerland). The filters were washed with 0.05% Tween 80 (Merck), 70% ethanol, and distilled H_2O, and left in open Eppendorf tubes for 24 h to dry. Dimethylformamide (700 μl) (BDH Laboratory Supplies, Poole, UK) was added to release the color from the microspheres. The samples were then, with 10-min intervals, twice vortex mixed for 30 s and centrifuged for 5 min at 6000 rpm. Absorbency of dye was measured in a spectrophotometer (Hewlett–Packard 8453, diode array spectrophotometer, Hewlett–Packard, USA) and analyzed using computer software (HP 845x UV-Visible Chemstation Systems, Hewlett–Packard). Tissue blood flow rate and cardiac output was calculated as previously described [9, 10].

Myeloperoxidase Activity

Myeloperoxidase was assayed according to previously described methods [11]. Briefly, tissue samples (100–500 mg) were frozen at −85°C in 50 mM potassium phosphate buffer, pH 6.0. Following thawing, the tissue samples were cut and dissolved in 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer (5 ml) and frozen once more. The suspensions were then centrifuged at 40,000*g* for 15 min. Myeloperoxidase activity in the supernatant was measured spectrophotometrically: 0.1 ml of the supernatant was mixed with 2.9 ml 50 mM phosphate buffer pH 6.0 containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. A Shimadzu UV-visible spectrophotometer (Shimadzu Corp., Tokyo, Japan) measured the absorbance difference at 460 nm at 30 and 90 s. The results are expressed as units MPO/100 mg tissue (wet weight). One unit MPO activity is defined as that degrading 1 μmol hydrogen peroxide per minute at 25°C.

High-Energy Phosphates

At different time points, tissue specimens from the jejunal loops were immediately frozen in liquid nitrogen and thereafter freeze-

dried. The specimens were later homogenized and extracts were made prior to the analysis of contents of ATP, ADP, and AMP by a standard high-performance liquid chromatography method as described by Sellevold *et al*. [12].

Permeability

A jejunal loop of 30 cm, closed at each end with ligatures, was filled with 100 ml ^{14}C polyethylene glycol 4000 (PEG-4000) (10.0 μCi diluted with 0.9% NaCl to 100 ml, final volume) (Amersham Bioscience, Buckinghamshire, UK). Samples containing ^{14}C PEG-4000 from the solution instilled in the intestinal loop, from venous blood, and from urine were counted in a β-scintillation counter. The net count rate in each sample was assumed to be proportional to the concentration of ^{14}C PEG-4000. Excretion of PEG-4000 in the urine was used as an index for permeability of this probe across the intestinal wall. Because radioactivity of the solution instilled into the intestinal loop showed some variations between batches, the excretion of ^{14}C PEG-4000 in the urine was expressed as a percent of the amount PEG-4000 instilled into the intestinal loop [13].

Morphology

Specimens of the intestinal wall used for morphometric analysis of the surface epithelium were fixed in 4% formaldehyde-buffered solution. Specimens for quantitation of mast cells were fixed in Carnoy's solution for at least 12 h and thereafter in 80% alcohol. All specimens were further processed and embedded in paraffin. Sections of 5 μm were stained with Hematoxylin-Erythrosin-Safran for morphometric analysis of the surface epithelium, and Astrablue-Safranin O for quantitation of mast cells.

An investigator blinded to the experimental group and the time in the experiment at which the biopsy had been taken examined the slides used for light microscopical morphometric analysis of the surface epithelium and mast cells. The morphometric analysis was carried out using an eyepiece graticule and a magnification of $\times$400 (i.e., with an ocular of $\times$10 and an objective of $\times$40).

Mast cells were counted in each slide in 10 microscopic fields in the mucosa and 20 microscopic fields in the submucosa. Mast cells were readily identified due to their blue cytoplasmatic granules.

The mucosal surface epithelium was subclassified into normal, *in situ* damage, denuded basement membrane, or simple cuboidal cells (see Results). In each slide 15 microscopic fields were examined, and the subclasses were quantified as a percentage of the surface length examined.

Villus height in five microscopic fields of well-oriented villi was measured in each slide. Villus height was measured along a perpendicular line drawn from the basement membrane at the villus tip to the muscularis mucosa with the aid of the eyepiece graticule.

For electron microscopy, tissue samples from the intestine were cut in 1-mm^3 cubes and fixed for 12 h at 4°C in 2% glutaraldehyde in 0.1 M phosphate buffer and postfixed for 1 h in 2% OsO_4 in 0.1 M phosphate buffer. After fixation, the cubes were rinsed with 0.1 M phosphate buffer, dehydrated through graded ethanol, embedded in Epon, and examined in a JEOL 100CX electron microscope after contrasting with uranyl acetate and lead citrate.

Experimental Protocol

After stabilization for 60 min and thereafter a baseline period of 45 min, nine pigs had their thoracic aorta cross-clamped for 60 min. Then the thoracic aorta was declamped and a reperfusion period of 120 min followed. One pig died immediately after declamping, leaving eight animals for further analysis. Portal blood flow was continuously measured and tissue blood flow was measured at baseline, 30 min after cross-clamping and 10 min after release of the clamp. ^{14}C PEG-4000 was instilled into an intestinal loop at the start of the experiment for assessment of intestinal permeability. Intestinal loops were removed at baseline, 50 min after cross-clamping, and at 60 and 105 min after release of the clamp to yield specimens for morphological analysis, for measurements of high-energy phosphates, and myeloperoxidase. Five control animals were treated according to the same protocol except for cross-clamping the thoracic aorta.

Statistics

Statistical significance of differences was determined by using independent sample t tests or analysis of variance (ANOVA). If ANOVA indicated a significant difference, results were further analyzed by using multiple-comparisons procedures (LSD). Linear correlation and linear regression analysis were used for demonstrating the relationship between the area of denuded basement membrane and the excretion of polyethylene glycol in urine, and between the degree of ATP repletion and the area of denuded basement membrane. Values of $P < 0.05$ were considered significant. Data are expressed as means ± SEM.

RESULTS

Central Hemodynamics

Cross-clamping of the thoracic aorta caused an immediate increase in mean arterial blood pressure (MAP) of about 75% ($P < 0.001$) (Table 1). MAP remained at this level throughout the 60-min clamping period. After removal of the vascular clamp, MAP fell to about 20% below baseline level for 5 min, but thereafter increased to and remained at a level similar to baseline for the rest of the experiment. Heart rate also responded to aortic cross-clamping with about 100% increase ($P < 0.001$). Unlike MAP, heart rate remained elevated after removal of the vascular clamp. Cardiac output, as measured 30 min after aortic cross-clamping, was found to be about 80% above baseline level ($P = 0.012$). Cardiac output had returned to baseline level at 10 min after removal of the vascular clamp.

In control sham-operated pigs no change in MAP, heart rate, or cardiac output was noted.

Portal and Regional Tissue Blood Flow

Cross-clamping of the thoracic aorta caused an immediate and sustained fall of portal blood flow to about 7% of baseline level (Table 1). When the vascular clamp was removed, the portal blood flow increased to and stayed at a level approximately 50% above baseline level for the remaining part of the experiment.

Tissue blood flow rates in the jejunal wall, stomach mucosa, pancreas, and cortex of the kidneys as measured 15 min before cross-clamping, 30 min after clamping, and 10 min after removal of the clamp are shown in Table 2. The blood flow in jejunum, stomach mucosa, and pancreas decreased after cross-clamping to 10, 20, and 20% of baseline level, respectively. Jejunal and stomach mucosal, but not pancreatic blood flow, increased to baseline level after removal of the

TABLE 1

Central Hemodynamic Variables and Portal Blood Flow in Pigs with Cross-Clamping of the Thoracic Aorta

Groups	Baseline	Cross-clamping		After release of the aortic clamp			
		15 min	30 min	5 min	10 min	60 min	120 min
Mean arterial pressure (mmHg)							
Aortic cross-clamp	82 ± 3	135 ± 7*	135 ± 8*	62 ± 3*	78 ± 2	78 ± 1	84 ± 2
Control	99 ± 7	99 ± 5	100 ± 6	108 ± 5	101 ± 6	97 ± 5	95 ± 8
Heart rate (beats/min)							
Aortic cross-clamp	69 ± 7	142 ± 14*	147 ± 13*	145 ± 10*	136 ± 15*	134 ± 14*	127 ± 16*
Control	65 ± 5	63 ± 5	68 ± 7	70 ± 8	70 ± 8	71 ± 10	84 ± 13
Cardiac output (ml/min)							
Aortic cross-clamp	3735 ± 323	—	6790 ± 1052*	—	4974 ± 818	—	—
Control	2897 ± 529	—	3734 ± 426	—	2545 ± 361	—	—
Portal blood flow (ml/min)							
Aortic cross-clamp	520 ± 74	39 ± 8*	38 ± 5*	813 ± 103*	982 ± 121*	902 ± 86*	725 ± 73*
Control	401 ± 25	455 ± 25*	396 ± 53	464 ± 37	454 ± 54	435 ± 39	442 ± 36

Note. Mean values ± SEM. The thoracic aorta was either cross-clamped for 60 min ($n = 8$), or exposed but not clamped (control) ($n = 5$).
*Significant difference from baseline.

clamp. No significant difference between right and left renal blood flow could be detected. Both right and left renal blood flow decreased to nearly zero after aortic cross-clamping and increased, but did not reach baseline level after declamping.

Morphology

Examination of the intestinal mucosa at 50 min after aortic cross-clamping, or at 60 or 105 min after removal of the aortic clamp, did not reveal any visible lesions. Preliminary microscopic evaluation, however, showed distinct changes almost exclusively confined to the surface epithelium. Such changes could be labeled into four categories (Fig. 1). The first category consisted of the normal columnar epithelium. The second category comprised a columnar epithelium with irregular shape and basal vacuolization of the cytoplasm (Fig. 1B). Such epithelium was often separated from the basement membrane by a narrow space. This space was predominantly at the tip of the villi, but could also extend toward the crypts. This epithelium was in the present study classified as *in situ* damage and corresponds to grade 2 and 3 in the classification reported by Chiu *et al*. [14]. The third category was actually not an

TABLE 2

Effect of Cross-Clamping of the Thoracic Aorta for 60 min on Tissue Blood Flow in Pigs

Groups	Baseline	Cross-clamp	Release of the clamp
Jejunum			
Aortic crossclamp	0.655 ± 0.105	0.055 ± 0.018*	0.809 ± 0.135
Control	0.548 ± 0.178	0.385 ± 0.106	0.399 ± 0.108
Stomach mucosa			
Aortic crossclamp	0.262 ± 0.028	0.053 ± 0.021*	0.306 ± 0.042
Control	0.170 ± 0.032	0.160 ± 0.021	0.145 ± 0.019
Pancreas			
Aortic crossclamp	0.423 ± 0.076	0.093 ± 0.037*	0.235 ± 0.041*
Control	0.173 ± 0.028	0.190 ± 0.043	0.229 ± 0.059
Right kidney			
Aortic crossclamp	2.228 ± 0.329	0.012 ± 0.007*	0.817 ± 0.166*
Control	1.670 ± 0.412	1.542 ± 0.365	1.461 ± 0.386
Left kidney			
Aortic crossclamp	2.042 ± 0.365	0.007 ± 0.005*	0.848 ± 0.158*
Control	1.739 ± 0.178	1.778 ± 0.205	1.615 ± 0.227

Note. Mean values ± SEM in ml/min/g. Blood flow was determined 15 min before aortic cross-clamping (baseline), 30 min after aortic crossclamping, and 10 min after release of the clamp ($n = 8$). In control experiments the thoracic aorta was exposed, but not clamped ($n = 5$).
*Significant difference from baseline.

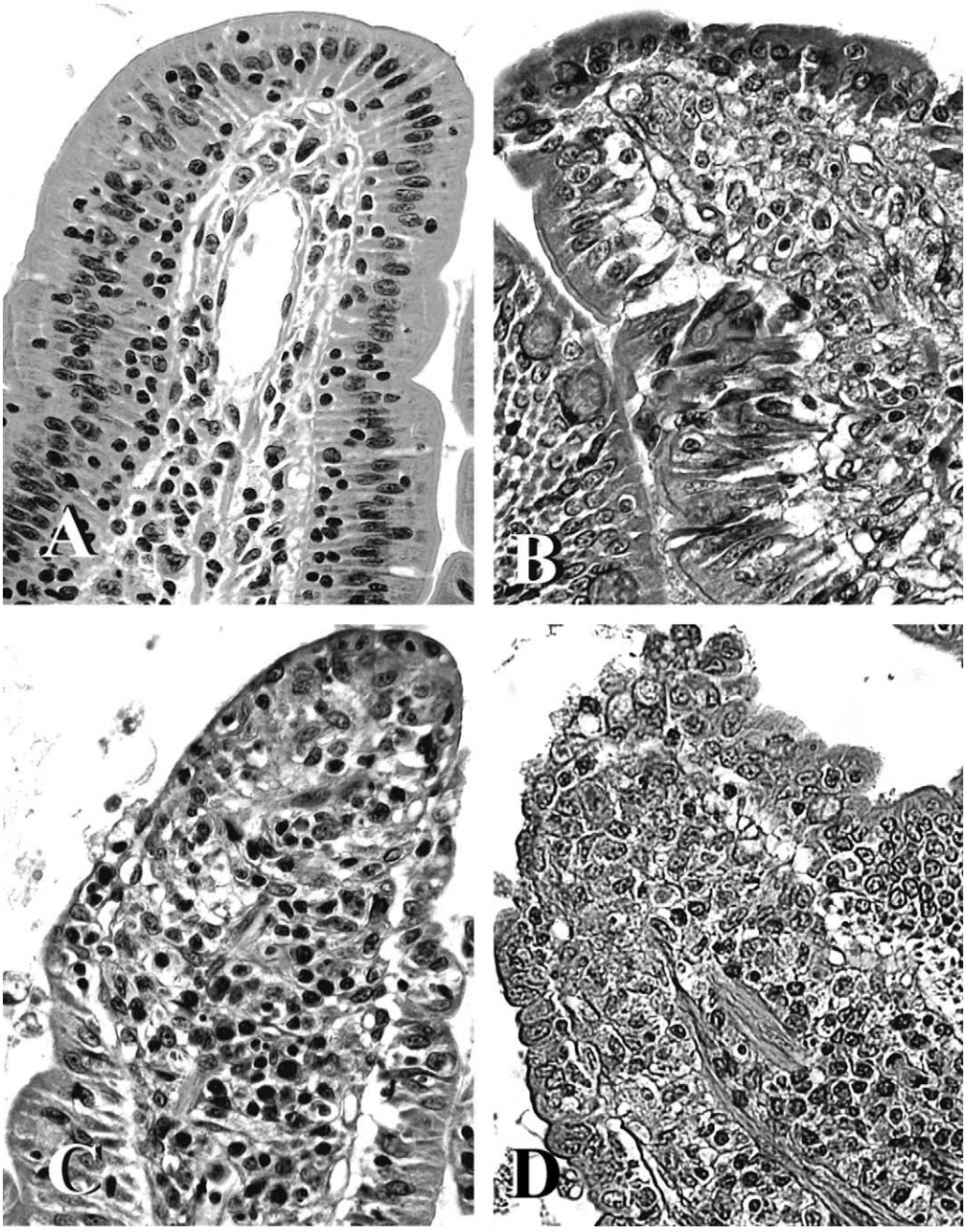

FIG. 1. Micrographs of jejunum mucosa from pigs subjected to aortic cross-clamping for 60 min, thereafter declamping and reperfusion for 120 min. (A) Section at the end of a 45-min baseline period. An intestinal villus with normal columnar epithelium is apparent. (B) A typical villus after 50 min of aortic cross-clamping showing *in situ* damage of the surface epithelium. Note columnar epithelium with irregular shape and basal vacuolization of the cytoplasm. (C) Typical villus at 60 min after aortic declamping. The surface epithelial cells have sloughed off at the tip of the villus, leaving the basement membrane denuded. (D) Typical villus 105 min after aortic declamping. The cells on the surface are cuboidal and irregularly arranged but form a complete lining.

epithelium, but on the contrary, denuded areas where the surface epithelium had been sloughed off the basement membrane (Fig. 1C). The denuded areas were confined to the tip of the villi. This type of damage corresponds to grade 4 in the Chiu classification. Transmission electron microscopy confirmed the presence of an intact basal lamina at denuded villi tips. At these sites flattened goblet cells with lammelipodia were frequently found. These cells were usually seated on an infolded basal lamina (Figs. 2 and 3). Grade 5 in the Chiu classification (disintegration of the lamina propria, hemorrhage, and ulcerations) was not detected in the present study. The fourth category, not described in the Chiu classification, comprised a mono-

