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Sequence of morphological alterations in a small intestinal ischaemia/reperfusion model of the anesthetized rat. A light microscopy study

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Summary. Previous studies have shown serious mucosal damage and destruction to be associated with intestinal ischaemia/reperfusion. As both destruction and concomitant regeneration can be observed together in this potentially lethal condition we have studied the development and sequence of events by evaluating morphological changes of the small intestine in an ischaemia/reperfusion model in anaesthetized rats. Forty-five minutes of ischaemia was followed by 4 hours of reperfusion. Tissue samples of the small intestine were examined by light microscopy in normal and semithin sections. Samples were collected at the end of ischaemia, at 10 min, and at 1, 2, 3 and 4 hours of reperfusion, respectively. Survival was assessed in a parallel group of anaesthetized rats. The morphological changes were described and they were analysed by a semi-quantitative method using five different markers of histological alteration. The mortality rate of a control survival group was 100%. Mucosal destruction at the end of ischaemia and during reperfusion was diffuse and steadily increased as a function of reperfusion time. At the same time the epithelium showed intensive regenerative growth which covered the denuded mucosal surface by the third hour of reperfusion. A secondary epithelial desquamation followed this process and was accompanied by heavy inflammatory cell infiltration. The infiltration may be the cause of the secondary epithelial injury.

Keywords: mucosal necrosis, epithelial regeneration, circulatory shock, secondary damage to mucosa

Intestinal ischaemia (occlusion of the superior mesenteric artery: SMA) is a serious hazard to life. Circulatory shock develops even faster if the ischaemic period, when long enough, is followed by reperfusion (Hamar *et al.* 1987). Consequences of the disease and also the major pathological changes in the damaged gut have been investigated intensively (Deer & Noer 1949; Andersson *et al.* 1984; Parks & Granger 1986; Megison *et al.* 1990). Most experimental studies that describe the morphological changes of the damaged gut in shock have been carried out in dogs or cats.

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Relatively few studies have focused their attentions on the same changes in the rat. Two major theories have been developed to explain the deterioration of the intestinal mucosa. One of them is the serious lack of oxygen (Lundgren & Haglund 1978) and the other is the accumulation and formation of free radicals that attack the different cellular structures making the damage worse (Granger *et al.* 1981).

The mucosal epithelium of the gut shows a remarkable rate of turnover in physiological conditions (LeBlond & Stevens 1948; Bertalauffy & Nagy 1958; McMinn & Mitchel 1954). Rapid regeneration of the epithelial layer has also been reported following intestinal hypoxia and/or ischaemia (Wagner 1979; Menge & Robinson 1979; Juhász *et al.* 1984). Wagner (1979) and Glotzer *et al.* (1962) followed the regeneration in an ischaemia/reperfusion model for several days and focused their attention on the later developmental phases. They avoided the development of circulatory shock in their studies by analysing the effect of ischaemia/reperfusion of only a short segment of the small bowel. Park and co-workers (1990) on the other hand concentrated on the early destruction of the mucosa in reperfusion (the first hour only). Because circulatory shock as a consequence of SMA occlusion/reperfusion develops in the early hours after revascularization, we wanted to study the morphological changes of the intestinal mucosa during the first few hours following reperfusion. In the present series of experiments an irreversible shock model (45 min of ischaemia followed by 4 hours of reperfusion) was used and the pathological changes of the total mucosa (epithelium and also lamina propria) were analysed in rats.

Materials and methods

Forty-eight Wistar rats (males and females, 250–300 g bw) were used for the studies. The animals had free access to food and water before the experiments. They were anaesthetized by pentobarbital-Na (35 mg/kg). A PE 90 cannula was introduced into the external jugular vein for saline administration. The SMA was exposed through a midline laparotomy and it was occluded by an atraumatic clip at its root. Haematocrit (Htc) values were determined with blood collected by tail incisions every hour and saline was given through the jugular vein (5 ml each time) when the Htc value exceeded 50. After 45 min of occlusion the clip was removed and the supply area of the SMA was allowed to be reperfused. The abdomen was closed by a suture. The temperature of the animals was maintained at 37°C by a heating pad.

