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Induction of heat shock protein 70 (HSP70) by zinc bis (DL-hydrogen aspartate) reduces ischemic small-bowel tissue damage in rats

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Induktion von Hitzeschockprotein 70 (HSP70) durch Zink-di-DL-Hydrogenaspartat reduziert Ischämieschäden des Dünndarmes im Ratten-Model

Abstract The aim of the study was to determine whether the induction of HSP70 by Zn^{2+} is able to protect the small bowel of rats against ischemia. Twenty-four male Wistar rats (weight 200–300 g) were divided into four groups: (1) saline treatment for 24 h (n=4); (2) Zn^{2+} treatment for 24 h (n=4); (3) Saline pretreatment for 24 h and ischemia (n=8); (4) Zn^{2+} pretreatment for 24 h and ischemia (n=8). Pretreatment with Zn^{2+} was carried out by intraperitoneal administration of 50 mg/kg zinc bis (DL-hydrogen aspartate)=10 mg/kg Zn^{2+}. Ischemia in a defined segment of the small bowel was produced by ligation of the mesenteric vein and artery and ligation of both ends of the segment. Tissue samples were collected before and 2, 4 and 6 h after ligation and investigated by histology, immunohistochemistry and Western blotting. Twenty-four h after i.p. Zn^{2+} injection, the small bowel expressed increased HSP70 tissue levels. Histology with subsequent grading of ischemic tissue injury showed significantly decreased tissue necrosis after Zn^{2+} pretreatment and HSP70 induction compared with saline pretreated controls. In conclusion, this study proves that Zn^{2+} is inducing HSP70 in the small bowel in vivo and hereby able to protect the small bowel against ischemia.

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Zusammenfassung Ziel dieser Studie war es zu klären, ob die HSP70-Induktion durch Zn^{2+} den Dünndarm von Ratten gegen Ischämie schützen kann. Es wurden 24 männliche Wistar-Ratten (Gewicht 200–300 g) in 4 Gruppen aufgeteilt: I. NaCl-Behandlung über 24 h (n=4); II. Zn^{2+}-Behandlung über 24 h (n=4); III. NaCl-Vorbehandlung über 24 h und Ischämie (n=8); IV. Zn^{2+}-Vorbehandlung über 24 h und Ischämie (n=8). Die Zn^{2+}-Vorbehandlung erfolgte durch intraperitoneale Gabe von 50 mg/kg Zink-di-DL-Hydrogenaspartat=10 mg/kg Zn^{2+}. Die Ischämie in einem definierten Segment des Dünndarms wurde durch eine Ligatur der V. und A. mesenterica sowie der beiden Segmentenden erzeugt. Gewebeproben wurden jeweils vor und 2, 4 und 6 h nach der Ligatur histologisch, immunohistochemisch und durch „Western blotting" untersucht. 24 h nach intraperitonealer Zn^{2+}-Injektion zeigten sich erhöhte HSP70-Dünndarmgewebespiegel. Histologische Untersuchungen mit nachfolgender Klassifizierung des Ischämieschadens ergaben geringere Gewebenekrotisierung nach Zn^{2+}-Vorbehandlung sowie HSP70-Induktion im Vergleich zu den mit NaCl vorbehandelten Kontrolltieren. *Schlußfolgerung*: Diese Studie weist nach, daß Zn^{2+} im Dünndarm in vivo (bei Ratten) HSP70 induzieren kann und daß damit der Dünndarm gegen Ischämie geschützt werden kann.

Schlüsselwörter Ratten · Dünndarm · Zink · Hitzeschockprotein 70

Introduction

Ischemia of the small bowel is still a major problem in modern intestinal surgery and intensive-care medicine. In elective intestinal surgery, anastomotic insufficiency in most cases is the result of local ischemia due to circulatory disturbances within the anastomotic area. Besides this

classical form of local ischemia of the gut, intensive-care patients in traumatology are threatened by several forms of shock that can lead to ischemia of the gut, with subsequent bacterial translocation. However, bacterial translocation secondary to ischemia of the gut caused by traumatic and hemorrhagic shock is a major source of posttraumatic sepsis. Enteral bacterial translocation has been implicated as a possible cause of subsequent organ failure. In several series, only a third of patients with clinical manifestations of sepsis and multiorgan failure had an identifiable focus of infection [2, 7]. In these patients the gut frequently plays an important role as a shock organ involved in the development of sepsis [20]. According to Fine et al. [4], the intestine is a major source of endotoxin. This is in accordance with the study of Meakins and Marshall [14], who state that the motor of multiorgan failure is the gastrointestinal tract.