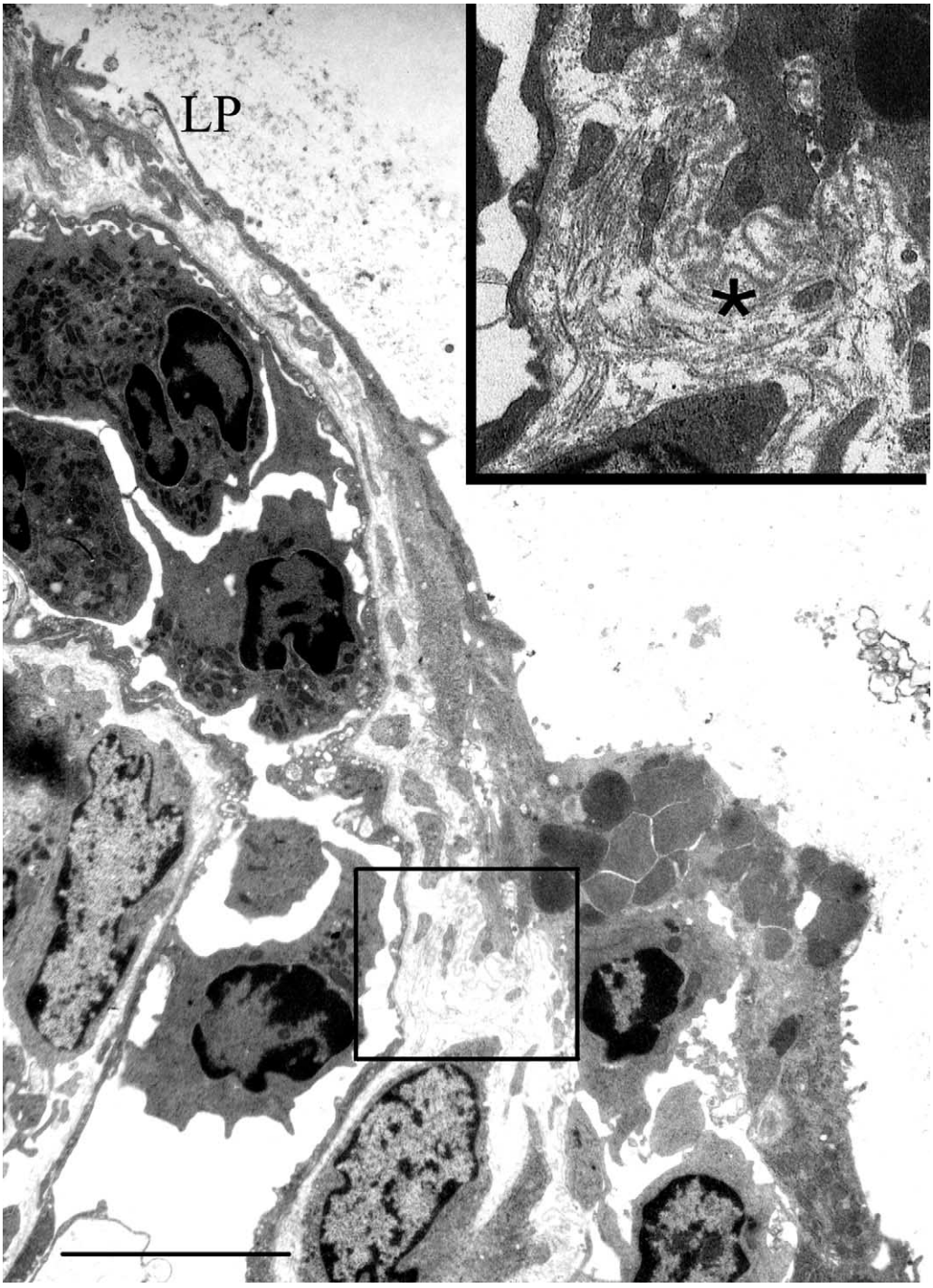

FIG. 2. Transmission electron micrograph of the pig intestinal mucosal surface at 60 min after aortic declamping. The villus tip is covered by a slender goblet cell lamellipodium (LP). A higher magnification demonstrates extensively infolded basal lamina (asterisk). Bar represents 5 μm.

layer of irregularly arranged cuboidal cells attached to the basement membrane along the sides of and at the tip of the villi (Fig. 1D). PAS staining revealed that such cells contained abundant mucus. Electron microscopy showed that junctional complexes were frequently present between cuboidal surface epithelial cells and confirmed that the cuboidal cells contained numerous mucus granula (Fig. 4). Included in the fourth category was also a minority of flattened spindle-like cells partly covering the denuded areas.

Figure 5 shows the distribution of the different categories of the surface epithelium detected by the blinded

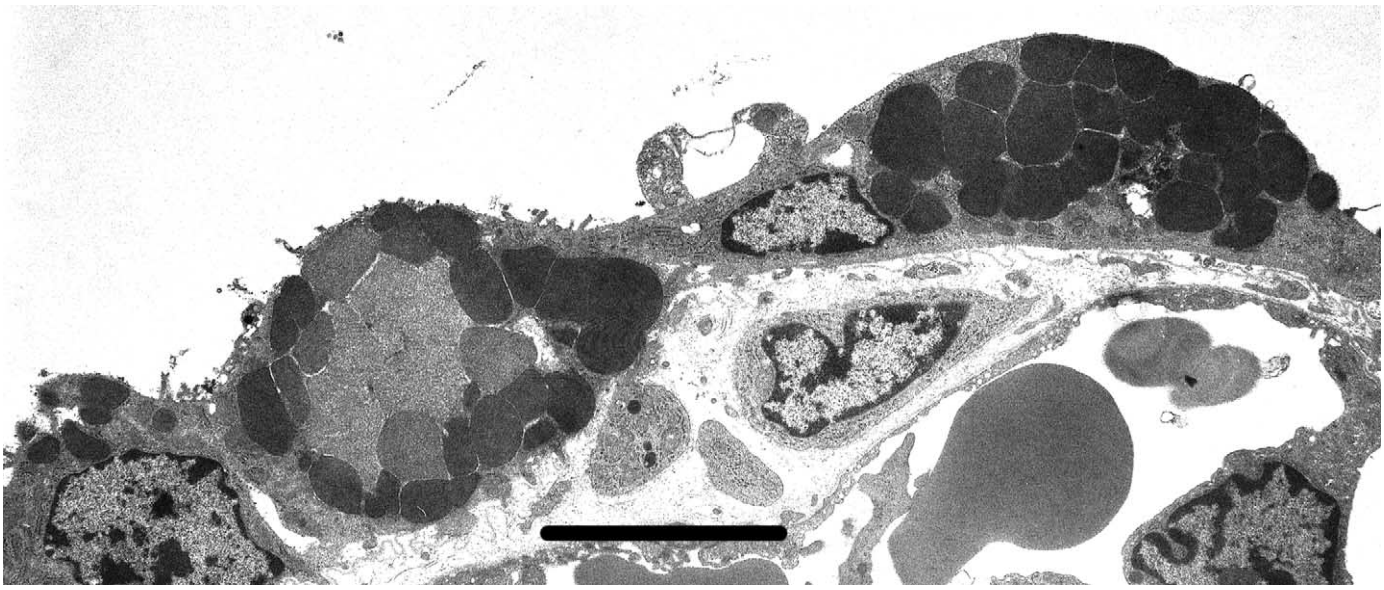

FIG. 3. Transmission electron micrograph of the pig intestinal mucosal surface at 60 min after aortic declamping showing a procession of goblet cells with squamous shape covering a villus tip. Bar represent 5 μm.

light microscopic examination. Intestinal specimens removed before aortic cross-clamping (baseline) had normal surface epithelium. At 50 min of the cross-clamping period about 45% of the surface epithelium showed *in situ* damage. The remaining part of the surface epithelium was either normal or the basement membrane was to a small extent denuded (~6%). At 60 min after removal of the aortic clamp, the basement membrane appeared much more denuded (~45%) than at 50 min of the cross-clamping (ischemic) period (P = 0.002). The percentage of normal epithelium was much less at 60 min after removal of the clamp than at 50 min of the ischemic period (P = 0.007). This finding most likely indicates that a considerable part of normal surface epithelial cells, during the first phase of reperfusion, underwent changes causing such cells to fall into the category with *in situ* damage at 60 min after removal of the aortic clamp. At 105 min after removal of the aortic clamp ~20% of the basement membrane was covered with cuboidal cells in contrast to the only negligible percentage of this cell type 45 min earlier (P = 0.009). This finding was reflected in a reduction of the percentage of denuded surface area in comparison to that found 45 min earlier (P = 0.008). In control animals no detectable changes of the surface epithelium were noted throughout the experiment (data not shown).

Measurement of the height of the intestinal villi revealed a gradual reduction throughout the experiment. The height of the villi at 105 min after removal of the clamp was ~80% of baseline value (P = 0.008).

Because degranulation of mast cells has been implicated in ischemia/reperfusion injury, the morphometric analysis also included counting of mast cells in the intestinal wall. However, with regard to both mucosal mast cells and submucosal connective tissue mast cells, no significant change in density was detected (Table 3).

Finally, because rapid infiltration of neutrophilic granulocytes in the intestinal tissue after ischemia/reperfusion is a prominent feature in some studies, the tissue concentration of a marker of such cells, myeloperoxidase, was measured. As shown in Table 3, no significant change in this enzyme was evident during the course of the experiment.

Intestinal Permeability

In the control group ^{14}C count rate in venous blood was throughout the entire experiment not different from background rate in venous samples taken before instillation of ^{14}C PEG-4000 into the intestinal loop. Similar observations were noted during the baseline period in animals subjected to aortic cross-clamping. Also during the 60-min aortic cross-clamping period with near zero intestinal blood flow, the increase in venous count rate was low, and at the end of this period, less than twice of background rate, indicating that only negligible amounts of PEG-4000 had entered the systemic circulation. After release of the aortic clamp, the venous concentration of PEG-4000 increased rapidly and had within 30 min reached a level about five times higher than that measured at the end of the clamping period. The venous concentrations of PEG-4000 thereafter remained remarkably stable within each animal for the remaining part of the experiment (the coefficient of variation ranging from 2.5 to 22.3%, median 11.6%).

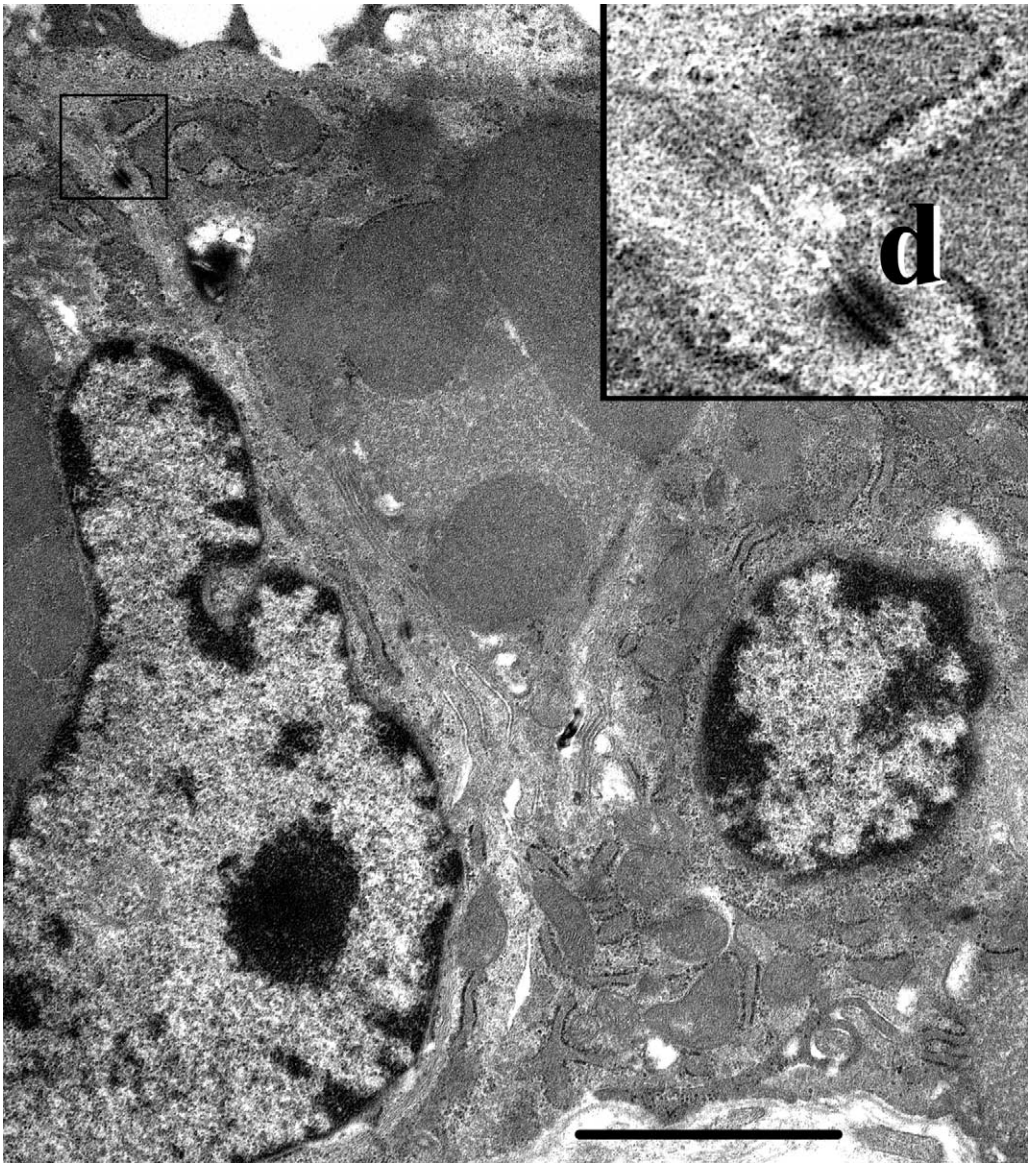

FIG. 4. Transmission electron micrograph of the pig intestinal mucosal surface at 105 min after aortic declamping. The micrograph shows cuboidal surface epithelial cells. Desmosomes (d) were numerous between such cells (insert). Bar represents 2 μm.

Urine output was rapidly restored to a level at least equal to baseline at 60 min after aortic declamping in each animal. The excretion of PEG-4000 in the urine during the reperfusion period in such animals was 4.3 $\pm$ 1.2% of that instilled into the intestinal loop, as compared to 0.33 $\pm$ 0.1% excreted in the urine during the corresponding time period in control animals (P = 0.021). (Note: Due to a technical error, data were only obtained from seven animals with aortic cross-clamping.)

To assess whether intestinal permeability was related to intestinal damage, the average amount of denuded basement membrane at 60 and 105 min of reperfusion was plotted against excretion of PEG-4000 in urine. A close and highly significant correlation between these parameters was obtained (r = 0.87, P = 0.01) (Fig. 6).

High-Energy Phosphates

Intestinal ischemia caused by aortic cross-clamping was as expected associated with a marked decrease in intestinal ATP content (Table 4). After removal of the clamp, at 60 min of reperfusion, the ATP level was about 85% of baseline level (P = 0.029). ATP was completely restored at 105 min of reperfusion. ADP remained unchanged throughout the experiment. In contrast, the AMP level was markedly elevated during ischemia ($P < 0.001$) and then fell to baseline values already at 60 min of reperfusion. One factor that might

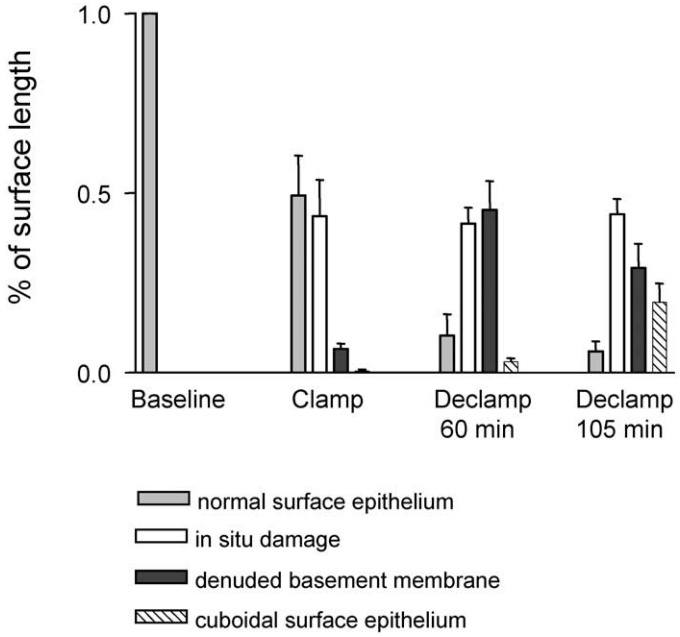

FIG. 5. Distribution of different categories of surface epithelium of the intestinal mucosa in eight pigs having their thoracic aorta cross-clamped for 60 min. The categories are normal columnar surface epithelium, columnar epithelium with irregular shape labeled *in situ* damage, denuded basement membrane, and irregularly arranged low cuboidal cells. Intestinal specimens were removed 10 min before clamping (baseline), 50 min after clamping, and at 60 and 105 min after declamping. Values are means ± SEM.

be decisive for the ability of the surface epithelium to maintain or restore its integrity after an ischemic insult could be the capacity to restore high-energy phosphates. To obtain more information about this possibility, the difference between the intestinal content of ATP at 60 min of reperfusion and at baseline was plotted against percentage of denuded basement membrane (judged by morphometry) at 60 min of reperfusion. As shown in Fig. 7, there was a reasonably good correlation between these parameters.

DISCUSSION

The present study revealed that aortic cross-clamping for 1 h produced a well-defined and reproducible damage of the intestinal mucosa, largely confined to the surface epithelium. The damage was apparently worse at 60 min after removal of the aortic clamp. However, reperfusion of the vascular bed for an additional 45 min was not associated with further morphological damage. On the contrary, distinct signs of mucosal repair were detected. About 20% of the villi surface was at that time covered with a layer of cuboidal cells, causing a reduction of the denuded surface area.