The rats were divided into two groups. One group served as a control from which no tissue samples were collected; only the survival times of these animals were noted ($n=24$). The experimental counterparts were divided into six subgroups (four animals in each). The different subgroups were killed at various timepoints: at the end of ischaemia (Group I), within 5–10 min (Group II), and at 1 (Group III), 2 (Group IV), 3 (Group V) and 4 h (Group VI) of reperfusion, respectively.

The entire small intestine was removed and cut into three segments (proximal, middle, and distal) of equal lengths. A 1-cm-long piece from the middle of each segment was dissected, rinsed in ice cold saline, and fixed in 4% buffered formaldehyde. From the mid part of each piece, several small tissue samples were collected. These samples were postfixed in glutaraldehyde and osmium tetroxide and embedded in Epon for semithin sectioning. The semithin sections were stained with Giemsa. The remaining pieces were paraffin embedded, conventionally sectioned, and stained with haematoxylin-eosin (H & E). The H & E and the semithin sections were examined together by light microscopy. The paraffin embedded preparations served to diagnose the pathology of the alterations while the semithin sections revealed cellular changes in more detail.

The extent of the following histological changes was noted. (1) Epithelial desquamation, that is, the extent of denudation of the

mucosal surface. (2) Extent of villous destruction. (3) Presence of inflammatory cells (granulocytes, lymphocytes, mast cells and macrophages). (4) Cell necrosis in the lamina propria. (5) Regenerative overgrowth of the epithelial cells. Each of these histological changes was scored 0 when there was a normal tissue structure, 1 when it was already present but not dominant, and 2 if it was a dominant feature. From each experiment three measuring points were gathered (proximal, middle and distal points of the gut), on each of four rats, so that at each timepoint there was a total of 12 individual values (scores) for every histological parameter. Mucosal injury was uniform in each animal group. There were no qualitative differences among the different parts of the gut (proximal, middle and distal sections) in their histological appearance. However, big differences were found between the different times of sampling. As a summary, a semi-quantitative value of the reperfusion injury as a function of time was calculated as the sum of the five parameters.

For statistical analysis mean values ± s.e.m. were calculated. A one-way analysis of variance (ANOVA) was used to detect changes within one histological parameter and the *t*-test was used to show significant differences between values of two timepoints within a group.

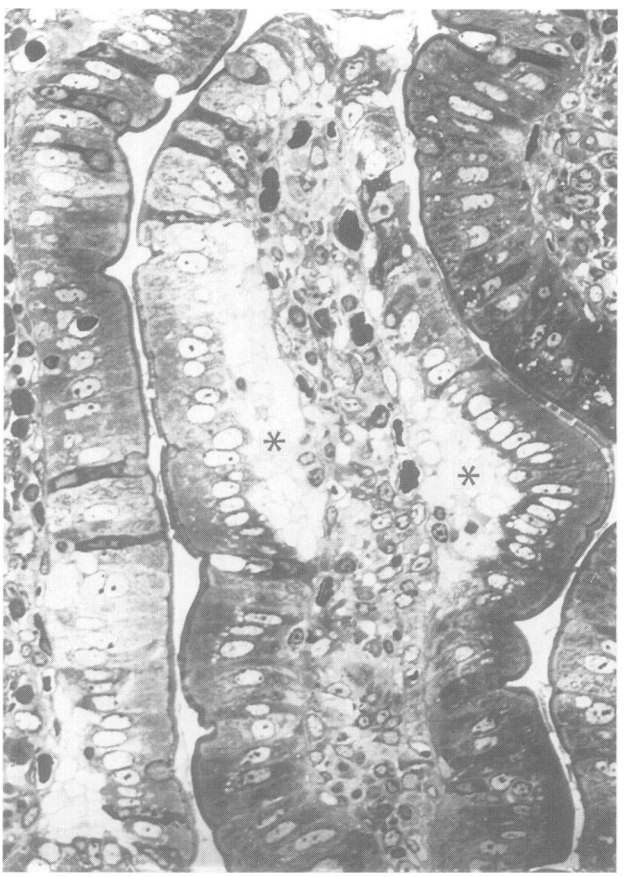

Fig. 1. Intestinal villous at the end of ischaemia. Subepithelial oedema is marked by asterisks. Semithin section. Giemsa ×400.