Pathophysiologically, small-bowel ischemia is caused by a relative or absolute hypoxia that progressively leads to necrosis depending on its degree. However, nature has evolved a defense mechanism, commonly referred to as the stress response, that allows virtually all organ systems to tolerate stress that might otherwise be lethal. The general feature of the stress response in all organisms is the rapid and almost exclusive synthesis of intracellular proteins, the so-called stress proteins, including heat shock proteins (HSPs).

HSPs, however, have been proven in many in vivo and in vitro studies to have beneficial effects in ischemic heart, skeletal muscle and brain disease [1, 5, 11, 12, 15, 16, 22] and septic shock [19, 21]. Published literature indicate that stress protein expression is induced in the intestines as well, though the possible protective effect of these proteins in the course of ischemia in the small bowel has not been examined. Therefore, the study presented was designed to determine whether induction of HSP70 with Zn^{2+} protects the small bowel of rats against experimental ischemia.

In the case of an evident improvement in intestinal ischemic tolerance with the use of preceding prophylactic zinc stimulation, it is conceivable that the clinical use of these prophylactics would be cost-effective in elective surgery on critical anastomosis, e.g. resections of the esophagus and low-anterior resections of the rectum.

Materials and methods

Animal model

Experiments were performed on 24 male Wistar rats weighing 250–300 g. They were housed at an ambient temperature of 22°C, placed on a 12:12-h light-dark cycle, and provided rat chow and water ad libitum. Food, but not water, was removed on the day before the experiment. For all manipulations, the animals were anesthetized with intraperitoneal (i.p.) ketamine (80 mg/kg) and xylazine (8 mg/kg). The study was performed in adherence to the NHI guidelines for the use of experimental animals.

Animals were divided into four groups: (1) only saline treatment for 24 h (n=4); (2) only Zn^{2+} treatment for 24 h (n=4); (3) saline pretreatment for 24 h and 6 h ischemia thereafter (n=8); (4) Zn^{2+} pretreatment for 24 h and 6 h ischemia thereafter (n=8). Pretreatment with Zn^{2+} was carried out by i.p. administration of 50 mg/kg zinc bis (DL-hydrogen aspartate)=10 mg/kg Zn^{2+} (UNIZINK, Köhler Pharma, Germany). Ischemia in a defined segment of the small bowel (10 cm) was produced by ligation of the corresponding mesenteric vein and artery as well as ligation of both ends of the defined small-bowel segment.

Tissue samples of each isolated small-bowel segment were collected before and 2, 4 and 6 h after ligation. The specimens were finally investigated by conventional histology with hematoxylin and eosin (H&E) stains and immunohistochemistry against HSP70 by the indirect immunoperoxidase reaction. The histological alterations of the tissue specimens were classified according to a modified classification of Park [17; Table 1] used in our institute in routine surgical pathology.

Antibodies

Antibodies used included polyclonal rabbit anti-heat shock protein 70 A500; 1:200 (DAKO, Hamburg, Germany) and monoclonal anti-HSP70/HSC70 SPA-820; 1:200 (BIOMOL, Hamburg, Germany). Anti-HSP70 reacts strongly with the two major HSP70s (HSP72 and HSP73).

Table 1 Modified classification of Park [17] used in this study to categorize the extent of ischemic tissue necrosis into defined degrees

Injury type	Grade	Histological features
No injury	0	Normal mucosal villi
Superficial mucosal injury	1	Development of subepithelial Gruenhagen's space, usually at the apex of the villus; often with capillary congestion or extension of the subepithlial space with moderate lifting of epithelial layer from the lamina propria
	2	Massive epithelial lifting downsides the villi. Denuded villi with lamina propria and dilated capillaries exposed. Increased cellularity of lamina propria may be noted
	3	Digestion and disintegration of lamina propria; hemorrhage and ulceration
Deep mucosal injury	4	Injury of the crypt layer with incomplete or complete necrosis
	5	Complete transmucosal necrosis including the muscularis mucosae
Mural injury	6	Incomplete transmural necrosis
	7	Complete transmural necrosis
	8	Complete transmural necrosis with intramural gas formation