Our morphological analysis indicated that a considerable portion of the surface epithelial cells, which at the end of the ischemia period showed features of damage but still remained attached to the basement membrane, had sloughed off during the first 60 min of reperfusion. Furthermore, surface epithelial cells that at the end of the ischemia period had a normal appearance underwent changes fulfilling our criteria for *in situ* damage during the first phase of reperfusion. These data are consistent with the concept of reperfusion injury of the intestine which has been a focus of intense research interest during the last two decades [15].

The mechanisms responsible for the increased mucosal injury during the first phase of reperfusion in the present experimental model are less clear. Reperfusion injury is largely ascribed to the cytotoxic effect of reactive oxygen metabolites, especially superoxide produced by xanthine oxidase [6, 16]. A primary and rich source of xanthine oxidase is neutrophilic granulocytes, which adhere to the endothelial surface of postcapillary small venules during and after ischemia, and subsequently emigrates into the tissues [17]. Mast cells may also be involved in this cascade of events, becoming activated and degranulated by superoxide during

TABLE 3

Myeloperoxidase and Mast Cells in Pigs with Cross-Clamping of the Thoracic Aorta

Groups	*n*	Baseline	Cross-clamp (50 min)	After release of the clamp	
				60 min	105 min
Myeloperoxidase (mU/g tissue)					
Aortic crossclamp	8	86 ± 34	130 ± 49	121 ± 35	110 ± 26
Control	5	48 ± 8	63 ± 13	37 ± 9	64 ± 13
Mucosal mast cells					
Aortic cross-clamp	7	6.0 ± 0.9	4.3 ± 1.2	5.9 ± 1.6	6.8 ± 1.0
Control	4	8.7 ± 1.4	6.6 ± 2.1	8.9 ± 2.0	8.5 ± 1.3
Connective tissue mast cells					
Aortic cross-clamp	7	2.8 ± 0.7	2.8 ± 1.0	2.4 ± 0.7	2.3 ± 0.5
Control	4	3.2 ± 0.9	1.5 ± 0.8	1.9 ± 0.5	2.0 ± 0.5

Note. Mean values ± SEM. Units for mast cells are counts/square in a counting lattice. The thoracic aorta was either cross-clamped for 60 min, or exposed but not clamped (control).

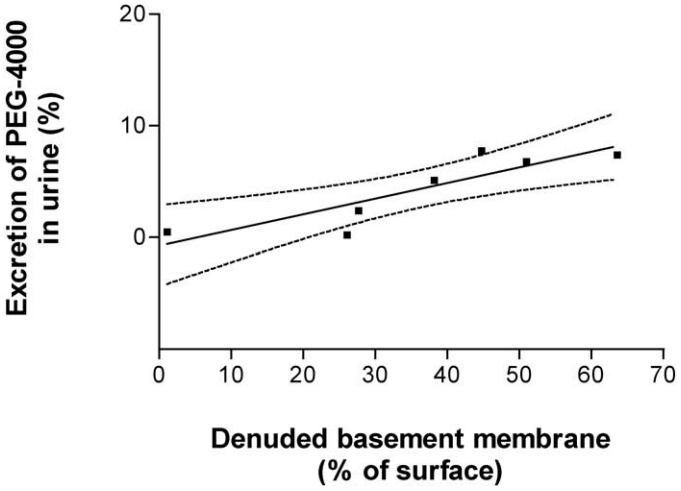

FIG. 6. Regression analysis of intestinal permeability as a function of intestinal surface epithelial injury in pigs after thoracic aortic cross-clamping for 60 min. ^{14}C polyethylene glycol 4000 (PEG-4000) was instilled into an intestinal loop before aortic cross-clamping. Ordinate is the percentage of PEG-4000 excreted in urine during reperfusion for 120 min after declamping of the aorta. Abscissa is the average amount of denuded basement membrane at 60 and 105 min of the 120-min reperfusion period. $r = 0.87$, $P = 0.01$. $y = 0.87x - 0.68$. Dotted lines denote 95% confidence interval.

ischemia and reperfusion. By release of mediators, the mast cells contribute to neutrophilic granulocyte recruitment, endothelial adhesion, and emigration into the tissue [18–20].

In the present study, myeloperoxidase, a marker of tissue neutrophil granulocyte content, did not increase in response to ischemia or reperfusion, indicating a lack of notable neutrophil granulocyte infiltration in the intestinal wall. Second, neither submucosal nor mucosal mast cell density in the intestinal wall appeared to be influenced during the experiment. Still we show that 60 min of reperfusion after ischemia is associated with a substantial increase in mucosal injury. This observation is consistent with the view that xanthine oxidase could be released from resident neutrophilic granulocytes and other sources in sufficient amounts to cause mucosal damage also in the porcine intestine. The lack of increased myeloperoxidase activity in the intestinal wall during a reperfusion period of 2 h after ischemia in the present experiments is in contrast to studies in feline and rat models [17, 20], but is in accordance with data obtained from a porcine model [8]. In the latter study reperfusion after 1 h of ischemia of the ileum was not associated with aggravation of mucosal injury. This observation was ascribed to low levels of xanthine oxidase/xanthine dehydrogenase levels in the porcine small intestine; thus, the importance of reperfusion injury for mucosal damage after ischemia in the porcine intestine was questioned. The reasons for the discrepancy between the study referred to and ours may be several. First, mucosal damage produced by ischemia alone was apparently less pronounced in our study, thereby rendering aggravation during the reperfusion period easier to detect. Second, in the cited study [8], ischemia was produced by clamping vessels close to segments of the intestinal wall, but no attempt was made to measure blood flow during the course of the experiment. In our study, we show that blood flow at the microcirculatory level of the intestine, which is a key factor in reperfusion injury caused by reactive oxygen metabolites, was completely restored as early as 10 min after release of the aortic clamp.

The energy metabolism during ischemia has been widely studied particularly in the heart and has revealed that the recovery rate of the reduced ATP during reperfusion could be used as an indicator of tissue damage and viability [21]. In this study ATP recovered to 85% of the normal level within 60 min after removal of the clamp. The repletion of ATP in our experiment may appear remarkably fast in comparison to that

TABLE 4

High Energy Phosphates

Groups	Baseline	Cross-clamp (50 min)	After release of the clamp	
			60 min	105 min
ATP				
Aortic cross-clamp	13.250 ± 0.851	3.500 ± 0.591*	10.725 ± 0.965*	12.388 ± 1.002
Control	16.120 ± 0.271	15.580 ± 1.153	14.780 ± 0.500*	15.200 ± 0.184*
ADP				
Aortic cross-clamp	2.488 ± 0.185	2.463 ± 0.151	2.238 ± 0.149	2.263 ± 0.113
Control	2.320 ± 0.120	2.380 ± 0.150	2.520 ± 0.092	2.340 ± 0.098
AMP				
Aortic cross-clamp	0.875 ± 0.465	6.888 ± 0.299*	0.888 ± 0.465	0.888 ± 0.491
Control	0.220 ± 0.020	0.280 ± 0.058	0.340 ± 0.051	0.280 ± 0.058

Note. Mean value ± SEM in μmol/mg dry weight. The thoracic aorta was either cross-clamped for 60 min ($n = 8$), or exposed but not clamped (control) ($n = 5$).

*Significant difference from baseline.

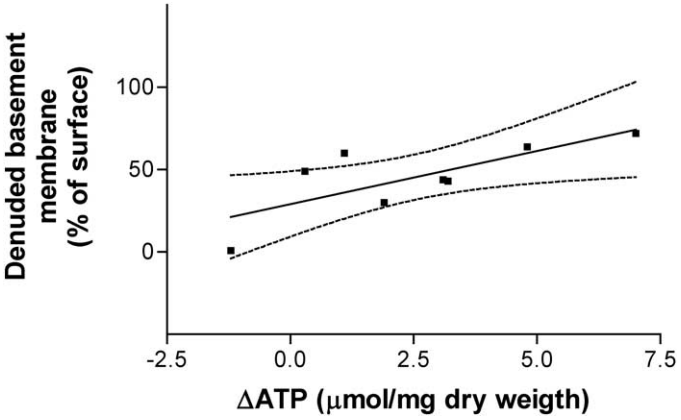

FIG. 7. Regression analysis of intestinal surface epithelial injury as a function of restored intestinal ATP in pigs after thoracic aortic cross-clamping for 60 min. Ordinate is percentage of villi surface having denuded basement membrane at 60 min of reperfusion. Abscissa is the difference between intestinal ATP content at baseline and at 60 min of reperfusion. $r = 0.75$, $P = 0.032$. $y = 0.751x + 0.29$. Dotted lines denote 95% confidence interval.

reported during reperfusion in several rat models after clamping of the superior mesenteric artery [21–23]. In those studies, in which the time frame of ischemia and reperfusion were similar to our model, the reduction of ATP during ischemia was about the same as in our model. In contrast to our results, the repletion of ATP as observed 30 to 120 min after reperfusion was only about 50%.

Preservation of ATP or ability of the cells to restore its ATP level is obviously important for preservation of cell integrity and intestinal barrier function [24–26]. That ATP may play a key role in the ability of the intestinal mucosa to withstand damage caused by ischemia/reperfusion was also supported by the findings in our study. A reasonably strong correlation was found between the extent of mucosal damage and the repletion of tissue ATP during the first phase of reperfusion (Fig. 7). One reason for the fast and complete repletion of intestinal tissue ATP during reperfusion could be related to the complete restoration of intestinal blood flow in our study. In most studies on repletion of ATP after intestinal ischemia, the data about tissue blood flow are not reported. However, in a rat model where the superior mesenteric artery had been occluded for 30 min, the recovery of ATP was much lower than in our model and the intestinal tissue blood flow was only about 50% of preocclusion level [21]. If such discrepancies in restoration of microvascular perfusion after intestinal ischemia reflect species differences, one might speculate that one reason could be more pronounced microvascular injury caused by reactive oxygen metabolites in rats than in pigs. Whatever the reason may be, our findings emphasize the importance of comparison between different species, and the importance of models mimicking clinical situations.

Ischemia/reperfusion injury of the gut invariably leads to loss of intestinal barrier functions. In our study transport of polyethylene glycol with a molecular weight of 4000 Da across the intestinal wall was used as a marker of permeability. It mimics the size of endotoxins and is known not to penetrate the intact intestinal mucosal barrier. PEG-4000, detected both in venous blood and in the urine, was substantially increased after release of the aortic clamp, reflecting a marked increased intestinal permeability. This is a classical response to ischemia/reperfusion injury repeatedly described [27]. However, our study showed in addition a highly significant and close correlation between the area of denuded basement membrane and permeability (Fig. 6).

These data confirm and emphasize the importance of the surface epithelium for maintenance of intestinal barrier functions. Furthermore, they may be of particular relevance in view of other characteristics that were present in our morphological analysis.

A prominent feature in the present study, almost exclusively detected at the end of the experiment, was that irregular cuboidal cells and a few flattened spindle-like cells partly covered the previously denuded villi tips. Additionally, villus height progressively decreased during the reperfusion period. At the light microscopic level these characteristics fulfill the morphological criteria for surface epithelial restitution [28]. Also our ultrastructural analysis showing surface epithelial cells extending lamellipodia over a wavy basal lamina and presence of junctional complexes between cuboidal cells is similar to that reported during restitution after chemical injury of the gastric mucosa [29]. Furthermore, the ultrastructural analysis also showed that such cells contained numerous mucus granula, confirming that they had their origin from surface epithelial cells and not other cell types located deeper in the mucosa.

Restitution is a process that may occur when damaged surface epithelial cells are shed and the viable epithelial cells that remain attached to the mucosa at the margin of the wound become flattened and rapidly migrate over the denuded basal lamina. The superficial epithelium is reestablished when migrating cells touch and form new junctions [30]. Contraction of the subepithelial myofibroblasts is thought to be responsible for the shortening of villi, leading to shrinking of the size of the epithelial defect [31]. Restitution of gastrointestinal surface epithelium as a phenomenon was originally described in the frog gastric mucosa more than 20 years ago [29]. Later it has been studied in a number of species and along the entire gastrointestinal tract and has been shown to start within minutes after injury of the surface epithelium [32–34]. Studies thus far have mainly focused on requirements for, or factors that may facilitate, this process [35], whereas knowledge

about the significance of the early restituted cellular lining in terms of its barrier function is sparse. It may, however, be of interest in context of the present study that the restituted surface epithelium of the feline gastric mucosa conferred tolerance against injury caused by luminal acid challenge and lowered blood flow not different from that of the normal surface epithelium [36].

Surprisingly little attention has been paid to restitution of the surface epithelium in studies on ischemia/reperfusion injury of the gut. Park and Haglund [37] showed that the villi surface of the rat small intestine was covered with a normal epithelium 6 h after 45 min of warm ischemia. This event was not accompanied by increased mitosis as judged by cellular incorporation of thymidine, and they assumed that the repair process had occurred by restitution. Recently, it was reported that goblet cells might be of importance in restitution of the surface epithelium after ischemia/reperfusion of the small bowel in a rat model [38]. Blikslager *et al*. noted a remarkably high degree of mucosal repair at 6 h after ischemia in the porcine ileum *in vivo* [8], and they have also reported on recovery of electrical parameters in the porcine intestine after ischemia by *in vitro* studies [39, 40]. Apart from the studies referred to above, there is a paucity with regard to information about restitution of the surface epithelium of the gut after ischemia/reperfusion injury. However, these data and the data from the present study showing a substantial amount of restituted surface epithelium less than 2 h after intestinal ischemia suggest that this phenomenon should be considered when evaluating injury in ischemia/reperfusion models. They further suggest that strategies for examination of, and influence on, this phenomenon may be important avenues in future studies on recovery of the intestinal mucosa after ischemia/reperfusion injury.

ACKNOWLEDGMENTS

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Paper II

Paper III

Postischaemic restituted intestinal mucosa is more resistant to further ischaemia than normal mucosa in the pig

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Short title: Resistance of restituted intestine

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ABSTRACT

Aim: Ischaemic preconditioning may protect the intestine against subsequent prolonged ischaemia. This study evaluates whether a much longer initial ischaemia time, encountered clinically, may modify the intestinal resistance to further ischaemia in a pig model.

Methods: After cross-clamping of the superior mesenteric artery (SMA) for 1 hour, the intestine was either reperfused for 8 hours, or a second cross-clamping for 1 hour was performed at 4 hours of reperfusion. Based upon microarray analysis of intestinal samples at 1, 4, and 8 hours of reperfusion, mRNA of selected genes was measured with QRT-PCR.

Results: The first ischaemic period caused exfoliation of surface epithelial cells from the basement membrane comprising about 90 % of the villi tips, a marked increase of permeability, and depletion of ATP. The second ischaemic challenge caused about 30 % less denudation of the basement membrane ($p=0.008$), no increase of permeability ($p=0.008$), and less depletion of ATP ($p = 0.039$). mRNA's for superoxide dismuthase 2, heat shock proteins, and signal transducer and activator of transcription 3, which may protect against ischaemia/reperfusion injury, were upregulated throughout the reperfusion period. mRNA's for matrix metalloproteinase 1, connexin 43, and peripheral myelin 22 which may be associated with cell migration or tight junctions showed a particular upregulation at 4 hours of reperfusion.

Conclusion: One hour of initial ischaemia followed by 4 hours of reperfusion is associated with increased intestinal resistance to further ischaemia. Differential regulation of genes identified in this study provide working hypothesis for mechanisms behind this observation.

Key Words: ATP, histology, intestine, ischaemia, microarray, permeability, reperfusion

INTRODUCTION

Brief sublethal periods of ischaemia followed by reperfusion may render a tissue relatively resistant to a subsequent prolonged period of ischaemia and reperfusion. This phenomenon, termed ischaemic preconditioning (IPC), was first described in the heart [1], and has later been shown to be present in a variety of tissues [2], including the small intestine [3-9]. In the intestine IPC is produced either by short repeated cycles of ischaemia/reperfusion or short single periods of ischaemia/reperfusion yielding a few min of total ischemia time. Such studies provide basis for strategies that may be used to improve survival or function of intestinal grafts, or protect the intestine during planned vascular surgical procedures involving temporary intestinal ischaemia. However, in hypovolaemic and septic shock states, during emergency aortic surgery, and a variety of other clinical situations, the intestine may suffer total or near total ischaemia periods which are several times longer than those used to produce IPC. Furthermore, such patients are at risk for new episodes of intestinal ischaemia after initial resuscitation or surgery.

Available studies do not reflect whether a relatively long period of intestinal ischaemia affords protection against structural damage and dysfunction caused by a subsequent ischaemic event or not. This is an important question not least in a clinical perspective.

Restitution of the intestinal surface epithelium is one factor that must be taken into consideration in this context. This term refers to the fact that after exfoliation of intestinal surface epithelial cells caused by various forms of injury, ischaemia included, the wounds at the villi tips are rapidly, within hours, sealed by migration of viable cells that remain attached to the basement membrane at the margins of the wound [10,11].

The objectives of the current study were 1) to evaluate the postischaemic restituted intestinal mucosa in terms of its resistance to structural injury and barrier failure caused by further ischaemia, and 2) to identify, on the mRNA level, changes in expression of genes encoding for proteins that may modify the response of the postischaemic mucosa to further ischaemia.

MATERIALS AND METHODS

The experimental protocol for this study was approved by the Norwegian State Commission for Animal Experimentation.

Twenty male pigs (Norwegian Landrace/Yorkshire) (20-27 kg) were used. The pigs were kept on standard laboratory chow and a 12:12-h light-dark cycle. The animals were deprived of food, but not water, for 24 h before the experiment.