Results

Mortality of the control group (24 rats) was 100%. All animals died within 5 hours after the release of the occluded SMA. Circulatory shock generally developed during the second to third post-release hour. At autopsy we found the characteristic macroscopic appearance of the gut: enlarged bowel loops filled with sanguineous fluid containing desquamated mucosal debris. The wall was bluish and demonstrated haemorrhages in several segments.

Histology

Small intestinal pieces were dissected and examined macroscopically. The mucosal surface was diffusely hyperaemic in Group I, had haemorrhagic infiltration in Groups II and III, and the segments were darkly (blue-reddish) coloured in Groups IV–VI. This colour was uneven in its distribution.

The following diffuse and progressive alterations in the histology of the small bowel were found.

Group I (45 min of ischaemia). Subepithelial blebs (Fig. 1), slight detachment of the epithelium from the villi (mainly from their tips) and randomly arranged necrotic epithelial islets were demonstrated.

Group II (5–10 min reperfusion). More epithelial lifting and necrosis was observed and dilated capillaries filled with red cells were present. A few granulocytes in the

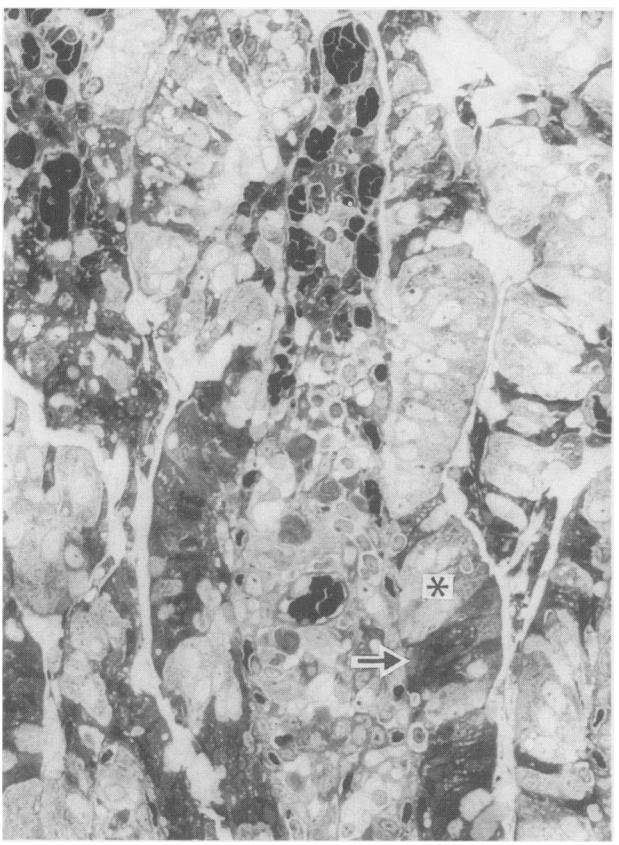

Fig. 2. Intestinal villous, 5–10 min reperfusion. Necrotic (arrow) and viable (asterisk) epithelial cells. Semithin section. Giemsa ×400.

villous lamina propria, and subepithelial oedema was seen (Figs 2 and 3). There was an increased number of epithelial cell mitoses and irregular proliferation of epithelial cells in the crypt region (the process demonstrated in Fig. 4 had already started at this earlier time) (Figs 2 and 3).

Group III (1 h reperfusion). Most villi were denuded and partly destroyed. The epithelial cells (with basophilic staining, and decreased cytoplasm to nucleus ratio) in the crypts and on the villous margins showed irregular growth patterns and there was increasing infiltration of the lamina propria by the granulocytes (Fig. 4).

Group IV (2 hours of reperfusion). Shorter villi with irregular shapes and with denuded tips and intensively regrowing epithelial cells on the villous sites were observed.

Group V (3 hours of reperfusion). There was an intensive regenerative overgrowth of the epithelium which almost totally covered the mucosal surface. At the same time macrophages, granulocytes, necrotic cells, cellular debris and haemorrhages were found in the lamina propria (Figs 5 and 6).

