Intestinal paracellular permeability during malnutrition in guinea pigs: effect of high dietary zinc

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Abstract

Background—Zinc has been shown to have beneficial effects in vitro on epithelial barrier function, and in vivo to reduce intestinal permeability in malnourished children with diarrhoea.

Aims—To determine whether malnutrition alters intestinal paracellular permeability, and whether zinc prevents such alterations.

Methods—Guinea pigs were fed a normal protein diet (NP group), a low protein diet (LP group), or a low protein diet enriched with 1800 ppm zinc (LPZn group) for three weeks. Intestinal permeability was measured on jejunal segments mounted in Ussing chambers by measuring ionic conductance and mucosal to serosal fluxes of ^{14}C-mannitol, ^{22}Na, and horseradish peroxidase. Tight junction morphology was assessed on cryofracture replicas.

Results—Mannitol and Na fluxes and ionic conductance increased in the LP group compared with the NP group but remained normal in the LPZn group. Accordingly, jejunal epithelia from the LP group, but not from the LPZn group, showed a small decrease in number of tight junctional strands compared with epithelia from the NP group. Neither malnutrition nor zinc treatment modified horseradish peroxidase fluxes.

Conclusions—Malnutrition is associated with increased intestinal paracellular permeability to small molecules, and pharmacological doses of zinc prevent such functional abnormality.

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Keywords: malnutrition, zinc, tight junction, paracellular permeability, intestine, guinea pig.

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Malnutrition, similar to intestinal diseases such as inflammatory bowel disease, gastrointestinal allergy, coeliac disease, and toxigenic diarrhoea, is associated with intestinal dysfunction. In children with diarrhoea, oral zinc supplementation was shown to improve intestinal permeability[1] and to reduce the severity and duration of diarrhoea.[2] The effects of zinc were more pronounced in the subgroup of malnourished children, suggesting that the intestinal dysfunction linked to malnutrition responds positively to zinc treatment.

In both human and animal malnutrition, intestinal transport of macromolecules in-

creases,[3–6] and intestinal ionic conductance and small solute permeability are also enhanced[7][8] indicating that function of the intestinal barrier is altered during malnutrition. These findings raise the question of morphological integrity of the intestinal epithelium during malnutrition, as intestinal morphology is strongly implicated in the barrier function.[9] In the intestinal epithelium the paracellular permeability is mainly governed by tight junctions. They provide a continuous seal around the apical region of adjacent cells, restricting the free passage of molecules and ions across the paracellular pathway. They are regulated at the cellular level by the cytoskeleton[10] and are physiologically modulated by nutrients.[11] Cytokines such as interferon γ (IFNγ)[12] or tumour necrosis factor α (TNFα),[13] and pathological events such as the transmigration of polymorphonuclear cells,[14] have been shown to alter the intestinal epithelial barrier in vitro, as indicated by the increase in the paracellular permeability of filter grown intestinal cells, and by morphological changes in the tight junction network. Interestingly, in a model of endothelial monolayers, zinc was mandatory to normal barrier function[15] and prevented the disruption of this barrier by TNFα.[16]

The first objective of this study was to gain insight into the mechanisms by which the intestinal barrier is altered during malnutrition. We therefore analysed the morphological and functional alterations that occurred in the jejunal epithelium of malnourished guinea pigs. The second objective was to assess the ability of pharmacological doses of zinc to prevent intestinal barrier dysfunction in our malnourished guinea pigs.

Methods

ANIMALS AND DIETS

Thirty weanling Dunkin-Hartley guinea pigs (Charles River, Saint-Aubin les Elbeuf, France) weighing 178 (SD 8) g at day 0, were used.

They were maintained at room temperature under a 12 hour light:12 hour dark cycle, fed ad libitum with three different diets for three weeks, and given free access to water. The diets were based on the known nutritional requirements of these animals[17] and were purchased from INRA/APAE, Jouy en Josas, France. Table I shows details of their composition. As guinea pigs are prone to

TABLE I *Composition of the diets*

Ingredients (g/100g diet)	NP	LP	LPZn
Soya proteins	30·0	4·0	4·0
L-Methionine	0·3	0·04	0·04
Saccharose	14·4	23·15	22·88
Corn starch	28·8	46·31	45·78
Corn oil	5·0	5·0	5·0
Cellulose	14·0	14·0	14·0
Vitamin mixture*	1·0	1·0	1·0
Mineral mixture†	6·5	6·5	6·5
Additional zinc (ZnSO₄ 7H₂O)	–	–	0·8