Surgical preparation

Anaesthesia was induced and maintained as previously described in detail [10,12]. The animals were tracheotomized and mechanically ventilated with 40 % oxygen at a rate of 20 breaths/min. Catheters were inserted into the left cardiac ventricle via the right carotid artery for injection of coloured microspheres, and into the abdominal aorta via the right femoral artery for monitoring blood pressure, heart rate, arterial blood gas sampling, and withdrawal of reference samples for tissue blood flow measurements. Another double-lumen catheter was inserted into the inferior caval vein via the right femoral vein for blood tests and infusions. The animals received Ringer's acetate 10-15 mL/kg/h, and a heating blanket and warm fluids were used to maintain normothermia.

A midline laparotomy was performed and a catheter inserted into the urinary bladder. An ultrasonic transit-time flow probe connected to a flow calculator (Transonic Systems Inc., Ithaca, NY, USA) and a vessel loop were placed around the superior mesenteric artery. After completion of surgery the pigs were allowed to stabilize for 60 min before start of the experiment, and at the end of the experiment the animals were killed with an overdose of pentobarbital.

Experimental Protocol

After a baseline period of 45 min, the superior mesenteric artery (SMA) of 7 pigs was cross-clamped for 1 h, then the artery was declamped and a reperfusion period for 8 h followed (single early clamping). The same protocol was followed for a group of 9 pigs except that the SMA was cross-clamped once again for 1 h at 4 hours of reperfusion (double clamping). A third group of 4 pigs (single late clamping) had cross-clamping of the SMA at a time point corresponding to late clamping in the double clamping group (Fig 1). Adjacent jejunal loops were removed at the following time points in the double clamping group: At baseline, at the end of the first and second cross-clamping, during reperfusion at 1 h and 4 h after the first declamping, and 1 h and 3 h after the second declamping of the SMA. Jejunal loops were removed at corresponding time points in the two other groups. From these loops adjoining specimens were taken for morphological analysis, analysis of high energy phosphates, and myeloperoxidase, respectively. In 7 animals with single early clamp of the SMA full-thickness samples of intestine were taken at baseline, 1, 4 and 8 h after declamping, snap frozen and stored on liquid nitrogen for subsequent isolation of total RNA.

Regional Blood Flow

Tissue samples for blood flow measurements were taken from the jejunum and the renal cortex after termination of the experiment. A mucosa specimen of jejunum was obtained by stripping off the muscularis/serosa. Tissue blood flow and cardiac output were determined by means of the distribution of colour labelled microspheres at baseline, 10 min after the first cross-clamping, and 10 min after the first and second declamping [10].

Morphology

Morphology specimens of the intestinal wall used for morphometric analysis of the surface epithelium were fixed in 4% formaldehyde-buffered solution, and further processed and embedded in paraffin. Sections of 5 μm were stained with Hematoxylin-Erythrosin-Safran. An investigator, blinded to the experimental group and the time in the experiment at which the biopsy had been taken, examined the slides. The morphometric analysis was carried out using an eyepiece graticule and a magnification of x100 (i.e., with an ocular of x10 and an objective x10). In each slide, 10 randomly chosen fields were examined. The epithelial cells within each observation grid square (containing approximately 10 epithelial cells) were categorized as mainly containing normal, in situ damage, denuded basement membrane, or simple cuboidal cells as previously described in detail [10] (Figure 1). A total of 100 grid squares were classified in each of the seven bowel segments, and the epithelial subclasses were quantified as percentage of the surface length examined.

Myeloperoxidase Activity

The tissue activity of a marker of neutrophilic granulocytes, myeloperoxidase, was assayed according to the method described by Krawisz et al [13].

Permeability

Intestinal plasma-to-lumen permeability was assessed by measuring the clearance of fluorescein isothiocyanate dextran (FD-4) (Sigma Chemical, St.Louis, MO). A jejunal loop of 20 cm was occluded at each end with ligatures and cannulated at its proximal and distal ends with the line of an infusion set and a Foley catheter, respectively. The exit sites were secured with watertight purse-string sutures. The loop was flushed with

500 mL of Ringer acetate warmed to 39˚C and then continuously perfused with 39˚C Ringer acetate at 60 mL/h. A solution of FD-4 in Ringer acetate was prepared fresh each day at a concentration of 15 mg/mL. A suitable plasma concentration of FD-4 was maintained by infusing a loading dose (10 mg/kg) in an auricular vein over 5 min followed by a continuous infusion (5 mg/kg/h) for the rest of the experiment. Beginning at 60 min before cross-clamping of the SMA, 0.5 mL of arterial blood was sampled every 30 min throughout the experiment. Fluorescence of FD-4 in plasma and intestinal perfusate was measured with a fluorescence spectrophotometer (Fluoroskan II, Labsystem). Flux and clearance of FD-4 were finally calculated [14].

High Energy Phosphates

At different time points, tissue specimens from the small intestine were immediately frozen in liquid nitrogen and thereafter freeze-dried. The specimens were later homogenized and extracts were made prior to the analysis of contents of ATP, ADP, and AMP by a standard high-performance liquid chromatography method as described by Sellevold *et al*. [15].

RNA extraction

Frozen tissue was homogenized using an Ultra-Turrax rotating knife homogenizer, total RNA extracted using Rneasy midi kit (Qiagen Inc., Valencia, CA, USA), and RNA quality evaluated by Bioanalyzer (Agilent Inc. Palo Alto, CA) and NanoDrop (NanoDrop® Technologies Inc. Wilmington, De). High quality RNA (RIN value >7, A260/A280 and A260/A230 ratios ~2) was used for microarray and real time PCR. High quality RNA was obtained for 6 of 7 animals.

Microarray analysis

Two individual samples from each time point (1, 4 and 8 hour reperfusion) were hybridized against RNA from intestinal samples taken immediately prior to ischaemia. Dye-swap hybridizations were done for each time point. Oligonucleotide (Operon Biotechnologies, Cologne, Germany) spotted arrays representing 11,300 tentatively identified porcine genes from The Microarray Core Facility, Texas A&M University. From each sample, 5 µg total RNA was reverse transcribed and labelled with Cy3 or Cy5 attached dendrimers, using the Genisphere 3DNA kit and the manufacturer's two-step protocol (Genisphere, Montvale, NJ, USA). Hybridization was performed on a Tecan HS4800 hybridization station with agitation at 38°C for 15 and 3 hours respectively, and washing at 38°C with 2X SSC and 0.2% SDS (4 min), at 38°C with 2X SSC (4 min), and at room temperature with 0.2X SSC (30 sec). Arrays were scanned with fixed laser power (100%) and PMT gain balancing Cy3 and Cy5 intensities, and images analyzed using GenePix 2.0 (Axon Instruments, Union City, CA, USA).

Raw data were pre-processed by automatic removal of weak spots and intensity based normalization (lowess). The expression of a given gene was identified as up- or downregulated if both arrays from each time point showed a log 2 ratio of ± 0.5 (indicating an approximately 50% up- or down regulation) in the same direction. The number of biological samples analyzed with microarray at each time-point did not allow traditional significance or clustering analysis. Rather, the microarray results served to generate hypotheses on the molecular mechanisms behind the observed ischemic preconditioning. These hypotheses were combined with biological background knowledge to select genes for quantitative real-time RT-PCR in a larger number of samples as described elsewhere. Microarray data were prepared according

to the “minimum information about microarray experiment” recommendations (MIAME), and deposited in ArrayExpress (www.ebi.ac.uk/arrayexpress/), with these accession numbers; A-MEXP-648 (platform) and E-TABM-978 (experiment).

Quantitative Real Time PCR (QRT-PCR)

PCR primers were designed from TC sequences of the DFCI Sus scrofa Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig), the same source used by Operon to design the microarray oligos. Optimization of reaction conditions for each primer pair was done on the SmartCycler System (Cepheid, Sunnyvale, USA) using the iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Reverse transcription was done in 40 µL reactions (2 µg total RNA, 1,8 µL iScript Reverse Transcriptase, 7,1 µL 5x iScript Reaction Mix and nuclease-free water up to a total volume of 40 µL), and after cDNA synthesis the samples were diluted 1:2 with nuclease-free water.

QRT-PCR was performed on a Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA), with per reaction iQ SYBR Green Supermix (12.5 µL), sense primer (2.5 µL, 4µM), antisense primer (2.5 µL, 4 µM), sterile water (5.5 µL) and cDNA template (2 µL). Temperature cycling was once for 2 min at 95°C (activation), then 40 reaction cycles of 20 sec at 95°C, 30 sec at optimized annealing temperature, 40 sec at 72°C and finally 5 min at 72°C (elongation) and melting point determination. Each QRT-PCR run contained 21 samples in duplicates for the target gene, and each sample was normalized to the reference gene β-actin. Quantification in Real-Time PCR was done using the delta-delta method [16], all relative ratios were converted to

Log_2 ratios before comparison to microarray results. All PCR products were verified by sequencing using standard methods.

Statistics

Continuous variables are presented as mean ± 1 SD for descriptive purposes, and as mean with standard error of the mean (SEM) when group differences are of primary interest. Non-parametric procedures with exact implementation in case of ties were employed throughout; i.e. Friedmans test for two-way classification, Wilcoxons' signed rank test for pairwise comparisons, and Kruskal-Wallis' and Mann-Whitney's rank sum test for comparison of independent samples. Morphometric outcome is reported as the mean proportion of epithelium cell categories, with 95 % confidence interval obtained through non-parametric bootstrap using the *boot* package of the software R [17], which also was used for the exact tests. For the purpose of comparison, the amount of damaged cells was combined, i.e. denuded basement membrane and *in situ* damage versus normal and cuboid epithelium. SPSS® version 12 (SPSS Inc., Chicago, IL) was used in the remainder of analyses. The comparisons were planned in advance and limited in number, thus no correction for multiple testing was applied. Reported p values are two-sided, values less than 0.050 are considered to indicate statistical significance.

RESULTS

Central Haemodynamics

Mean arterial blood pressure was 67 ± 10 mmHg at baseline. Cross-clamping of the superior mesenteric artery (SMA) caused about 15 % increase in blood pressure ($p<0.001$). After removal of the clamp, mean arterial blood pressure immediately fell to baseline level, and remained so until the artery was cross-clamped again. Heart rate was 74 ± 13 beats/min at baseline, increased to 104 ± 34 beats/min during clamping ($p<0.001$ to $p=0.041$ for all groups), and returned towards baseline during reperfusion. Blood flow of the SMA was 543 ± 141 mL/min at baseline, decreased to zero during cross-clamping and reached baseline within 15 min after declamping. SMA blood flow thereafter increased about 30 % above baseline ($p=0.001$), lasting for 60 min before it returned to baseline.

Cardiac Output and Regional Tissue Blood Flow

Cardiac output did not change significantly during the experiment (Table 1). Cross-clamping of the SMA caused jejunal mucosal blood flow to fall close to zero. After the first declamping, jejunal mucosal blood flow returned to baseline level, whereas after the second declamping flow was modestly increased compared to baseline ($p= 0.027$). Renal blood flow decreased about 20 % during clamping ($p = 0.011$), but thereafter returned rapidly to baseline, and no difference between the two kidneys was detected.

Morphology

Macroscopic examination of the intestinal mucosa did not reveal visible lesions at any time throughout the experiment. Microscopic examination, however, showed distinct changes confined to the surface epithelium (Fig 2).

Figure 3 shows the distribution of the different categories of the surface epithelium. Intestinal specimens removed before cross-clamping of the SMA had normal surface epithelium. After the basal period, cross-clamping of the SMA for 1 h showed that 90 % (95 % CI: 64-98 %) of the surface area had denuded basement membrane at the villi tips (Fig 3). During the following reperfusion period such denuded areas were gradually covered with cuboidal cells. At 4 hours after removal of the arterial clamp about 80 % of the villi tips were covered with cuboidal cells. Cross-clamping of the SMA for 1 hour once more was associated with 63 % (95 % CI: 42-79 %) denudation of the basement membrane and 10 % (95 % CI: 1-26 %) in situ damage (Fig 3B). This area of mucosal damage (denuded basement membrane and in situ damage) was clearly less both compared to the extent of damage found after the first cross-clamping in the same experimental groups ($p = 0.008$), and compared to animals with single late cross-clamping showing 80 % (95 % CI: 22-99 %) denudation of the basement membrane and 18 % (95 % CI: 0-53 %) in situ damage at the corresponding time point ($p = 0.035$)(Fig 3C). As shown in fig 3A animals with single early clamping of the SMA showed already at 5 hours after declamping almost complete covering of the basement membrane with cuboidal cells, and at 6 and 8 hours about 40 % of the cells had a normal cylindrical shape.

Myeloperoxidase

No significant change in activity of this enzyme was evident during the course of the experiments.

Intestinal Permeability

Using fluorescein isothiocyanate dextran (FD-4) as marker of plasma-to-lumen permeability revealed that cross-clamping of the SMA caused a marked increase of the FD-4 –clearance during the reperfusion period (Fig 4), peaking about 2 hours after removal of the arterial clamp. Then the clearance gradually decreased to a level not different from baseline at the end of the experiment. Interestingly, cross-clamping of the SMA for a second time was without effect on FD-4 clearance. Animals with single late clamping of the SMA showed a similar increase in FD-4 clearance as observed in early clamping.

High Energy Phosphates

Intestinal ischaemia caused by cross-clamping of the superior mesenteric artery was associated with a marked decrease in intestinal ATP content and increase in AMP. ADP remained unchanged (Table 2). After removal of the clamp, at 1 h of reperfusion, the ATP level was about 87 % of baseline level ($p = 0.008$). ATP was completely restored at 4 h of reperfusion. During the second cross-clamping period ATP decreased and AMP increased again, but to levels that were significantly higher than after the first ischaemic insult for ATP ($p = 0.039$), and lower for AMP ($p = 0.027$).

Microarray

Using the data filtering approaches described previously, the microarray analysis identified 184 (101 upregulated, 83 downregulated), 393 (139 upregulated, 254 downregulated) and 280 (130 upregulated, 150 downregulated) differentially expressed genes after 1, 4 and 8 h reperfusion, respectively. Genes were considered particularly interesting if they were regulated similarly at all reperfusion time points or alternatively at both 1 and 4, or both 4 and 8 h reperfusion. From these genes a selection for further analysis was done based upon whether the gene products are known to influence reperfusion injury or intercellular junctions. Using this approach 12 genes were subjected to QRT-PCR, and for this analysis RNA from all available samples at all time points was used (Table 3). A complete list of the microarray data is available in a supplementary table published at www.ebi.ac.uk/arrayexpress/.

DISCUSSION

The present study revealed that; 1) the intestinal mucosa subjected to complete ischaemia for 1 hour, and then allowed to restitute for 4 hours, exhibited a resistance to structural damage caused by further ischaemia that was better than that of the normal mucosa; 2) the restituted mucosa was much more resistant to increased permeability caused by further ischaemia than the normal mucosa; 3) the drop in intestinal ATP content in response to ischemia was less pronounced in the restituted mucosa. Finally, microarray- and QRT-PCR analysis showed differential expression of a number of genes encoding for proteins in the intestinal wall that may be involved in, or explain these observations.

In contrast to many other ischaemia/reperfusion models [18-21], the current study did not reveal any sign of worsened damage of the intestinal surface epithelium or other types of damage (i.e. ulcerations) throughout the reperfusion period. This finding fits with a recent study with this experimental model [12] and with another porcine model [22]. In the latter study it was ascribed to much lower levels of xanthine dehyrogenase/xanthine oxidase in the small intestine of pigs than that found in other animal species (i.e. rats) commonly used in ischaemia/reperfusion studies. The present study points to another possibility, because among the differentially expressed genes superoxide dismuthase (SOD2) was upregulated at all time points throughout the reperfusion period. By its ability to scavenge superoxide, this enzyme is of fundamental importance for protection of tissues against reactive oxygen species [19]. This is consistent with unchanged level of mRNA for vascular adhesion molecule 1 (VCAM-1), lack of increased myeloperoxidase activity, and complete restoration of intestinal mucosal blood flow after declamping of the superior mesenteric artery. In

other models the “no reflow phenomenon” (i.e. inhibition of full return of nutritive perfusion after ischaemia), and increased emigration of neutrophils from the postcapillary venules caused by reactive oxygen species are prominent features [21]. In the present experiments it is possible that properties of the restituted cuboidal surface epithelial cells itself could account for part of the protection after the second ischaemic insult because such cells are to a large part recruited from the proliferative zone at the villi necks, and therefore are younger, less differentiated, and probably more resistant to ischaemia than mature normal surface epithelial cells [23]. Hyperpermeability, as judged by clearance of FD-4, was after the second ischaemic insult more reduced than structural injury as judged by microscopy. There are at least two conceivable and likely explanations for this apparent discrepancy. First, the barrier impeding permeation of this marker molecule consists not only of the intestinal surface epithelium, but also of the endothelium and interstitial tissue. Second, it is likely that function of tight junctions between endothelial cells and between surface epithelial cells still in situ along the sides of and at the bottom of the villi crypts quantitatively is the most important determinant for permeability of the marker molecule from blood to lumen in the present experimental model. Although it may not be without exceptions [24], there are substantial evidence showing that a hydrophilic marker such as FD-4 to a large part is transported via the paracellular route which again is regulated by tight junctions [25-27].

The upregulation of mRNA levels for heat shock proteins 70 and 90 before the second ischaemic insult is of considerable interest. There is good evidence to support a pivotal role of particularly HSP70 and 72 for protection against ischaemia/repefusion injury for example in the heart [28], kidney [29], and the small intestine [30,31].