Group VI (4 hours of reperfusion). Intensive subepithelial bleb formation with detachments of irregularly regrown and partly necrotic epithelium of the villi were found. In the crypt layer there was also epithelial necrosis. Necrotic cells, oedema and disintegration of the lamina propria were seen (Figs 7 and 8).

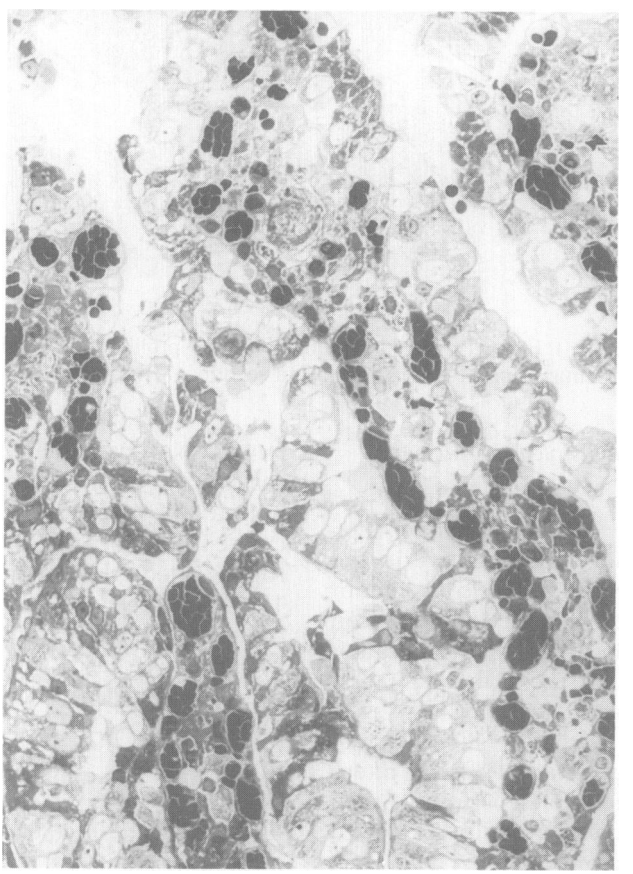

Fig. 3. Intestinal villous. 5–10 min reperfusion. Epithelial desquamation at the tip and lifting on the side. Oedema and hyperaemia are apparent in the lamina propria. Semithin section. Giemsa ×400.

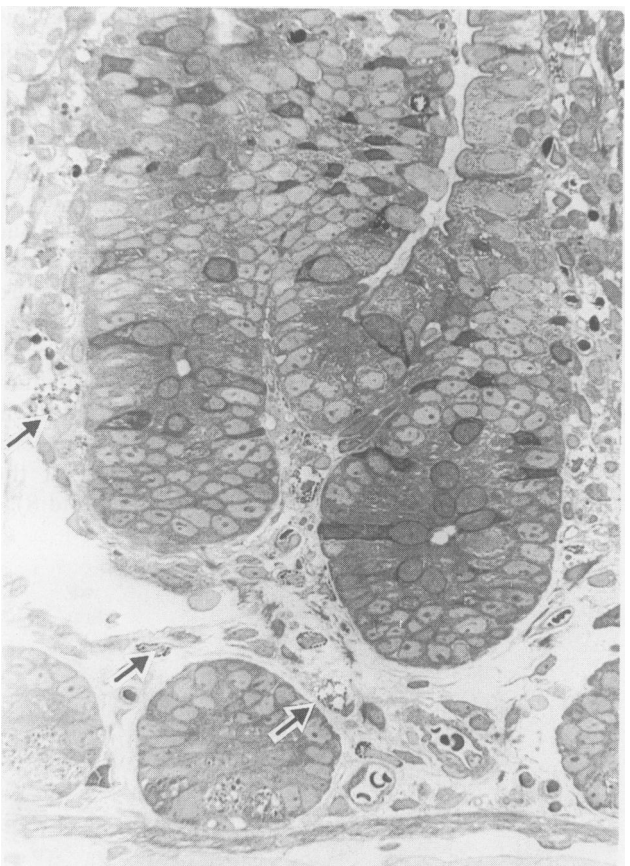

Fig. 4. Intestinal crypts, 1 h reperfusion. Irregular epithelial proliferation in the crypt region, and granulocytes in the lamina propria (arrows). Semithin section. Giemsa × 200.