NP=normal protein diet; LP=low protein diet; LPZn=low
protein diet with 1800 ppm zinc.
*Vitamin mixture (IU or mg/kg diet): retinyl palmitate, 25 000
IU/kg; cholecalciferol, 2000 IU/kg; dl-tocopheryl acetate, 135
IU/kg; menadione, 20 mg/kg; thiamin HCl, 20 mg/kg;
riboflavin, 20 mg/kg; nicotinic acid, 200 mg/kg; calcium
D-pantothenate, 40 mg/kg; pyridoxine HCl, 15 mg/kg;
inositol, 2000 mg/kg; D-biotin, 0·8 mg/kg; folic acid, 10 mg/
kg; cyanocobalamin, 0·04 mg/kg; ascorbic acid, 2 g/kg;
paraaminobenzoic acid, 10 mg/kg; saccharose, 2·5 g/kg.
†Mineral mixture (g or mg/kg diet): CaHPO₄-2H₂O, 8·33 g/kg;
CaCO₃, 14·56 g/kg; NaCl, 2·81 g/kg; KCl, 4·52 g/kg;
CH₃COOK, 27·10 g/kg; FeSO₄ 7H₂O, 0·40 g/kg; MnSO₄
H₂O, 0·40 g/kg; MgSO₄ 7H₂O, 1·50 g/kg; MgO, 5·00 g/kg;
CuSO₄-5H₂O, 0·04 g/kg; NaF, 0·04 g/kg; KI, 0·03 g/kg;
(NH₄)₆Mo₇O₂₄ 4H₂O, 0·90 mg/kg; CoSO₄ 7H₂O, 0·03 g/kg;
Na₂SeO₃, 0·90 mg/kg; ZnSO₄ 7H₂O, 0·20 g/kg (45 ppm Zn).

develop sensitisation to milk proteins, soya
proteins were used as the source of dietary
proteins. The guinea pigs were divided into
three dietary groups, fed a normal protein diet
(NP, 30% soya protein, n=12), a low protein
diet (LP, 4% soya protein, n=12), and a low
protein diet enriched with 0·8% (1800 ppm)
ZnSO₄ 7H₂O (LPZn, n=6). The LP and NP
diets contained adequate zinc (45 ppm). As
zinc has been reported to be non-toxic even
at doses of more than 100 times the
recommended dietary allowance,[18] especially
in the guinea pig,[17] we chose a dose of 1800
ppm zinc on the assumption that its effects
would be apparent in the LPZn group. This
large amount of zinc constitutes a
pharmacological dose that may not apply to
supplementation in human malnutrition.
Isocaloric dietary adjustments were made with
saccharose and corn starch (1/3:2/3 w/w). As
soya proteins are deficient in amino acids
containing sulphur, diets were supplemented
with methionine (1% of the soya content).
Because low protein diets are known to induce
anorexia,[19] animals given such diets probably
have protein energy malnutrition rather than
strict protein deficiency.

EXPERIMENTAL DESIGN
After 18–20 days on their respective diets,
guinea pigs were fasted for 18 hours,
anaesthetised intraperitoneally with a lethal
dose of sodium pentobarbitone (90 mg/kg).
The jejunum was removed and carefully rinsed
free of intestinal content with cold Ringer's
solution containing (in mM) 140 Na⁺, 5·2 K⁺,
120 Cl⁻, 25 HCO₃⁻, 1·2 Ca²⁺, 2·4 HPO₄²⁻, 0·4
H₂PO₄⁻, 1·2 Mg²⁺, and 2 glutamine. The
jejunum was then stripped of the outer muscle
layer and fixed for electron microscopy. Other
pieces of jejunum were opened along the
mesenteric border and immediately mounted
in Ussing chambers for permeability studies.
Plasma samples and some jejunal segments
were also collected and stored at –80°C for zinc

and protein assays by flame atomic absorption
and Folin reagent respectively. All these
experiments were done according to the
principles of laboratory animal care.