The signal transducer and activator of transcription 3 (STAT 3) proteins exert protective effect against reperfusion injury in the heart [32], and recently strong evidence suggests that they are crucial for both ischaemic and pharmacological preconditioning in this tissue [33]. In the liver, STAT 3 activation by interleukin 6 is probably essential for potentiation of protective pathways during ischaemia/reperfusion [34]. In the intestine there is only one study showing upregulation of this protein in response to ischaemia/reperfusion [35], but its eventual protective role in this tissue remains unknown.

In view of the strongly attenuated permeability during the second reperfusion period in the present experiments it is intriguing to note that the mRNA level of connexin 43, the essential protein of gap junctions, was robustly upregulated immediately before this time period. Stimulation of connexin 43 after ischaemia/reperfusion injury has been shown to attenuate permeability increase in the gastric mucosa [36], and recently evidence was provided that connexin 43 is closely associated with tight junction proteins of endothelial cells in the porcine brain and lung, and also influenced paracellular flux of permeability markers in such tissues [37]. Another protein with functions related to tight junctions is peripheral myelin protein 22 (PMP-22) for which the mRNA level was upregulated similarly to that of connexin 43. Aside from the peripheral nervous system this protein shows a particularly high expression in the gastrointestinal tract and good evidence suggests it is a component of intercellular junctions in gastrointestinal epithelium [38]. It is also noteworthy that PMP-22 shares homology with claudins which are the essential proteins in tight junctions that play a major role in controlling paracellular solute movement across epithelia [27].

Matrix metalloproteinases (MMPs) play an important role in wound repair for example by removal of devitalized tissue and remodelling of connective tissue [39].

MMP-1 has been shown to be essential for epithelial migration in skin wounds [40], and recently it was shown that MMP-1 is expressed in epithelial cells migrating across superficial wounds of the intestinal mucosa [41]. These data are noteworthy in the context of the present study because restitution of surface epithelial cells (i.e. cell migration) occurred simultaneously with an upregulation of the mRNA levels of MMP-1 and its endogenous inhibitor tissue inhibitor of matrix metalloproteinase 1 (TIMP-1).

The activator protein 1 (AP-1) transcription factor is known to be activated early by ischaemia/reperfusion and to induce proinflammatory gene expression in most tissues including the intestine [42,43]. The strong and early upregulation of c-jun and JunD mRNA, which are part of the AP-1 complex, is consistent with these observations. Furthermore, of all the effects of c-jun and JunD, it is should be noted that strong evidence suggests that these proteins are essential for regulation of MMP-1 and TIMP-1 [44-46].

Downregulation of mRNA for Aldolase B may reflect an adaptive response in the present experiments, because this enzyme catalyze the cleavage of fructose-1,6-biphosphate. Good evidence suggest that this high energy glycolytic intermediate is particularly effective in prevention of ischemia/reperfusion injury [47-49], and may be involved in intestinal preconditioning [50].

A limitation of the present study is that changes on the protein level induced by the differentially expressed genes were not measured. However, it has been shown that for example heat shock protein 70 exhibits a time pattern and expression of proteins that correlate well with mRNA in ischaemia/reperfusion models [31,51,52]. For most of the other differentially regulated genes in the current study this type of information

is not available, at least not in experimental models relevant for the present one, and such studies are warranted.

Nevertheless, the present study is to our knowledge the first to show that an initial period of intestinal ischaemia relevant for many clinical situations can confer protection against a subsequent ischaemic event. Microarray and subsequent QRT-PCR analysis identified changes in the expression of genes encoding for proteins that in some cases have been shown to possess protective properties against ischaemia/reperfusion injury of both the intestine and other tissues. However, several of the proteins have only scarcely, if ever, been reported to be associated with such protection of the intestine. These genes and the time pattern of their regulation may provide working hypotheses that should be tested in order to elucidate mechanisms for the enhanced resistance of postischaemic intestinal mucosa to further ischaemia reported in the present study.

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FIGURE LEGENDS

Figure 1

Experimental design. Clamp means clamping of the superior mesenteric artery for 1 hour.

Figure 2

Micrographs of jejunum mucosa from pigs subjected to cross-clamping of the superior mesenteric artery for 1 hour, thereafter declamping and reperfusion for 4 hours. (A) Section at the end of a 45-min baseline period. An intestinal villus with normal columnar epithelium is apparent. (B) A typical villus after 1 h of arterial cross-clamping showing *in situ* damage of the surface epithelium. Note columnar epithelium with irregular shape and basal vacuolization of the cytoplasm. (C) Typical villus 1 h after declamping. The surface epithelial cells have sloughed off at the tip of the villus, leaving the basement membrane denuded. (D) Typical villus 4 hours after declamping. The cells on the surface are cuboidal and irregularly arranged but form a complete lining, representing restitution of the surface epithelium. The micrographs have equal magnification. Bar represents 100 μm.

Figure 3

Distribution of different categories of surface epithelium of the intestinal mucosa in pigs having the superior mesenteric artery cross-clamped for 1 h, then reperfused for 8 h (Single early clamping, n=7) (A). Double clamping (n=9) (B) means cross-clamping for 1 h, then reperfusion for 4 h, cross-clamping for 1 h once more, and finally reperfusion for 3 h. Single late clamping (n=4) (C) means cross-clamping for 1 h

corresponding to the 2nd cross-clamping in the double-clamping group, and then reperfusion for the remaining 3 h. The categories are normal columnar surface epithelium, columnar epithelium with irregular shape labelled *in situ* damage, denuded basement membrane, and irregularly arranged low cuboidal cells. Intestinal specimens were in the double clamping group removed at baseline, at the end of the first cross-clamping period, during the first reperfusion at 1 and 4 h after release of the clamp, at the end of the second cross-clamping period, and during the second reperfusion at 1 and 3 h after the release of the clamp. In the two other groups intestinal specimens were removed at time points corresponding to double clamping.

Figure 4

FD-4 clearance from blood to the intestinal lumen in pigs with single early clamping (n=7), double clamping (n=8), and single late clamping (n=4) of the SMA. Values are mean ± SEM.

Note: Due to a technical error, data were not obtained from one animal in the double clamping group. CL = clamping. DC = declamping.

Table 1. Cardiac output and effect of double or single cross-clamping of the superior mesenteric artery for 60 min on intestinal mucosal blood flow in pigs.

Groups	Baseline	1. Cross-clamp 10 min	1. Reperfusion 10 min	2. Reperfusion 10 min
Cardiac output				
Single early clamping	6.0 ± 1.7	5.3 ± 1.3	5.7 ± 1.8	6.1 ± 1.6
Double clamping	7.1 ± 2.1	5.7 ± 2.4	6.3 ± 1.9	6.7 ± 3.8
Single late clamping	7.0 ± 1.1	6.9 ± 2.9	6.9 ± 2.8	8.2 ± 2.0
Jejunum, mucosa				
Single early clamping	0.391 ± 0.087	0.031 ± 0.034*	0.480 ± 0.232	0.497 ± 0.256
Double clamping	0.471 ± 0.247	0.016 ± 0.017*	0.597 ± 0.343	0.720 ± 0.282*
Single late clamping	0.874 ± 0.534	0.524 ± 0.192	0.561 ± 0.142	0.931 ± 0.229

Mean values ± SD for cardiac output (L/min) and jejunum mucosa (mL/min/g). The superior mesenteric artery was cross-clamped for 60 min, then reperfused for 240 min, cross-clamped for 60 min once more, and finally reperfused for 180 min (Double clamping, n=9). Single early clamping (n=7) means cross-clamping for 60 min and reperfusion for the remaining part of the experiment. Single late clamping (n=4) means cross-clamping for 60 min corresponding to the 2nd cross-clamping in the double clamping group, and then reperfusion for the remaining 180 min. * denotes significant difference from baseline.

Table 2. High Energy Phosphates

Groups	Baseline	1. Cross-clamp	1. Reperfusion		2. Cross-clamp	2. Reperfusion	
		1 h	1 h	4 h	1 h	1 h	3 h
ATP							
Single early clamping	15.13 ± 1.50	3.27 ± 0.69*	12.84 ± 0.87*	13.74 ± 1.58	13.73 ± 1.03	14.31 ± 1.25	13.46 ± 0.68
Double clamping	14.74 ± 1.11	3.22 ± 0.85*	12.85 ± 0.84*	14.25 ± 1.30	4.85 ± 1.77*†	12.15 ± 1.24	13.37 ± 1.43
Single late clamping	14.97 ± 1.34	15.28 ± 0.54	15.53 ± 0.35	15.18 ± 1.19	3.98 ± 0.77*	13.07 ± 1.34	14.39 ± 1.21
ADP							
Single early clamping	2.82 ± 0.16	3.23 ± 0.20	2.91 ± 0.35	3.29 ± 0.49	2.97 ± 0.27	3.17 ± 0.24	3.30 ± 0.35
Double clamping	2.79 ± 0.28	3.39 ± 0.27	3.00 ± 0.35	3.28 ± 0.29	3.89 ± 0.58	3.39 ± 0.58	3.61 ± 0.34
Single late clamping	2.84 ± 0.58	2.85 ±0.33	3.11 ± 0.29	2.95 ± 0.28	3.65 ± 0.42	2.75 ± 0.34	3.02 ± 0.40
AMP							
Single early clamping	0.5 ± 0.18	6.5 ± 0.74*	0.4 ± 0.13	0.5 ± 0.17	0.4 ± 0.18	0.5 ± 0.15	0.5 ± 0.19
Double clamping	0.4 ± 0.23	6.6 ± 0.75*	0.5 ± 0.18	0.5 ± 0.24	4.9 ± 1.85*†	0.6 ± 0.22	0.6 ± 0.21
Single late clamping	0.6 ± 0.15	0.6 ± 0.16	0.6 ± 0.17	0.5 ± 0.07	5.5 ± 2.02*	0.5 ± 0.09	0.5 ± 0.12

Mean ± SD in µmol/mg dry weight. See notes for table 1. *Significant difference from baseline. †significant difference from 1. cross-clamp.

Table 3. Gene expression in the intestinal wall confirmed by Quantitative Real Time PCR (QRT-PCR) after occlusion of the superior mesenteric artery for 1 hour

Description	Abbreviation	TC numbers	Human homolog	QRT-PCR (Log2 ± SD)		
				1 h	4 h	8 h
C-jun oncogene	CJUN	TC117376	NM_002228	2.00 ± 0.67*	-0.89 ± 0.43*	-1.06 ± 0.38
Jun D proto-oncogene	JUND	TC107819	NM_005354	2.79 ± 0.24*	-0.34 ± 0.64	0.07 ± 0.22
Superoxide dismutase 2	SOD2	TC105618	NM_000636	1.14 ± 0.85*	1.66 ± 0.65*	1.50 ± 0.88
Heat shock protein 70	HSP70	TC105379	NM_005347	3.19 ± 0.51*	1.62 ± 0.87*	0.49 ± 0.37
Heat shock protein 90	HSP90	TC244898	NM_007355	1.89 ± 0.87*	2.08 ± 1.04*	0.54 ± 0.26
Signal transducer and activator of transcription 3	STAT3	TC116801	ENST00000325011	-0.03 ± 0.56	0.86 ± 0.38*	0.70 ± 0.23
Gap junction protein alpha 1 (Connexin 43)	GJA1	TC262243	NM_000165	0.79 ± 0.20*	1.42 ± 0.37*	0.85 ± 0.08
Peripheral myelin protein 22	PMP22	TC106103	NM_153322	0.47 ± 0.27*	1.26 ± 0.50*	0.31 ± 0.60
Matrix metalloproteinase 1 (collagenase 1)	MMP1	TC107850	NM_002421	1.05 ± 0.18*	1.61 ± 0.53*	0.66 ± 0.85
Tissue inhibitor of metalloproteinase 1	TIMP1	TC116432	NM_003254	1.43 ± 0.79*	2.06 ± 0.32*	2.27 ± 0.79
Vascular cell adhesion molecule	VCAM1	TC104764	NM_001078	0.30 ± 0.35	0.23 ± 0.65	-0.22 ± 0.78
Aldolase B	ALDOB	TC265846	NM_000035	-0.66 ± 0.58*	-1.26 ± 1.14*	-1.35 ± 1.24

Listed is the ratio reperfusion/baseline within the same animals at various time points in the reperfusion period. The data are expressed as Log2 ratios relative to baseline. *=$p<0.050$. Note: Because data were available for only 4 animals at 8 hours of reperfusion, statistics were not calculated at this time point.

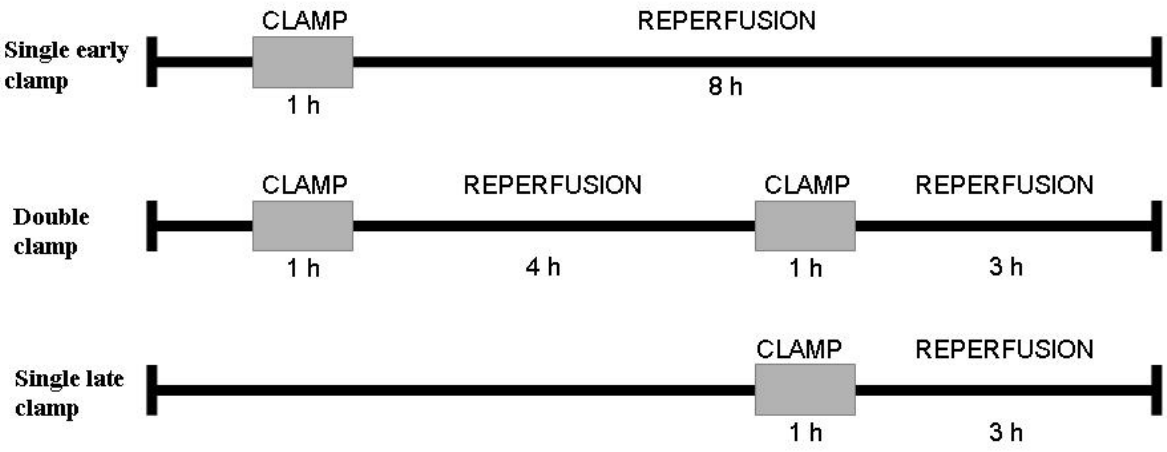
Single early clamp
CLAMP
REPERFUSION
1 h
8 h
Double clamp
CLAMP
REPERFUSION
CLAMP
REPERFUSION
1 h
4 h
1 h
3 h
Single late clamp
CLAMP
REPERFUSION
1 h
3 h

Figure 1

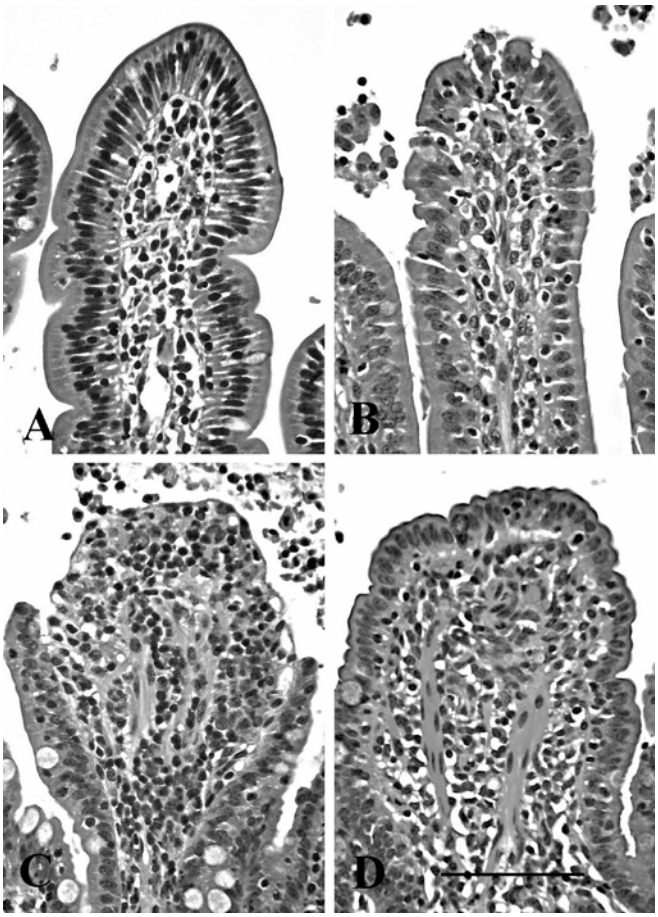

Figure 2

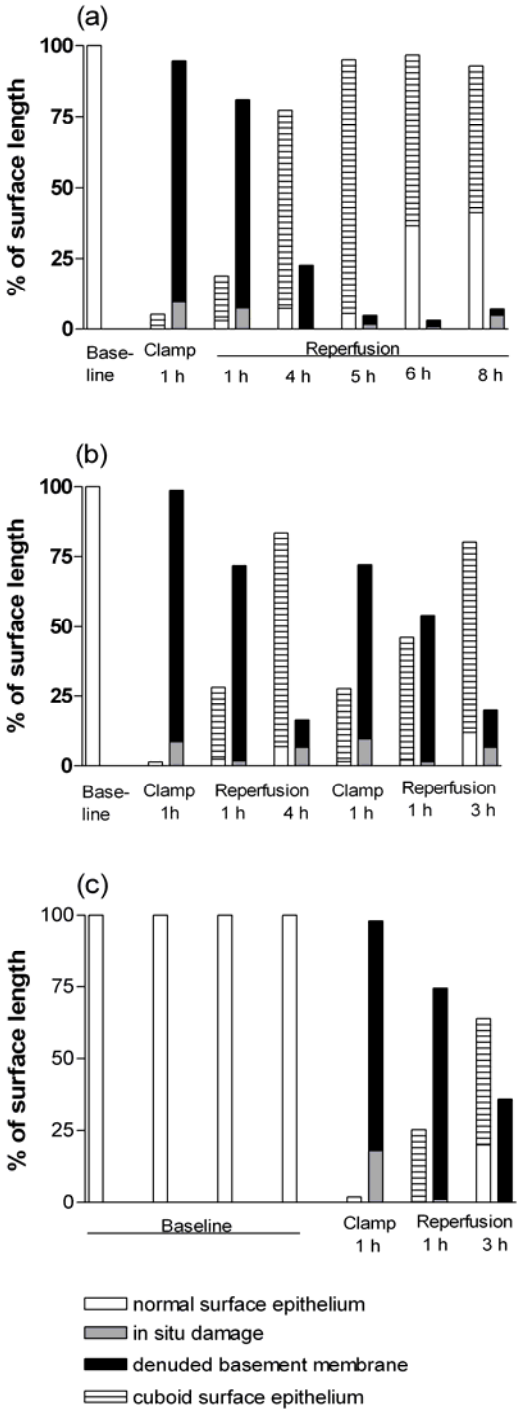
(a)
% of surface length
100
75
50
25
0
Base-line
Clamp 1 h
Reperfusion
1 h
4 h
5 h
6 h
8 h
(b)
% of surface length
100
75
50
25
0
Base-line
Clamp 1h
Reperfusion 1 h
Reperfusion 4 h
Clamp 1 h
Reperfusion 1 h
Reperfusion 3 h
(c)
% of surface length
100
75
50
25
0
Baseline
Clamp 1 h
Reperfusion 1 h
Reperfusion 3 h
normal surface epithelium
in situ damage
denuded basement membrane
cuboid surface epithelium