Quantification of the histological changes (Table 1)

Extent of mucosal surface denudation. Epithelial desquamation was intensive in the first hour of reperfusion. The concomitant increase in the rate of cell multiplication resulted in a significant re-epithelialization by the third hour. A second desquamation took place in the last hour.

Extent of villous destruction. This was intensive in the first hour of reperfusion, then stagnated for 2 hours and was then followed by a second deterioration of the villi.

Inflammatory cell infiltration of the lamina propria. No accumulation of inflammatory cells of any type occurred during the first few minutes of reperfusion. A progressive increase in number of granulocytes and macrophages took place in the following 2 hours. There appeared to be a further increase in the number of inflammatory cells in the last 2 hours of reperfusion but the scoring system was not sufficiently sensitive to quantify these changes.

Cell necrosis and destruction of the lamina propria. Reperfusion elicited a progressive destruction of the lamina propria with necrosis of the different cell components.

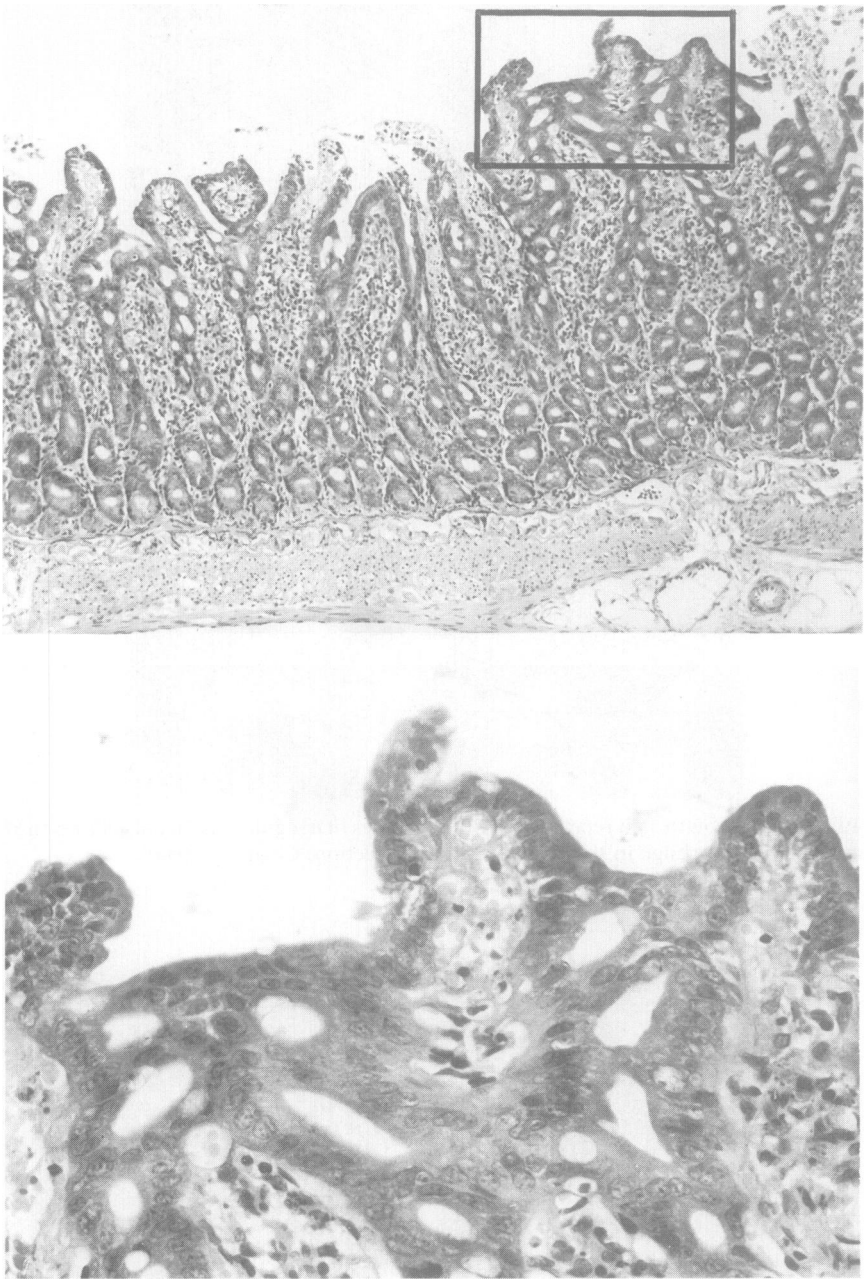

Fig. 5. Intestinal wall, 3 h reperfusion. a, Mucosal surface is almost covered by regenerating epithelium. H & E × 100. b, Magnified area of a, undifferentiated epithelium and infiltration of the lamina propria by inflammatory cells. H & E × 300.

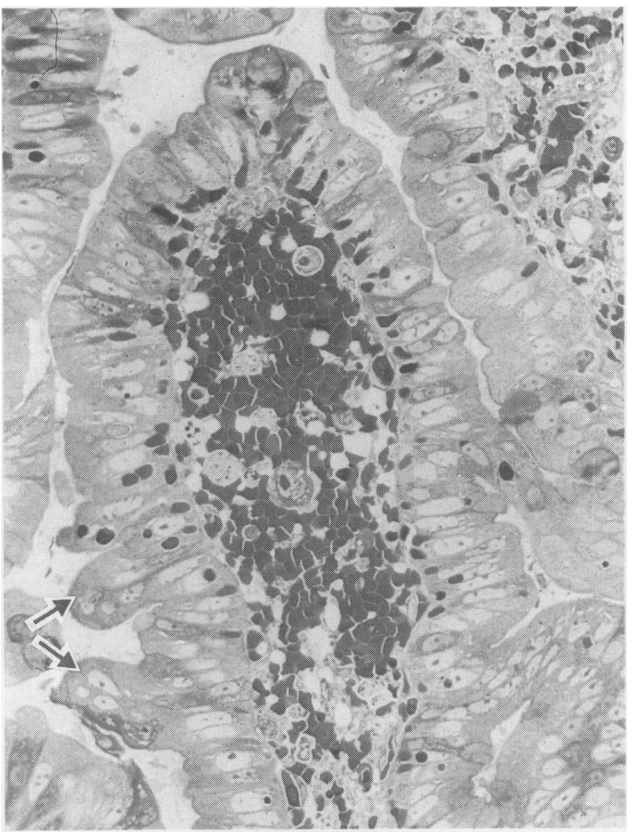

Fig. 6. Epithelial overgrowth, 3 h reperfusion. Bulky masses of irregular epithelial cells on the side of the villous (arrows), haemorrhage in the stroma. Semithin section. Giemsa ×400.

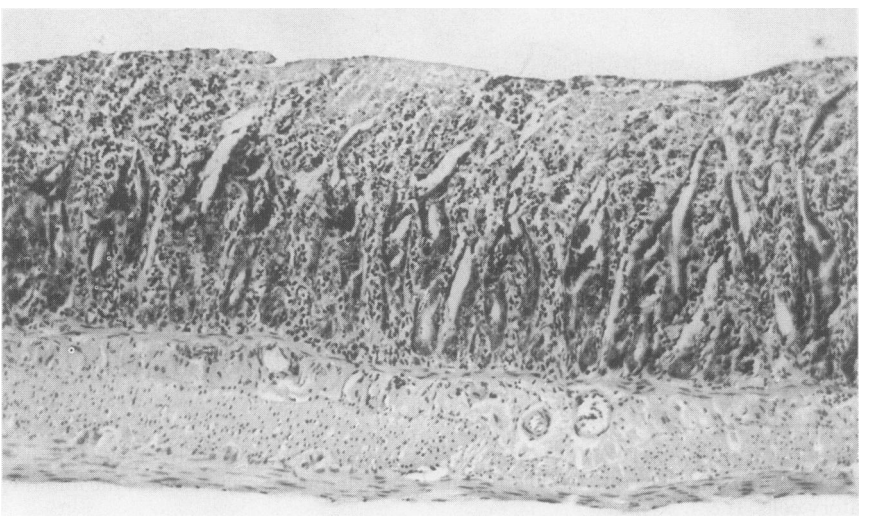

Fig. 7. Intestinal wall, 4 h reperfusion. Necrosis in the villous region. H & E ×100.