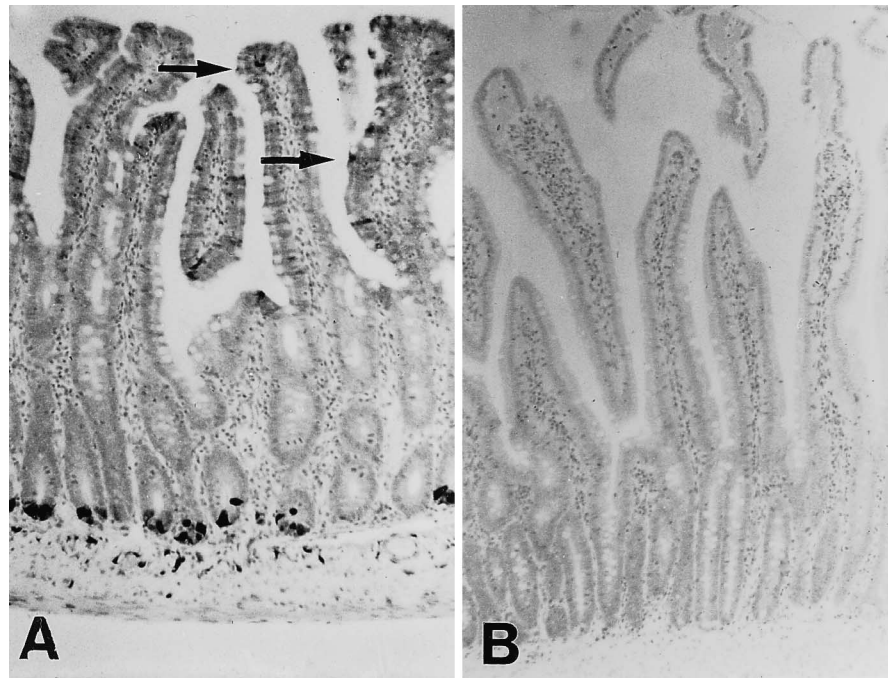

Fig. 1 Induction of HSP70 (**A**) in the tips (*arrows*) of the villi of the small bowel of group 2 (only Zn^{2+} pretreatment) after 24 h by zinc bis (DL-hydrogen aspartate); control group 1 (only saline pretreatment) immunohistochemically showed no expression of HSP70 (**B**); HSP70; ×200

Light microscopy and immunohistochemistry

One part of the tissue samples was fixed in 10% buffered formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was also performed on the paraffin-embedded material using the avidin-biotin complex method, with diaminobenzidine as a chromogen. The same staining method was used in all test and control animals. The immunohistological staining intensity was scored as areactive=–, low=+, moderate=++, and intense=+++ by two independent pathologists.

The other part of the tissue specimens was frozen immediately in liquid nitrogen and stored at –70°C.

Protein detection

HSP70 was detected by Western immunoblotting. The frozen tissue samples were subsequently thawed, homogenized in cold phosphate-buffered saline, and centrifuged at 10,000 *g* for 15 min. The supernatants were collected, and protein concentration was measured with a colorimetric reaction using bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, Ill., USA). The samples were then suspended in sodium dodecyl sulfate-glycerol sample buffer. Proteins were separated by sodium dodecyl sulfate polyacryamide gel electrophoresis, with 50 mg total protein loaded per lane. After gel electrophoresis, proteins were transferred to a nitrocellulose membrane and labeled with the primary antibodies HSP70, at 1:200 dilution. After secondary labeling with goat, alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG, respectively, at 1:2000 dilution, protein was visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Statistics

Statistical analysis was carried out using SPSS software. Student's *t*-test was used to examine the results of the histological determination of the ischemic small-bowel injury. *P* values <0.05 were considered to be significant. Results were expressed as mean±SD.

Results

All animals survived the whole experimental period. In particular, i.p. injection of Zn^{2+} at a dose of 10 mg/kg did not affect the animals. Histology of the organs in group 2 and 3 (only saline or Zn^{2+} treatment, respectively), especially the liver, lungs, kidneys, gut and peritoneum, showed no pathological changes.