ANALYSIS OF ZINC STATUS OF GUINEA PIGS
The zinc concentration was measured in the
plasma and intestine of guinea pigs from the
three dietary groups. Plasma was diluted 1:5
with doubly deionised water and intestinal
homogenates were mineralised in Teflon
bombs placed in a microwave enclosure
(MDS81D, CEM Corp, Matthews, USA) as
follows: 50 μl intestinal homogenates were
mineralised with 300 μl ultrapure nitric acid at
500 W for five minutes and then appropriately
diluted with doubly deionised water. Samples
were analysed for zinc by air-acetylene flame
atomic absorption (Perkin-Elmer spectro-
photometer model 2380, Norwalk, CT, USA)
at a wavelength of 213·8 nm.

INTESTINAL PERMEABILITY STUDY
Intestinal permeability was analysed in 24
animals only. Four adjacent jejunal segments
from each animal were mounted in Ussing
chambers as flat sheets with an exposed area
of 0·1 cm². They were bathed on both sides
with 1·5 ml Ringer's solution, which was
continuously circulated at constant tem-
perature, oxygenated, and maintained at
pH 7·4 with 5% CO₂ in 95% O₂. The mucosal
and serosal bathing solutions were connected
via agar bridges to calomel electrodes for
measurement of the transepithelial potential
difference and to Ag-AgCl electrodes for
current application. The tissue was kept under
short circuit conditions by an automatic
clamping device (World Precision Instruments
Inc, New Haven, CT, USA) that cancelled out
fluid resistance. Short circuit current was
constantly recorded and the tissue was pulsed
at 5 mV every 30 seconds. The short circuit
current deflection was used to calculate ionic
conductance (or its reverse, electrical
resistance) according to Ohm's law. Steady
state potential difference, short circuit current,
and ionic conductance 30 minutes after tissue
mounting were taken as baseline electrical
variables. After equilibration, 5 mM mannitol
was added to both the mucosal and serosal
compartments, and 20 kBq/ml ¹⁴C-mannitol,
20 kBq/ml ²²Na⁺ (both from NEN, Dupont de
Nemours, France), and 0·4 mg/ml of the
macromolecular marker horseradish peroxi-
dase (HRP type VI, Sigma) were all added
simultaneously on the mucosal side only. Their
appearance on the serosal side was monitored
by sampling 800 μl aliquots of this
compartment at 10, 30, 50, 70, and 90 minutes
with buffer replacement. Radioactivity was
measured on 500 μl samples by liquid
scintillation photometry (Kontron, SL4000)
using double label correction. Mannitol and
Na⁺ fluxes were calculated according to their
specific activity in the medium and expressed
in μmol/h.cm² and μEq/h.cm² respectively.
Fluxes of intact horseradish peroxidase

(ng/h.cm^2) were assessed by measuring the enzymatic activity of peroxidase in 200 μl serosal samples, as previously described.[20] Fluxes are given at steady state, as the mean of the fluxes at 70 and 90 minutes.

ELECTRON MICROSCOPY

Studies were carried out on enterocytes of the middle villous region of the jejunum.

Ultrathin sections

Jejunal segments were fixed for one hour in 2·5% glutaraldehyde in 0·1 M phosphate buffer, pH 7·2. Intestinal segments were postfixed in 1% OsO_4 in 0·1 M phosphate buffer, dehydrated, and embedded in Araldite resin. Sections 120 nm thick were double stained with aqueous 0·5% uranyl acetate and lead citrate and observed under a Philips EM201 electron microscope operating at 80 kV.

Freeze-fracture analysis of jejunal segments

Microvillus morphology and basolateral membrane plications were the variables used to identify mature and immature cells, for three reasons: the microvilli of undifferentiated crypt cells are shorter and less abundant than in differentiated absorptive cells, P face particles are more numerous in the microvillus membranes of villus cells than in those of crypt cells, and plications of the basolateral membrane are present near the tight junctions, especially between cells near the villus tip.[21] Here, glutaraldehyde fixed segments were cryoprotected with 25% glycerol in 0·1 M phosphate buffer for 30 minutes, cut, and mounted on replica hats. Samples were frozen in pasty nitrogen, fractured in a freeze-fracture device (Cryofract, Reichert-Jung), shadowed with platinum, and coated with carbon under ultrahigh vacuum. Replicas were examined by electron microscopy after cleaning. To allow for variations within and between animals, replicas from two animals per group and two sites per animal were studied. In each group, strand numbers were quantified using 15 to 20 electron micrographs. On these micrographs straight lines were drawn perpendicular to the axis of the tight junction network every 0·2 μm, and the number of intersections with the tight junctions was counted and represented the number of strands. A mean strands number was then calculated for each electron micrograph. Overall, the tight junction length measured accounted for at least 30 μm per group.