Table 1. Average scores of histological changes

Change	Time of scoring						ANOVA (*P*)
	0 (min)	10 (min)	1 (h)	2 (h)	3 (h)	4 (h)	
1	1.08 (0.23)	1.67*† (0.14)	1.33† (0.19)	1.50† (0.15)	0.92* (0.23)	1.50*† (0.15)	<0.05
2	1.00 (0.00)	1.08 (0.08)	1.75*† (0.13)	2.00*† (0.00)	2.00† (0.00)	2.00† (0.00)	<0.001
3	0.25 (0.13)	0.33 (0.14)	0.75*† (0.18)	1.08*† (0.08)	0.92† (0.23)	1.08† (0.08)	<0.01
4	0.00 (0.00)	0.00 (0.00)	1.08*† (0.23)	1.25† (0.13)	1.50*† (0.19)	1.58† (0.15)	<0.001
5	0.00 (0.00)	0.58*† (0.15)	1.17*† (0.11)	1.83*† (0.11)	1.83† (0.11)	1.75† (0.13)	<0.001

1, Epithelial desquamation; 2, extent of total villous destruction; 3, inflammatory cell infiltration; 4, cell necrosis of the lamina propria; 5, epithelial regeneration. Significance of the changes within a histology group (ANOVA) is indicated in the right column.

Values represent mean (s.e.m.)

* Significantly different ($P<0.05$) from the previous value.

† Significantly different ($P<0.05$) from the ischaemic (0 min) value (*t*-test).

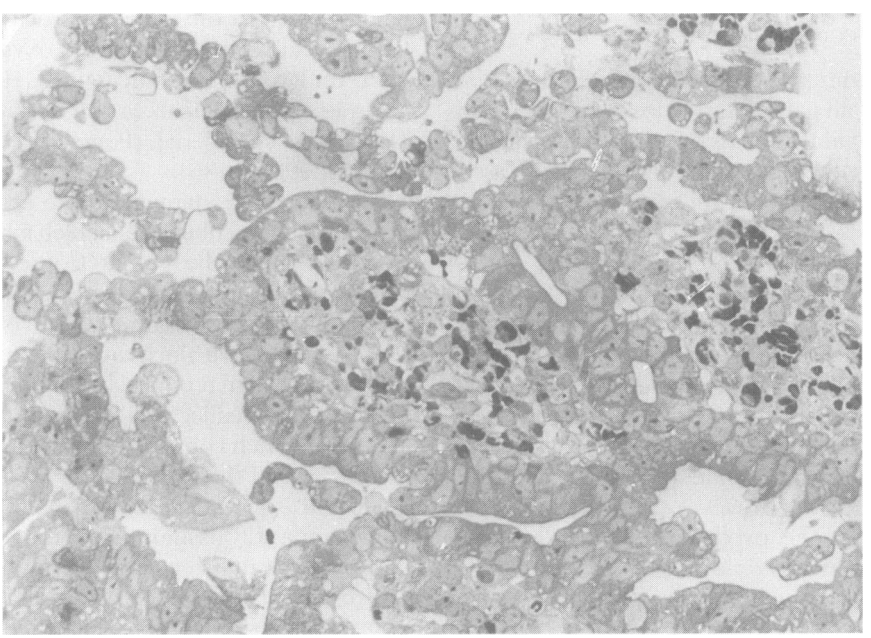

Fig. 8. Intestinal necrosis, 4 h reperfusion. Cross-section of necrotic villi and desquamated cell layers. Irregular and vacuolized epithelium with subepithelial blebs. Necrotic stroma. Semithin section. Giemsa ×400.