Immunohistochemistry of the animals of group 2 (only Zn^{2+} treatment) showed induction of HSP70 in the small bowel. HSP70 was expressed in the mucosa, particularly at the tips of the villi (Fig. 1A). In addition, the gut wall structures, including the smooth muscle, the vessel walls, endothelial and mesothelial cells, showed significantly increased staining patterns against the HSP70 antibody. The control animals (only saline treatment) showed no or only focal HSP70 antibody reaction by immunohistochemistry (Fig. 1B; Table 2). The immunohistochemical results were

Table 2 Summary of the immunohistological staining results after induction of HSP70 with zinc bis (DL-hydrogen aspartate); areactive=–, low=+, moderate=++, and intense=+++; group 1 (only saline treatment), group 2 (only Zn^{2+} treatment)

	Group 1	Group 2
Tips of villi	–	+++
Basal mucosa	–	+/++
Smooth muscle	+	+++
Vessel walls	+	+++
Endothelial cells	(+)	++
Mesothelial cells	–	++

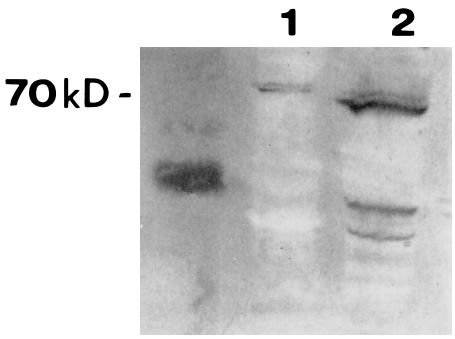

Fig. 2 Representative Western Blot analysis of small-bowel homogenates of group 1 (only saline pretreatment; *lane 1*) and group 2 (only Zn^{2+} pretreatment; *lane 2*) demonstrating the induction of HSP70 in the small bowel by Zn^{2+}

Table 3 Time profile of ischemic small bowel necrosis after 0, 2, 4 and 6 h experimental duration in group 3 (saline pretreatment and ischemia) and group 4 (Zn^{2+} pretreatment and ischemia); * $p<0.05$

Mean SD	0 hrs	2 hrs	4 hrs	6 hrs
Group III	0	4.8 ±1.46	6.34 ± 2.63	7.13 ± 2.05
Group IV	0	1.71 ± 0.62*	3.49 ± 1.37*	6.78 ± 2.8

confirmed by Western blotting (Fig. 2), which indicates a strong expression of HSP70 in the small bowel after Zn^{2+} treatment.

Histology of the tissue specimens of group 3 and 4 (saline or Zn^{2+} pretreatment and ischemia) showed no significant morphological alterations at t=0 hours. At t=2 hours group 3 already had complete mucosal necrosis, whereas group 4 showed only superficial mucosal lesions (Fig. 3A, B). At t=4 hours histology of group 3 (without Zn^{2+} pretreatment) revealed incomplete transmural necrosis (Fig. 3C), and histology of group 4 showed complete disturbance of the epithelial cell layer with denuded villi (Fig. 3D). At t=6 hours both groups showed the histological picture of incomplete or complete transmural necrosis (Fig. 3E, F). Whereas group 4 revealed a significantly decreased ischemic tissue injury in the small bowel within the first 4 h, no significant difference could be observed after 6 h between the two groups (with or without Zn^{2+} pretreatment; Table 3).

Discussion

In accordance with the results of the in vitro and in vivo [1, 5, 11, 12, 15, 16, 19, 21, 22] analyses and those found in the present study, HSP70 exhibits a significant tissue-protective effect in experimental small-bowel ischemia. The expression of HSP70 was induced by zinc bis (DL-hydrogen aspartate). In contrast to other heavy metals, in particular sodium arsenite ($NaAs0_2$), which has been used as an inducer in recent in vivo experiments [19], the injurious or toxic qualities of zinc bis (DL-hydrogen aspartate) are essentially lower. Sodium arsenite is described as very poisonous with an oral LD_{50} in rats of 41 mg/kg [19]. In the experiments of Ribeiro et al. [19], rats that received 10 mg/kg i.v. of sodium arsenite appeared lethargic and anorectic; some of them had diarrhea. The mortality at this dose was 20%. Toxicological features of zinc bis (DL-hydrogen aspartate) are determined for mice or rats, respectively (Köhler Pharma, Alsbach, Germany; unpublished results/personal communication). Acute toxicity is reported in mice with LD_0=50 mg/kg, LD_{50}=125 mg/kg and LD_{100}=500 mg/kg (observation period, 10 days). Rats which received a daily dose of 80 mg/kg zinc bis (DL-hydrogen aspartate) over a period of 32 weeks, showed no striking pathological alterations. In our study we used 50 mg/kg zinc bis (DL-hydrogen aspartate) i.p., a dose which can be considered safe.