Figure 3

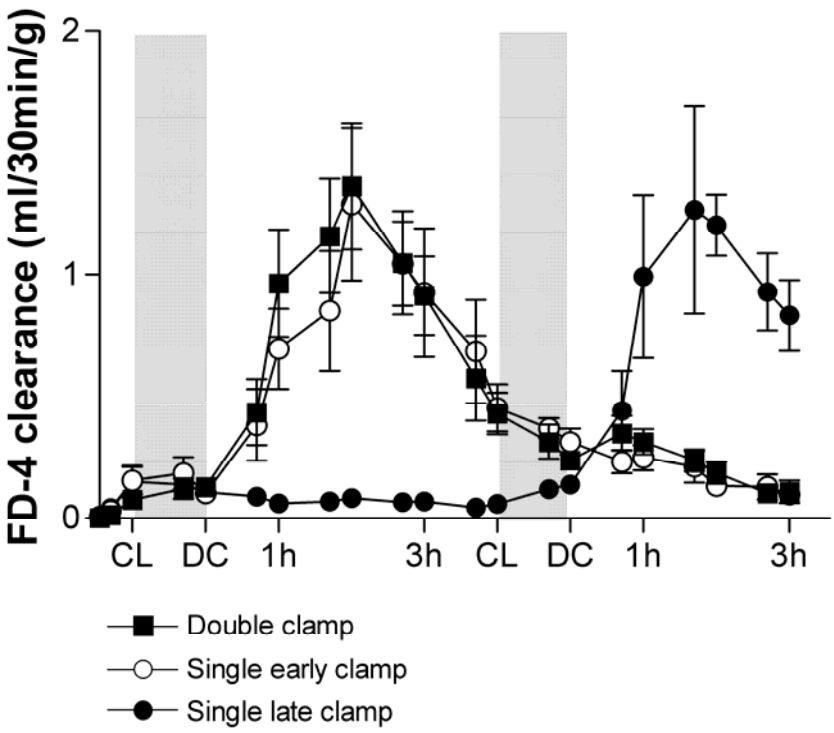
FD-4 clearance (ml/30min/g)
2
1
0
CL
DC
1h
3h
CL
DC
1h
3h
Double clamp
Single early clamp
Single late clamp

Figure 4

Paper IV

Lactate and glycerol released to the intestinal lumen reflect mucosal injury and permeability changes caused by strangulation obstruction

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ABSTRACT

Background: The present study evaluates whether microdialysis of glycerol and lactate reflects mucosal injury and permeability changes after strangulation obstruction of the pig small intestine. **Methods**: Strangulation obstruction was induced by tightening a rubber band around a small bowel loop until its venous pressure increased to a level just below diastolic aortic pressure (partial strangulation), or further until cessation of flow in the main feeding artery (total strangulation). Mucosal injury and permeability of marker molecules from blood to lumen and vice versa was compared to release of glycerol and lactate to the intestinal lumen. **Results**: Mucosal injury, hyperpermeability, and release of glycerol were more pronounced after total than after partial strangulation. In animals with partial strangulation there was a complete restitution of the surface epithelium, and luminal glycerol and lumen-to-blood permeability of polyethylene glycol 4000 remained low. Such animals showed a sustained elevation of lactate and blood-to-lumen permeability of fluorescein isothiocyanate dextran after 2 hours of partial strangulation, but a decline to baseline levels of these parameters in animals with 1 hour partial strangulation. **Conclusion**: Microdialysis of lactate and glycerol in the intestinal lumen may be used to assess structural and functional changes of the intestinal mucosa after strangulation obstruction.

INTRODUCTION

Strangulation obstruction of the small intestine remains to be a serious condition associated with much higher morbidity and mortality rates than that of simple intestinal obstruction [1,2]. Methods with clinical applicability such as laser Doppler flowmetry, pulse oximetry, and recently intraoperative visualization of the intestinal microcirculation with a portable microscope [3-6] have been evaluated experimentally, and may improve the precision of the intraoperative judgement of intestinal viability after release of a strangulation obstruction. However, the evaluation of such patients in the early postoperative period is still a major challenge due to persistent risk for perforation of the intestine, and because such patients are at risk for development of the multiple organ dysfunction syndrome due to intestinal barrier failure [7,8].

Microdialysis of anaerobic metabolites released to the intestinal lumen may potentially be used to assess mucosal dysfunction caused by mucosal ischemia [9-12]. Recently it was shown that microdialysis of glycerol derived from damaged cell membranes and released to the intestinal lumen can distinguish between histologic injury caused by different duration of complete intestinal ischemia in an experimental model [13]. However, this technique has not been evaluated either clinically, or in experimental models on strangulation obstruction of the intestine. The latter may, in addition to venous stasis present with varying degree of reduced or completely inhibited arterial blood supply to the strangulated bowel segment.

Many experimental models on strangulation obstruction are based upon ligation of the veins either alone or combined with varying degree of impaired arterial inflow to the intestinal segment under study [14-17]. Although such models may provide

information particularly on morphologic injury and other events during the strangulation period itself, their suitability for study of the reperfusion period after release of the strangulation is obviously limited. This concern was met in a model focusing on compensatory circulatory mechanisms in the intestinal wall during strangulation obstruction recently reported by Fevang et al [18-20]. In the present study, focusing on the reperfusion period after release of strangulation obstruction, a modification of this model was used.

The objective of the current study was 1) to assess morphologic injury of the intestinal mucosa and associated permeability changes after strangulation obstruction and the ensuing reperfusion period, and 2) to evaluate whether release of lactate and glycerol from the intestinal wall as measured with the microdialysis technique reflect such changes.

MATERIALS AND METHODS

The experimental protocol for this study was approved by the Norwegian State Commission for Animal Experimentation.

Surgical preparation

Thirty juvenile male pigs (Norwegian Landrace/Yorkshire) (22-42 kg) were used. The pigs were kept on standard laboratory chow and a 12:12 light-dark cycle. The animals were deprived of food, but not water, for 24 h before the experiment. Anaesthesia was induced and maintained as previously described in detail [13,21]. The animals were tracheotomized and mechanically ventilated with 40 % oxygen at a rate of 20 breaths/min. Catheters were inserted into the left cardiac ventricle via the right carotid artery for injection of coloured microspheres, and into the abdominal aorta via the right femoral artery for monitoring blood pressure, heart rate, arterial blood gas sampling, and withdrawal of reference samples for tissue blood flow measurements. Another double-lumen catheter was inserted into the inferior caval vein via the right femoral vein for blood tests and infusions. The animals received Ringer's acetate 10-15 ml/kg/h, and a heating blanket and warm fluids were used to maintain normothermia.

A midline laparotomy was performed. A Foley catheter (Bard Ltd., West Sussex, UK) was placed in the urinary bladder, secured with a purse-string suture, for monitoring urine output and for urine samples. Another rubber Foley catheter (Ch 22) was placed, without traction, around a segment (about 3m) of the distal ileum including the

mesentery, artery, vein, and bowel (Fig 1). A cannula (Hydrocath arterial catheter, 20 G, 6 cm, Becton Dickinson Critical Care Systems Pte Ltd, Singapore) was inserted into the main vein of the bowel loop for continuous recording of venous pressure. The main artery supplying the closed intestinal loop was dissected free and a Doppler ultrasound probe (I.D. 5 mm) was placed around the vessel. Strangulation of this bowel loop was performed by tightening the rubber catheter around the bowel and its mesentery. Within the closed bowel loop two separate ileal loops of about 30 cm and 20 cm were isolated for assessment of permeability across the intestinal wall, and in addition a microdialysis catheter was inserted into the intestinal lumen for measurements of lactate and glycerol. A loop of the middle part of the small intestine (about 3m) was used for control within the same animal.

After completion of surgery, the laparotomy was closed with towel clamps. The pigs were allowed to stabilize for 60 min before the start of the experiment. At the end of the experiment the animals were killed with an overdose of pentobarbital.

Experimental Protocol

The experimental set-up is outlined in fig 1. After a baseline period of 45 min, strangulation obstruction was induced by gradually tightening the rubber Foley catheter around the bowel loop until venous pressure in the loop was between 40 and 45 mmHg. This pressure level was selected because it is just below the diastolic pressure and therefore is associated with stable pulsative flow in the main supplying artery to the bowel loop. In 9 animals strangulation with this venous pressure was maintained for 1 hour and in 8 animals for 2 hours (partial strangulation). In a third group of 8 animals this venous pressure was maintained for 5 min, but thereafter

further strangulated until complete cessation of arterial blood flow for 2 hours (total strangulation). In all of these experiments the strangulation period was followed by 4 hours of reperfusion. Five animals served as control experiments.

Blood flow

Blood flow of the main mesenteric artery was measured with an ultrasonic transit-time flow probe connected to a flow calculator (Transonic Systems Inc., Ithaca, NY, USA). Tissue blood flow and cardiac output were determined by means of the distribution of colour labelled microspheres according to procedures described in recent studies from our laboratory [21].

Morphology

Specimens for microscopic evaluation were taken at the end of the baseline period, strangulation period, and the 4 hour reperfusion period. In addition specimens were also taken from an adjacent non-strangulated bowel loop in the same animals and in the five control animals at corresponding time points. This yielded 150 slides that were coded and examined in random order at the end of all experiments. Morphology specimens of the intestinal wall used for morphometric analysis of the surface epithelium were fixed in 4% formaldehyde-buffered solution, and further processed and embedded in paraffin. Sections of 5 μm were stained with Hematoxylin-Erythrosin-Safran. An investigator, blinded to the experimental group and the time in the experiment at which the biopsy had been taken, examined the slides. The morphometric analysis was carried out using an eyepiece graticule and a

magnification of x100 (i.e., with an ocular of x10 and an objective x10). In each slide, 10 randomly chosen fields were examined. The epithelial cells within each observation grid square (containing approximately 10 epithelial cells) were classified into four categories as previously described in detail from our laboratory [21]. The first category consisted of the normal columnar epithelium. The second category comprised a columnar epithelium with irregular shape and basal vacuolization of the cytoplasm. Such epithelium was often separated from the basement membrane by a narrow space. This space was predominantly at the tip of the villi, but could also extent toward the crypts. This epithelium was in the present study classified as *in situ* damage. The third category was denuded areas where the surface epithelium had been sloughed off the basement membrane at the villi tips (*denuded basement membrane*). The fourth category comprised a monolayer of irregularly arranged cuboidal cells attached to the basement membrane along the sides of and at the tip of the villi (*cuboidal epithelium*). A total of 100 grid squares were classified in each of the five bowel segments obtained from each animal. The result was summarized as the percentage of the surface length occupied by the epithelial subclasses. In addition, in the 10 randomly chosen fields of each slide, the amount of blood in the lamina propria outside the blood vessels was judged as 0 (nothing), 1+ (some) or 2+ (substantial).

Permeability

Lumen to blood permeability was determined as previously described [21,22]. Briefly, a jejunal loop of 30 cm was ligated at both ends, and 100 ml (10.0 µCi) ^{14}C polyethylene glycol (PEG-4000) (Amersham Bioscience, Buckinghamshire, UK), was injected into the lumen. Venous blood samples for determination of the concentration

of PEG-4000 in plasma were taken at 30 min intervals. Urinary excretion of PEG-4000 was expressed as % of PEG-4000 instilled into the intestinal loop.

Blood to lumen permeability was determined as previously described [23]. Briefly, a jejunal loop of 20 cm was ligated at both ends and cannulated at its proximal and distal ends with the line of an infusion set and a Foley catheter, respectively.

The loop was continuously perfused with 39˚C Ringer acetate at 60 ml/h. Intestinal plasma-to-lumen permeability was assessed by measuring the clearance of fluorescein isothiocyanate dextran (FD-4) (Sigma Chemical, St.Louis, MO). A suitable plasma concentration of FD-4 was maintained by infusing a loading dose (10 mg/kg) in an auricular vein over 5 min followed by a continuous infusion (5 mg/kg/h) for the rest of the experiment. 0.5 ml of arterial blood and intestinal perfusate were sampled every 30 min throughout the experiment. Fluorescence of FD-4 in plasma and intestinal perfusate was measured with a fluorescence spectrophotometer (Fluoroskan II,Labsystem). Flux and clearance of FD-4 were finally calculated.

Microdialysis

Microdialysis catheters (CMA 70, membrane length 20 mm, 20 kD, CMA Microdialysis AB, Stockholm, Sweden) were introduced into the lumen of the ileal loop and into the subclavian artery as earlier reported from our laboratory [11]. The microdialysis catheters were perfused at a flow rate of 1 µL/min with an isotonic perfusion fluid (CMA Perfusion Fluid T1 and microdialysis pump CMA 107). The catheters were perfused *in situ* for ≥ 75 min before baseline measurements. Samples were collected over 30 min and were analyzed immediately on site for glycerol and lactate by enzymatic fluorometric assays (CMA 600 microdialysis analyzer) using

peroxidise methodology. *In vitro* recovery at a flow rate of 1 μL/min has been tested for three microdialysis catheters in 1.1 mM, 5.5 mM, and 10.3 mM concentrations of sodium lactate in Ringer's acetate (64 [7] %), and 100 μM, 500 μM, and 1000 μM concentrations of glycerol in Ringer's acetate (37 [4] %) [13].

Statistics

Continuous variables are presented as mean ± 1 SD in text and table for descriptive purposes, and as mean with standard error of the mean (SEM) in the figures where group differences are of primary interest. Due to unequal variance of the observed variables at different time points, non-parametric procedures were employed throughout; i.e. Friedmans test for two-way classification, Wilcoxons' signed rank test for pairwise comparisons, Kruskal-Wallis and Mann-Whitney's rank sum test for independent samples. Morphometric outcome is reported as the mean proportion of epithelium cell categories, with 95 % confidence interval obtained through non-parametric bootstrap using the *boot* package of the software R [24], which also was used for the exact tests. In the analysis the proportion of damaged cells, i.e. denuded basement membrane and *in situ* damage, were combined. SPSS® version 12 (SPSS Inc., Chicago, IL) was used in the remainder of analyses. The comparisons were planned in advance and limited in number, thus no correction for multiple testing was applied. Reported p values are two-sided, values less than 0.050 are considered to indicate statistical significance.

RESULTS

Hemodynamics

For central hemodynamics, see Table 1. Cardiac output averaged 6819 ± 2448 ml/min at baseline, there were no difference between the experimental groups and cardiac output did not change during the course of the experiments in any of the groups.

Blood flow

Strangulation of the bowel loop until stable mesenteric venous pressure between 40 and 45 mmHg was associated with a decrease in mesenteric artery blood flow to about 20 % of baseline level (Fig 2). After release of the strangulation mesenteric artery blood flow rapidly returned to and remained at the baseline level in animals with 1 hour partial strangulation, whereas blood flow after 2 hour partial strangulation did not completely return to baseline level (p at least = 0.031). In contrast, animals with total strangulation of the bowel showed a temporary, but marked increase in mesenteric artery blood flow at 1 hour in the reperfusion period (p = 0.023) (Fig 2). Tissue blood flow as measured with microspheres in whole wall samples of bowel loops showed very similar response to partial strangulation as that measured with ultrasound transit time flowmetry in the mesenteric artery (Table 2). However, mucosal tissue blood flow in such animals was reduced to only 35 ± 23 % as compared to 16 ± 20 % of baseline level in the serosa/muscularis of the intestinal wall (p = 0.002). During the reperfusion period blood flow in the whole wall and in the mucosa rapidly returned to and remained at baseline level both in animals subjected to

partial and total strangulation of the bowel. The higher mucosal blood flow at the end of the experiments in animals with total strangulation compared to the other experimental groups ($p = 0.043$) is consistent with a similar difference at baseline ($p = 0.010$) (Table 2). Blood flow in the serosa/muscularis showed essentially the same pattern as in the mucosa during the reperfusion period. However, blood flow in this layer did not completely reach baseline level in animals with 1 hour partial strangulation either at 10 min ($p = 0.027$) or 4 hours ($p = 0.020$) reperfusion. Intestinal tissue blood flow did not show significant changes throughout the experiment in control animals.