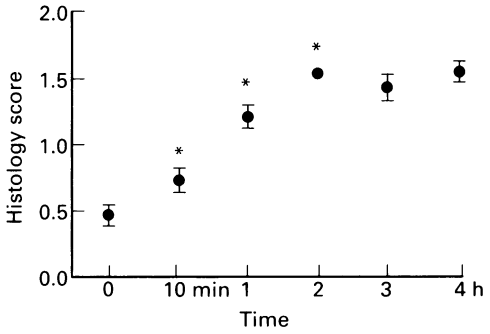

Fig. 9. Reperfusion injury. Total histology score (y axis) changes as a function of time (x axis) following reperfusion. *Significant differences ($P<0.05$) between the given value and the previous one.

Regenerative epithelial overgrowth. Proliferation of the mucosal epithelium started from the very beginning of reperfusion and at 3 hours the newly formed epithelium practically covered the denuded mucosal surface.

Quantification of the reperfusion injury

As a summary of the above changes the scores of all parameters were aggregated and averaged at the respective timepoints. Figure 9 shows that tissue injury increases as a function of time during reperfusion.

Discussion

In the present experiments we observed that two parallel processes characterized the tissue alterations during ischaemia and reperfusion. One of them is the continuous destruction of the mucosal lamina propria. The other is a marked tendency of the epithelium to regenerate. However, a secondary destruction of the newly formed cells occurs, probably at the time when the circulatory shock syndrome reaches an irreversible stage.

The epithelial cells, especially in the crypt region, remain viable for a remarkably long time. Although the phosphocreatine and ATP contents of the small intestine are reduced to a low level within 20 min during SMA occlusion (Blum *et al.* 1986), epithelial cells are still capable of thymidine uptake and mitoses in the crypts after 60 min of ischaemia (Wagner 1979). A few viable cells among the necrotic debris can still be recognized after several hours of SMA occlusion (Juhász *et al.* 1984; Glotzer *et al.* 1962).

A massive epithelial desquamation is initiated within the first few minutes of reperfusion. Subepithelial blebs are formed during ischaemia (Fig. 1 and Brown *et al.* (1970)), but the blebs do not always lift the epithelium from its base and the process is only partly associated with oedema formation (Brown *et al.* 1970). A rapid lifting of the cells from their base is accompanied by oedema in the subepithelial region when reperfusion starts. The microcirculation of the villi is damaged by hypoxia and the renewed haemodynamic load can lead to increased oedema by fluid loss across leaky capillary walls, and this then lifts the cells. This early destruction of the villi can in part be attributed to the above microcirculatory damage caused by the reduced oxygen supply (Lundgren & Haglund 1978). However, the role of free radicals in this situation has also to be considered (Parks *et al.* 1988; Granger *et al.* 1986).

The secondary damage to the epithelium also starts with subepithelial bleb formation. The process can be similar to the changes described earlier at the end of ischaemia. At this later time the intestinal stroma is already heavily loaded with activated inflammatory cells which can produce large numbers of free radicals (Vedder *et al.* 1989; Inaunen *et al.* 1989) which could attack the basolateral membranes of the epithelia.

Several successful attempts have been made to quantify tissue injury in the gut in low flow or ischaemia/reperfusion (Haglind *et al.* 1980; Chiu *et al.* 1970; Åhrén & Haglund 1973). These studies reported detailed analyses of the destruction only of the gut wall; none focused on the regenerative tendency of the epithelium. They also evaluated the

general appearance of the gut mucosa but did not characterize changes in different cell types. Our semiquantitative system is based on simultaneous assessment of five different parameters and also focuses on the epithelial regrowth. This system is adequate to illustrate the dynamics of early reperfusion injury. However, at later times (third and fourth hours of reperfusion), despite the increasing stromal destruction and cell infiltration, this type of quantification of the reperfusion injury is inadequate. The scoring system is not sensitive enough to characterize the damage which occurs at later times of reperfusion injury because the maximum score (2) had already been reached earlier.

In summary we conclude that the intestinal mucosa in SMA occlusion/reperfusion shock is characterized by a steady deterioration of the lamina propria. At the same time, the epithelium illustrates strong regenerative regrowth. This epithelial overgrowth, however, is intercepted by a secondary destruction of the cells which may be attributed both to a deterioration in microcirculation caused by shock and consequent low flow states, and to the inflammatory infiltration of the lamina propria.

Acknowledgements

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