The small intestine reacts very sensitively to hypoxia. Chiu et al. in 1970 [3] and Haglund et al. in 1987 [9] reported that superficial mucosal injury could be detected after 20 min due to total ischemia or in 60–120 min due to severe partial ischemia. However, even before morphologic injury is detectable, hypoxia causes increased permeability of the intestinal mucosa [9]. The main consequences of the increased permeability and the destruction of the morphological barrier between the lumen and the intestinal tissue are increased translocalization of bacteria and endotoxins. Several studies have indicated that the ischemic gut might be the source of a continuous release of bacteria and endotoxin, creating the background for the chronic septic condition often seen in patients with multiple organ failure syndrome [2, 13, 14].

The first morphological alterations in the small intestine can be detected on the tip of the villi and on the epithelial cell layer covering the villi. Chiu et al. in 1970 [3] subdivided the injury into five grades, a system which has been widely used in experimental small-bowel ischemia to describe the extent of tissue injury. Furthermore, Chiu and his colleagues [3] demonstrated that their grading system described degrees of injury in a continuous spectrum, the velocity of the process depending on the extent of ischemia and its duration. The disadvantage of the Chiu grading system is that total ischemia of prolonged duration (>90 min) leads to injury of the basal mucosal layer, which is not covered by this classification. To be able to describe this additional injury, Park et al. in 1990 [17] modified the Chiu grading system and added stages for deep mucosal and transmural tissue injury.

The rat model in this study is a model of total circulatory arrest by ligation of the mesenteric artery and vein of a defined, 10-cm-long small-bowel segment. As in previous investigations [3, 9], specimens of group 3 animals (saline pretreatment and ischemia) showed a continuous spectrum of small-intestinal ischemic injury: after 2 h of ischemia, deep parts of the mucosa were necrotic; after 4 h, incomplete transmural necrosis was observed. A prolonged total, combined venous and arterial ischemia for 6 h, re-