Renal blood flow averaged 2.24 ± 0.77 ml/min/g at baseline, with no difference between the groups, between the right and left kidneys, or throughout the course of the experiments.

Morphology

Strangulation of the bowel loop was associated with formation of oedema and a change to a bluish colour of varying intensity. However, it was not possible to distinguish partial from total strangulation by its macroscopic appearance. Fig 3 shows the distribution of the different categories of the surface epithelium at the end of the strangulation period and the 4 hours reperfusion period. Partial strangulation for both 1 and 2 hours caused denudation of the basement membrane at the villi tips comprising about 25 % of the surface area. At the end of the ensuing 4 hours reperfusion period the denuded basement membrane in animals with partial strangulation was almost completely covered with cells that either had a normal

columnar or a cuboidal shape. Total strangulation for 2 hours caused 89 % denudation of the basement membrane at the villi tips and 7 % in situ damage. This amount of mucosal damage was much more pronounced than in animals with both 1 and 2 hours partial strangulation ($p = 0.003$ and $p = 0.011$, respectively). At the end of the reperfusion period, more than half of the formerly denuded basement membrane had been covered with cuboidal cells (Fig 3). The remaining surface area with denuded basement membrane was clearly less than at the end of the strangulation period ($p = 0.031$). Structural damage apart from that of the surface epithelium was not detected. However, some intestinal bleeding in the lamina propria was present after strangulation (Table 3), but there was no significant difference between any of the experimental groups.

Permeability

Blood to lumen: Using FD-4 as marker of permeability revealed that total strangulation of the bowel loop caused a marked and sustained increase of the FD-4 clearance throughout the reperfusion period ($p = 0.008$) (Fig 4). Also partial strangulation for 1 and 2 hours was associated with increase in FD-4 clearance early in the reperfusion period ($p = 0.008$ for both groups). Permeability for this marker molecule later fell ($p = 0.016$) to baseline level in animals with 1 hour partial strangulation. Such a decrease was not evident in pigs subjected to 2 hours partial strangulation, in which the permeability was higher than found in control experiments at the end of the experiment ($p = 0.004$).

Lumen to blood: Similar to the findings for blood-to lumen permeability, the venous concentration of PEG-4000 increased markedly after release of total strangulation (p

= 0.008) (Fig 5A), and was still higher than baseline level at the end of the experiment (P = 0.008). In animals with partial strangulation there were also a slight increase of venous PEG-4000, however, the concentrations were not different from that found in control animals at any time point throughout the course of the experiment. The pattern of urinary excretion of PEG-4000 was very similar to that found for venous concentrations although the maximal increase occurred about 1 hour later than in venous blood (Fig 5 B).

Microdialysis of lactate and glycerol

Baseline concentrations of intestinal luminal lactate and glycerol in pigs in the present and other studies from our laboratory are 0.11 (range 0 -0.89) mmol/L and 16.2 (range 0-122) µmol/L, respectively (n = 82). Microdialysis data were excluded for further analysis due to obviously wrong "baseline" concentrations for lactate (26 mmol/L) and glycerol (350 µmol/L) in one animal experiment (partial strangulation for 2 hours). In addition microdialysate was not obtained from the luminal catheter in one animal experiment (partial strangulation for 1 hour) due to a non-functioning microdialysis pump.

The luminal concentration of lactate increased rapidly and had reached a plateau at 1 hour after both 1 hour (p = 0.008) and 2 hours partial (p = 0.031) as well as total strangulation of the bowel loop (p = 0.008) (Fig 6 A). During the reperfusion period, lactate fell (p = 0.016) to baseline level in animals with 1 hour partial strangulation, whereas it remained high in the groups with 2 hours partial and total strangulation; clearly different from that found in the control group (p = 0.010, p = 0.002, respectively). In contrast to lactate, the luminal glycerol concentration was only

modestly higher than control at 1 hour after partial strangulation ($p = 0.030$ and $p = 0.050$, respectively) (Fig 6 B). After total strangulation, however, there was a marked elevation of glycerol ($p = 0.008$) which was sustained throughout the reperfusion period.

In arterial blood the concentration of lactate apparently increased slightly in response to both partial and total strangulation for 2 hours, but the difference compared to baseline was not significant. Lactate later decreased towards the end of the experiment in all groups subjected to strangulation of the bowel, whereas it remained unchanged in control animals (Table 4). Arterial glycerol increased modestly in response to both 1 hour ($p = 0.008$) and 2 hours ($p = 0.016$) partial strangulation, but was not different from baseline throughout the reperfusion period. No significant changes in arterial glycerol were noted in the control and total strangulation groups (Table 4).

DISCUSSION

The present study revealed that microdialysis of lactate and glycerol, using catheters placed in the intestinal lumen, to a large extent mirrored changes in permeability and structural integrity of the intestinal mucosa induced experimentally by varying intensity and duration of small bowel strangulation.

Strangulation obstruction is associated with increased pressure in the mesenteric venous bed of the strangulated bowel. However, as shown in our experiments, when the strangulation is severe enough to cause cessation of the arterial blood supply, the venous pressure falls (Fig 2), which most likely is due to rapid extravasation of fluid from the venous system to the interstitial tissue and peritoneal cavity. Apart from extravasation of blood to the lamina propria, microscopic examination revealed a pattern of intestinal injury in this model that is much alike that found after impeded arterial blood supply to the intestine, without venous obstruction [13, 21, 25]. As shown previously and confirmed in this study, the surface epithelium of the villi tips was particularly susceptible to hypoxia and ischemia [13,21,25]. The ensuing repair of the mucosa after release of strangulation was also similar to that reported previously [13,21] with replacement of denuded areas of the basement membrane with cuboidal surface epithelium. This type of repair termed restitution, originally described in the frog gastric mucosa, has been shown to occur by migration of viable cells attached to the basement membrane at the margins of the wounds of the villi tips [21,26].

Reduction of arterial blood flow close to 80 % in bowel loops with partial strangulation was associated with a relatively limited damage of the mucosa. This finding may at least partly be explained by the redistribution of blood flow towards the mucosa noted in the present experiments and in similar experiments reported by

Fevang et al [19]. These observations most likely reflect autoregulatory mechanisms reported to be present both in the cat small intestine and in the dog colon [27,28], but it is noteworthy that they are intact also during strangulation obstruction and may be important for tissue protection in this situation.

Because both marker molecules for permeability used in the present study are hydrophilic and have a relatively high molecular weight it is likely that they are transported via the paracellular pathway [29,30], although we cannot completely exclude that dextrans, like FD-4, can traverse the intestinal epithelium, in part, via a transcellular process [31].

Permeability for the macromolecule FD-4 from blood to lumen in the initial period of reperfusion reflected fairly well the extent of mucosal injury caused by partial and total strangulation of the bowel. However, at the end of the experiment clearance of FD-4 still remained high and unchanged both in animals with total and partial strangulation for 2 hours. Both of these experimental groups showed at this time a substantial repair, the formerly denuded basement membrane in animals with partial strangulation was even completely covered with surface epithelium. The latter observation is compatible with reports suggesting that epithelial restitution per se and alone is not sufficient for recovery of barrier functions such as normalized permeability [32]. There is substantial evidence to suggest that formation of tight junctions between the restituted cells or repair of these paracellular bridges in areas with surface epithelial cells still in situ is the major determinant for normalization of barrier functions [33,34]. Incomplete repair of tight junctions could therefore account for the sustained elevation of FD-4 clearance also at the end of the reperfusion period when the surface area was almost completely covered with cuboidal or cylindrical epithelium.

The mucosal injury inflicted by partial strangulation was apparently not sufficient to cause increased permeability for PEG-4000. Although this probe had a similar molecular weight as FD-4, there is substantial evidence showing that permeability across the intestinal mucosa also depends on factors such as molecular size, configuration and hydrophilic properties [35]. In addition it is also likely that permeation of marker molecules is higher in the blood to lumen direction than in the opposite direction. Strong support for this view comes from in vitro studies on both the small and large bowel showing a much higher permeation of nearly all commonly used marker molecules in the serosa to mucosa direction than in the mucosa to serosa direction [36,37].

Although measurement of biomarkers with microdialysis catheters in the peritoneal cavity is feasible and has been shown to reflect splanchnic ischemia [38,39], we recently found that release of glycerol and lactate to the gut lumen after ischemia and during the reperfusion period correlated much better to mucosal dysfunction and histologic injury of the mucosa [13]. During surgery for intestinal strangulation obstruction, the intestinal lumen is accessible for placement of microdialysis catheters with techniques similar to those used for jejunal feeding catheters.

Glycerol released from the phospholipid membrane of the villous epithelial cells after cellular damage can be monitored with the microdialysis technique, both in the intestinal wall and lumen using experimental models with total intestinal ischemia [12,13,40].

However, the current study extends these reports by showing that release of glycerol from the mucosal side can distinguish moderate tissue injury associated with low mucosal blood flow and limited change in permeability from total ischemia with extensive tissue injury and a large change in permeability. This observation is of

particular importance in a clinical setting. For example, it may be difficult or even impossible to judge intraoperatively to what extent a strangulated intestinal loop has suffered total or near total ischemia or whether some arterial blood supply is present. Measurement of lactate in the intestinal lumen with microdialysis could not, as shown in figure 6, distinguish the hypoxic state of the mucosa in experiments with partial strangulation from the anoxic state in animals with total strangulation. This finding is consistent with another study showing a threshold for increase of intestinal luminal lactate at about 30 % reduction of baseline flow in the superior mesenteric artery and a marked increase at 15-20 % reduction [10], which is comparable to that in the present experiments with partial strangulation obstruction. However, the decline of lactate during the reperfusion period in animals with 1 hour - but not in animals with 2 hours partial strangulation most likely reflects less pronounced dysfunction of the intestinal mucosa in such experiments. This interpretation is supported by the more sustained permeability of FD-4 in animals with 2 hours partial strangulation. A pertinent question is whether glycerol and lactate released to the intestinal lumen could have their origin from other sites than the intestinal wall. We cannot exclude this possibility completely, particularly for lactate. However, there was no correlation between mean arterial and luminal concentrations either for lactate or glycerol (Table 4 and Fig 6).

In conclusion, the present study suggests that the microdialysis technique can be used to assess functional and structural changes of the mucosa after strangulation obstruction. A decline in luminal lactate as an indicator of normalized anaerobic metabolism in the mucosa after treatment for strangulation obstruction may indicate recovery of barrier functions, whereas persistent elevation of luminal glycerol may be a warning sign.

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FIGURE LEGENDS

Figure 1. Schematic drawing of the experimental set-up in pigs with strangulation of the small bowel.

Figure 2. Pressure in the main drainage vein and flow in the main feeding artery (measured with ultrasonic transit-time flowmetry) in a bowel loop of pigs with partial strangulation for 1 hour (n = 9) and 2 hours (n = 8), and total strangulation for 2 hours (n = 8) or control (n = 5). After release of strangulation a 4 hours period of reperfusion followed. Values are mean ± SEM.

Figure 3. Distribution of different categories of surface epithelium of the intestinal mucosa in pigs subjected to strangulation of small bowel. The categories are 1) normal columnar epithelium, 2) columnar epithelium with irregular shape, basal vacuolization of the cytoplasm, and often separated from the basement membrane by a narrow space, labelled *in situ* damage, 3) denuded basement membrane at the villi tips, and 4) irregularly arranged low cuboidal cells attached to the basement membrane at the upper parts and tips of the villi. Intestinal specimens were removed at the end of the strangulation period and at the end of the 4 hours reperfusion period. (A) Partial strangulation for 1 hour. (B) Partial strangulation for 2 hours. (C) Total strangulation for 2 hours. At baseline and in the control group there were no sign of mucosal injury as judged by microscopy (data not shown). Mean values. The bars represent 95 % confidence intervals.

Figure 4. FD-4 clearance from blood to the intestinal lumen in pigs with strangulation of small bowel. Values are mean ± SEM, Bas = baseline. S = strangulation.

Figure 5. (A) Concentration of PEG-4000 in venous blood, and (B) PEG-4000 in urine (expressed as % of the ^{14}C-PEG-4000 instilled into the ileum) as indexes of permeability from the intestinal lumen to blood in pigs with strangulation of small bowel. Values are mean ± SEM. Bas = baseline. S = strangulation.

Figure 6. Gut luminal lactate and glycerol detected by microdialysis in pigs with strangulation of small bowel. Values are mean ± SEM. Bas = baseline.

Table 1. Hemodynamic variables during strangulation and reperfusion of the small intestine in pigs

Groups	Baseline	Strangulation		Reperfusion			Friedman's test
		10 min	100 min	10 min	65 min	240 min	
Mean aortic pressure (mmHg)							
Partial strangulation, 1 h	75 ± 14	67 ± 13*	-	69 ± 16	69 ± 15	66 ± 9*	P = 0.003
Partial strangulation, 2 h	71 ± 11	62 ± 7*	61 ± 5*	66 ± 10	68 ± 9	67 ± 11	P = 0.001
Total strangulation, 2 h	66 ± 15	58 ± 7	75 ± 13*	71 ± 14	67 ± 10	65 ± 16	P < 0.001
Control	64 ± 8	65 ± 10	-	70 ± 8	70 ± 8	71 ± 20	P = 0.011
Heart rate (beats/min)							
Partial strangulation, 1 h	71 ± 26	73 ± 31	-	87 ± 47	92 ± 55	96 ± 49	P = 0.357
Partial strangulation, 2 h	86 ± 30	95 ± 34*	148 ± 67*	138 ± 64*	133 ± 63*	106 ± 25	P < 0.001
Total strangulation, 2 h	77 ± 15	83 ± 19	89 ± 17*	90 ± 15	96 ± 20*	126 ± 58*	P = 0.010
Control	58 ± 15	58 ± 16	-	55 ± 14	55 ± 14	60 ± 17	P = 0.462

Mean values ± SD. A loop of the ileum (3 m) was partially strangulated for 1 hour (n=9), 2 hours (n=8), or totally strangulated for 2 hours (n=8). Thereafter a reperfusion period of 4 hours followed. A group of five animals without strangulation served as controls. * denotes significant difference from baseline (p< 0.05).

Table 2 Intestinal tissue blood flow in pigs with strangulation of the small bowel

Groups	Baseline	Strangulation	Reperfusion	
			10 min	4 hours
Whole wall				
Partial strangulation, 1 h	0.48 ± 0.20	0.09 ± 0.11	0.34 ± 0.12	0.31 ± 0.12
Partial strangulation, 2 h	0.41 ± 0.17	0.08 ± 0.05	0.36 ± 0.17	0.30 ± 0.10
Total strangulation, 2 h	0.50 ± 0.19	0.01 ± 0.01	0.47 ± 0.34	0.53 ± 0.20
Control	0.43 ± 0.12	0.40 ± 0.14	0.42 ± 0.15	0.36 ± 0.14
Mucosa				
Partial strangulation, 1 h	0.27 ± 0.11	0.06 ± 0.04	0.26 ± 0.17	0.27 ± 0.14
Partial strangulation, 2 h	0.25 ± 0.06	0.10 ± 0.05	0.26 ± 0.11	0.28 ± 0.11
Total strangulation, 2 h	0.46 ± 0.12#	0.01 ± 0.01	0.25 ± 0.27	0.55 ± 0.22#
Control	0.26 ± 0.05	0.34 ± 0.09	0.28 ± 0.10	0.33 ± 0.10
Serosa/muscularis				
Partial strangulation, 1 h	0.97 ± 0.37	0.08 ± 0.08	0.68 ± 0.35*	0.52 ± 0.22*
Partial strangulation, 2 h	0.65 ± 0.39	0.09 ± 0.07	0.67 ± 0.42	0.52 ± 0.29
Total strangulation, 2 h	0.70 ± 0.46	0.01 ± 0.02	0.96 ± 0.74	0.67 ± 0.32
Control	0.66 ± 0.27	0.38 ± 0.16	0.63 ± 0.38	0.49 ± 0.19

Mean values ± SD in ml/min/g. See notes for table 1. * denotes significant difference from baseline. # denotes significant difference from all other groups.

Table 3

Bleeding score in the lamina propria in pigs with strangulation of the small bowel

	Strangulation	Reperfusion, 4 hours
Partial strangulation, 1 h	0.9 ± 0.9	0.6 ± 0.9
Partial strangulation, 2 h	1.1 ± 0.9	0.8 ± 0.9
Total strangulation, 2 h	1.8 ± 0.5	0.6 ± 0.7*
Control	0.0 ± 0.0	0.0 ± 0.0

Mean values ± SD. Bleeding was scored as 0, 1 (some), and 2 (substantial). * denotes significant difference from baseline.