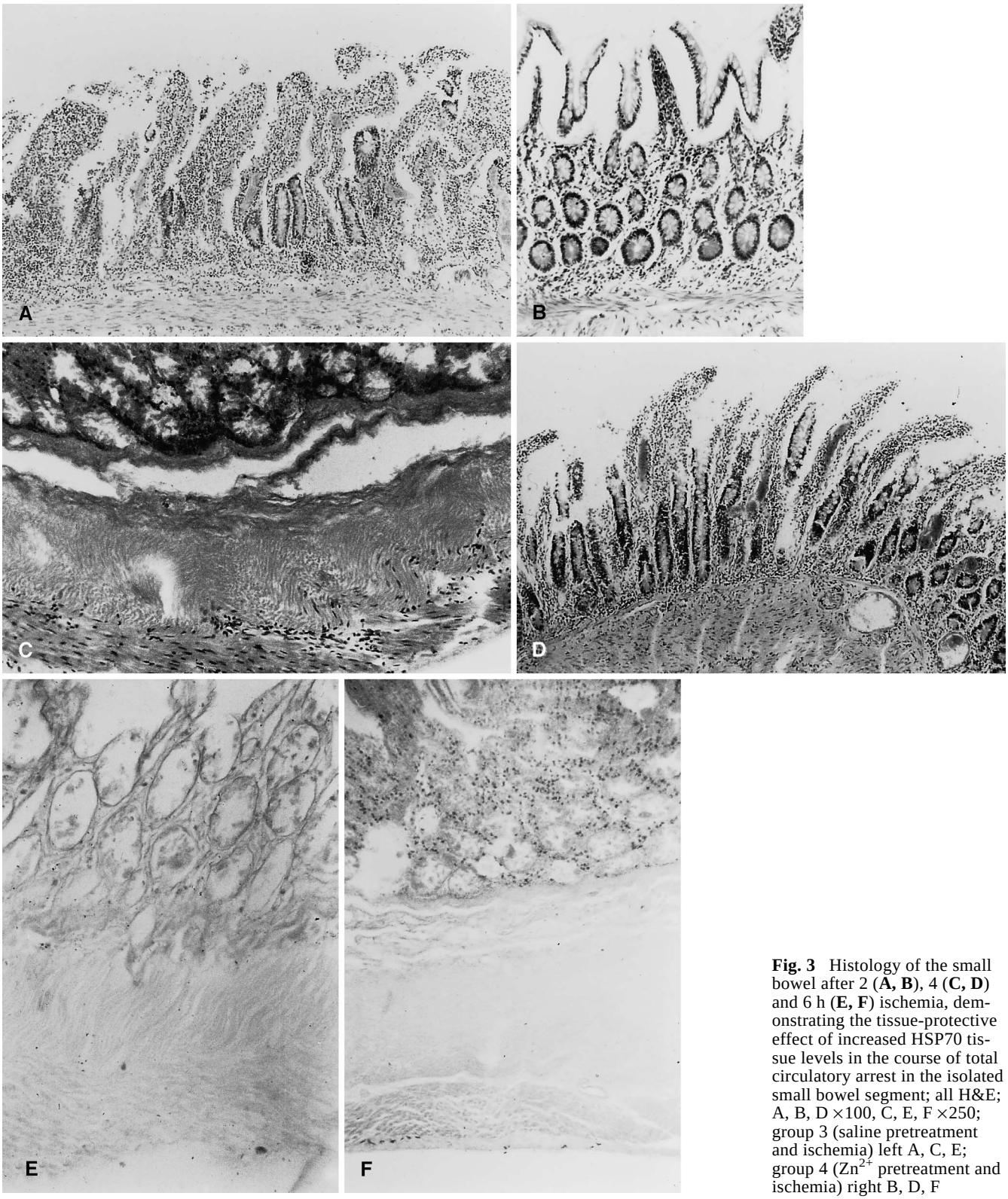

Fig. 3 Histology of the small bowel after 2 (**A, B**), 4 (**C, D**) and 6 h (**E, F**) ischemia, demonstrating the tissue-protective effect of increased HSP70 tissue levels in the course of total circulatory arrest in the isolated small bowel segment; all H&E; A, B, D ×100, C, E, F ×250; group 3 (saline pretreatment and ischemia) left A, C, E; group 4 (Zn^{2+} pretreatment and ischemia) right B, D, F

sulted in complete transmural necrosis. In comparison, specimens of group 4 (Zn^{2+} pretreatment and ischemia) exhibited a significantly decreased degree of tissue injury within the first 4 h of total combined ischemia. The tissue samples only showed superficial mucosal necrosis after 2 h of ischemia; after 4 h deep mucosal injury began to be observed. After 6 h, even group 4 animals showed transmural necrosis indicating that the overall hypoxic tolerance of the small bowel in rats in states of total circulatory arrest is limited to this time interval.

In its last consequence, hypoxia leads to increased levels of intracellular, denaturated protein structures that basically affect cellular hemostasis. The major HSP70 functions as a molecular chaperon, guiding the folding and refolding of proteins as they are synthesized and conveyed between cellular organelles. The precise mechanism by which HSP70 prevents hypoxia-mediated tissue necrosis in this study is uncertain. The general cytoprotective effect of HSP70 appears to be secondary to its ability to bind damaged proteins, thus preventing premature folding and denaturation of these proteins, as well as acting as a chaperon in other protein-protein interactions [6, 8, 10, 23]. The importance of these genes and their products for cellular hemostasis has been clearly demonstrated through "knockout studies", such as the microinjection of anti-heat shock protein 70 antibodies into individual cells, rendering them sensitive even to most minor pertubations [18]. High intracellular HSP70 levels, therefore, may be able to maintain hemostasis to a certain degree and period of hypoxia.

In conclusion, this study demonstrates that induction of HSP70 by zinc bis (DL-hydrogen aspartate) is an appropriate, preventive method to reduce ischemic small-bowel tissue damage in rats.

Whether an increased expression of HSP70 in the small bowel prevents the translocation of bacteria and endotoxin by an adequate reduction of permeability owing to ischemia and whether the actual administration of Zn^{2+} could also be useful in the prophylactic concept of elective surgery on critical anastomosis, as it is in resections of the esophagus or low-anterior resections of the rectum to prevent intestinal ischemia, needs further research.

Apart from purely prophylactic use, with further positive results, post-primary use in emergency situations like multiple trauma and shock for the possible reduction of translocations caused by ischemia would have to be proved in standardized models.

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