Table 4. Arterial concentrations of lactate and glycerol during strangulation and reperfusion of the small intestine in pigs

Groups	Baseline	Strangulation	Reperfusion			Friedman's test
			1 h	2 h	4 h	
Lactate, mmol/L						
Partial strangulation, 1h	0.8 ± 0.3	0.9 ± 0.6	0.9 ± 0.6	0.7 ± 0.2	0.5 ± 0.2	p = 0.012
Partial strangulation, 2 h	1.5 ± 0.8	2.0 ± 1.3	2.3 ± 1.5	1.2 ±0.8	0.6 ± 0.3	p = 0.001
Total strangulation, 2 h	1.1 ± 0.4	2.1 ± 1.6	1.1 ± 0.4	0.7 ± 0.3	0.6 ± 0.3	p = 0.002
Control	1.3 ± 0.5	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	p = 0.104
Glycerol, µmol/L						
Partial strangulation, 1h	20 ± 15	33 ± 20	30 ± 21	20 ± 14	24 ± 18	p = 0.027
Partial strangulation, 2 h	49 ± 86	73 ± 104	92 ± 103	45 ± 63	26 ± 25	p = 0.011
Total strangulation, 2 h	25 ± 45	48 ± 48	19 ± 22	18 ± 18	27 ± 25	p = 0.187
Control	38 ± 22	27 ± 7	21 ± 10	15 ± 3	21 ± 8	p = 0.058

Mean values ± SD. A loop of the ileum (3 m) was partially strangulated for 1 hour (n = 9), 2 hours (n = 8), or totally strangulated for 2

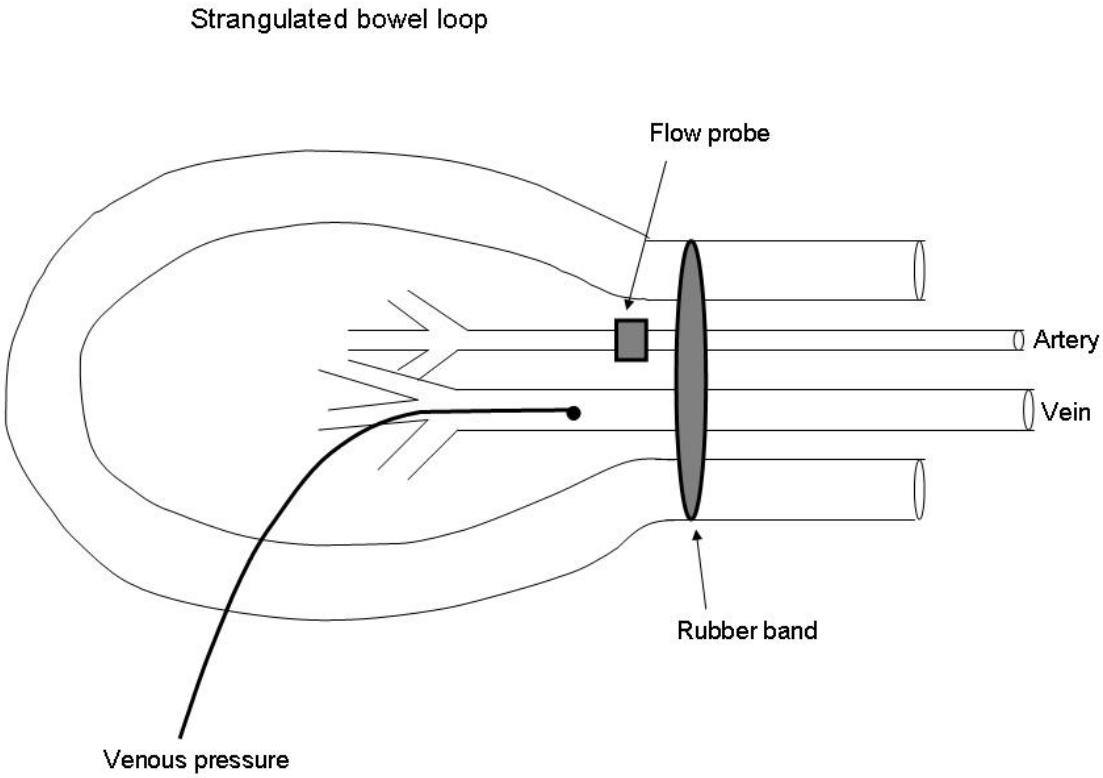
Strangulated bowel loop
Flow probe
Artery
Vein
Rubber band
Venous pressure

Figure 1

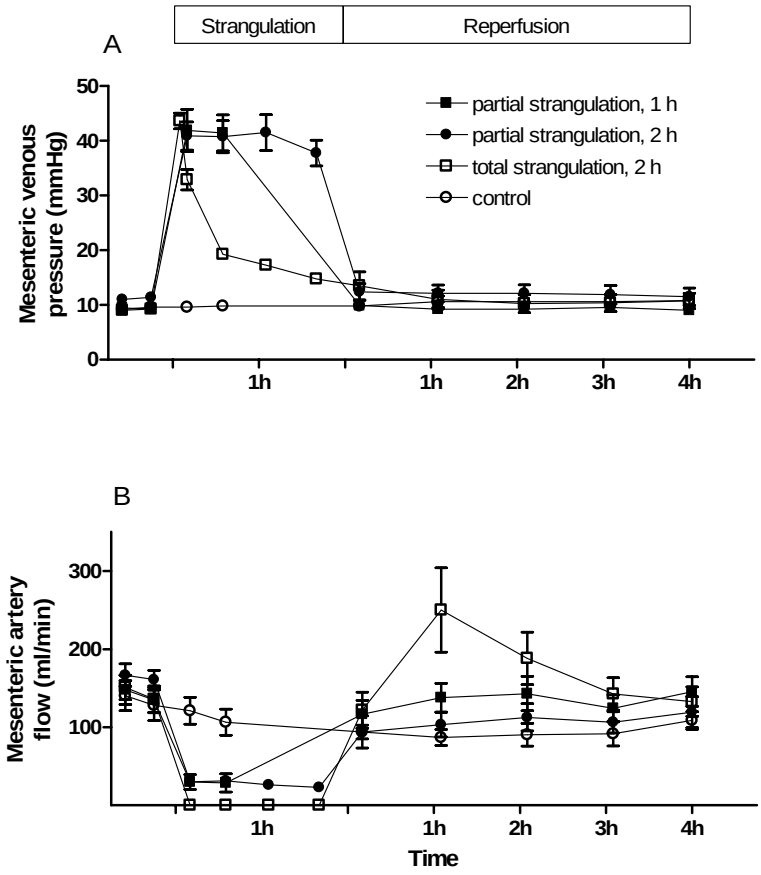
Strangulation
Reperfusion
A
Mesenteric venous pressure (mmHg)
50
40
30
20
10
0
partial strangulation, 1 h
partial strangulation, 2 h
total strangulation, 2 h
control
1h
1h
2h
3h
4h
B
Mesenteric artery flow (ml/min)
300
200
100
1h
1h
2h
3h
4h
Time

Figure 2

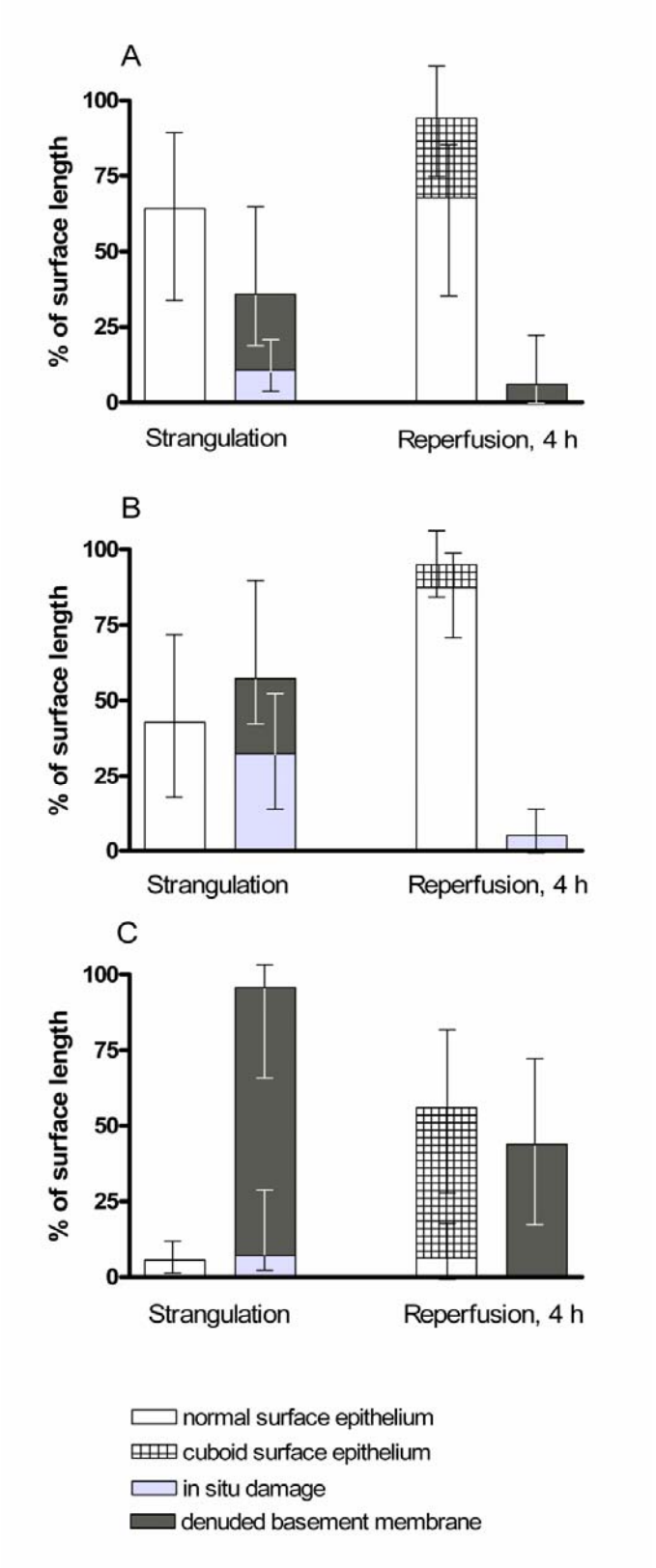
A
100
75
50
25
0
% of surface length
Strangulation
Reperfusion, 4 h
B
100
75
50
25
0
% of surface length
Strangulation
Reperfusion, 4 h
C
100
75
50
25
0
% of surface length
Strangulation
Reperfusion, 4 h
normal surface epithelium
cuboid surface epithelium
in situ damage
denuded basement membrane

Figure 3

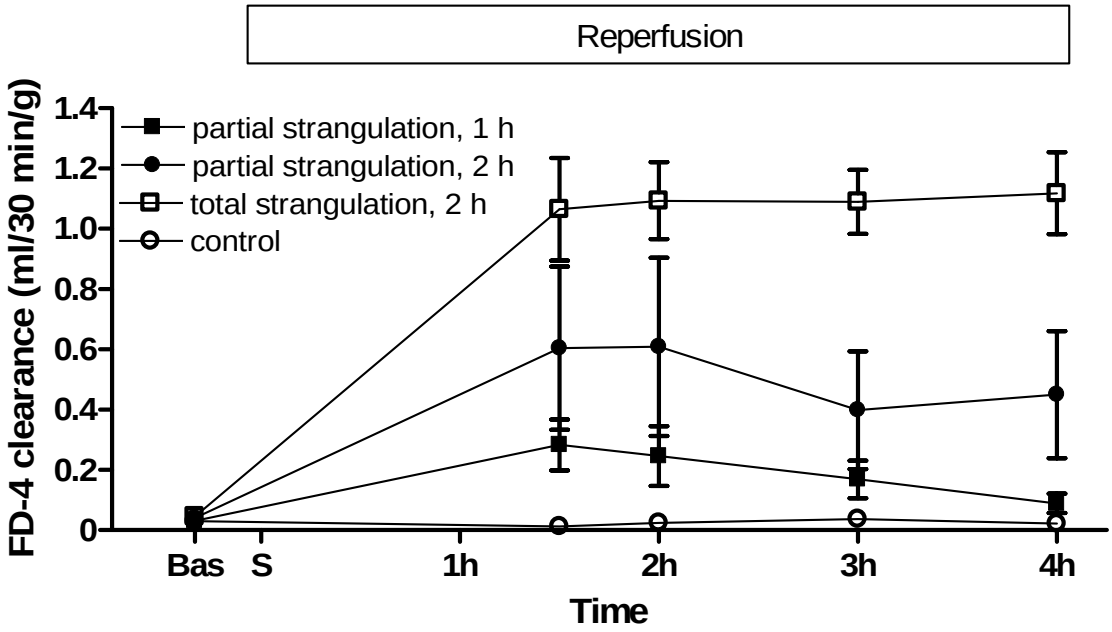
Reperfusion
FD-4 clearance (ml/30 min/g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
partial strangulation, 1 h
partial strangulation, 2 h
total strangulation, 2 h
control
Bas
S
1h
2h
3h
4h
Time

Figure 4

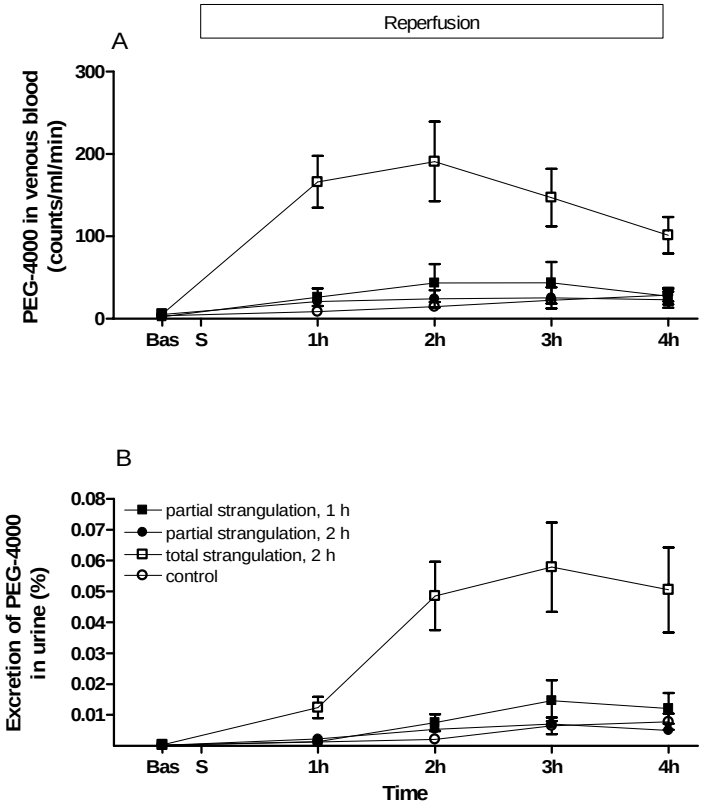
Reperfusion
A
PEG-4000 in venous blood (counts/ml/min)
300
200
100
0
Bas
S
1h
2h
3h
4h
B
Excretion of PEG-4000 in urine (%)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
partial strangulation, 1 h
partial strangulation, 2 h
total strangulation, 2 h
control
Time

Figure 5

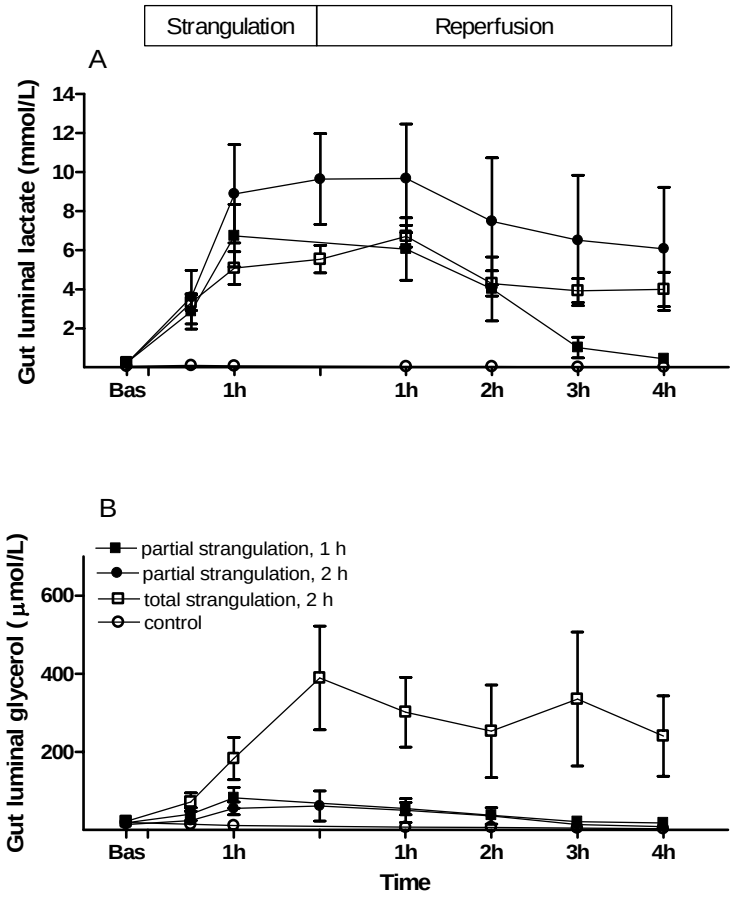
Strangulation
Reperfusion
A
Gut luminal lactate (mmol/L)
14
12
10
8
6
4
2
Bas
1h
1h
2h
3h
4h
B
partial strangulation, 1 h
partial strangulation, 2 h
total strangulation, 2 h
control
Gut luminal glycerol (µmol/L)
600
400
200
Bas
1h
1h
2h
3h
4h
Time

Figure 6

Dissertations at the Faculty of Medicine, NTNU

1977
1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978
3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979
5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980
6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981
8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983
9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984
11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AN STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985
18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986
24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987
27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988
30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.

33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.

68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.

168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

2001

178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

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