STATISTICAL ANALYSIS

Data were analysed with the SAS package (SAS Institute, Cary, NC, USA). Results are expressed as means (SD), with n as the number of animals or electron micrographs. Correlation and linear regression analysis were done to study the relation between numerical values. Means and ranges were compared by analysis of variance (ANOVA) using the general linear model procedure (least square means).

Results

NUTRITIONAL STATUS OF GUINEA PIGS

Throughout the three weeks of experimental feeding, control guinea pigs (NP group) gained weight as expected and exhibited normal plasma and intestinal protein concentrations (Table II). By contrast, the malnourished animals, in both the LP and LPZn groups, showed weight losses, which after 18 days resulted in a mean body weight that was below the initial level at weaning, and was half the mean body weight of the NP group. In the LP and LPZn groups, total plasma proteins were significantly reduced but intestinal mucosal proteins remained unchanged. As shown in the LPZn group, zinc treatment during malnutrition (LPZn) raised plasma and intestinal zinc concentrations. However, malnutrition alone did not modify zinc status, because in the LP group it remained close to normal.

INTESTINAL PERMEABILITY

Basal short circuit current was increased (p=0·06) in the malnourished group (LP: 50·49 (16·59) μA/cm^2, n=10) compared with the control group (NP: 39·27 (10·11) μA/cm^2, n=9) but remained unchanged when malnourished animals were given additional zinc (LPZn: 42·36 (12·18) μA/cm^2, n=5). Ionic conductance, calculated as described in methods, increased significantly in intestinal segments from the LP group compared with segments from the NP and LPZn groups (Fig 1A). This increase in conductance was concomitant with an increase in mannitol and Na fluxes (Fig 1B and C). There was a linear relation between mannitol and Na fluxes (Fig 2); the slope of the relation was 58, which was close to the theoretical value of 54 for the free diffusion of mannitol and Na.[22] Mannitol and Na fluxes were closely correlated (r=0·981, p<0·0001), and the intercept of this relation was 0·38 μEq/h.cm^2. When malnourished guinea pigs were given the high zinc diet (LPZn group), all the permeability indices remained unchanged compared with control values (p>0·5). Lastly, measurements of intact horseradish peroxidase fluxes disclosed no significant increase in the absorption of this macromolecular tracer (Fig 1D).

MORPHOLOGICAL FINDINGS

Initial examination of the apical region of the plasma membrane of enterocytes from NP, LP, and LPZn groups by thin section electron microscopy showed no morphological differences in this respect between the three dietary groups (not shown). Figure 3 shows freeze-fracture replicas of jejunal segments from the three groups. In these micrographs, a tight junction appeared as a network

TABLE II   *Weight gain, plasma and mucosal proteins, and zinc concentrations in the three dietary groups of guinea pigs*

	NP	No of guinea pigs	LP	No of guinea pigs	LPZn	No of guinea pigs
Weight gain (between days 0–18)	127 (28)	12	−39 (15)*	12	−31 (12)*	6
Plasma proteins (g/l)	42·6 (6·6)	12	30·6 (5·7)*	10	31·8 (5·6)*	6
Mucosal proteins (g/100g)	13·9 (1·4)	10	13·4 (3·3)	11	14·9 (2·2)	6
Plasma zinc (μmol/l)	22·7 (5·6)	12	21·4 (8·9)	10	48·8 (14·6)*†	6
Mucosal zinc (nmol/g)	215 (68)	12	298 (81)	10	930 (426)*†	6

Values are means (SD); NP=normal protein diet; LP=low protein diet; LPZn=low protein diet with 1800 ppm zinc.
*p<0·001 *v* NP; †p<0·001 *v* LP.

composed of strands shown by fracture of the cytoplasmic leaflet of the plasma membrane (P face), and grooves produced by fracture of the exoplasmic leaflet (E face). The number of strands and grooves in the tight junction network was counted to assess the effect of malnutrition and zinc on structure of the tight junctions. Despite the absence of obvious differences between the appearance of the tight
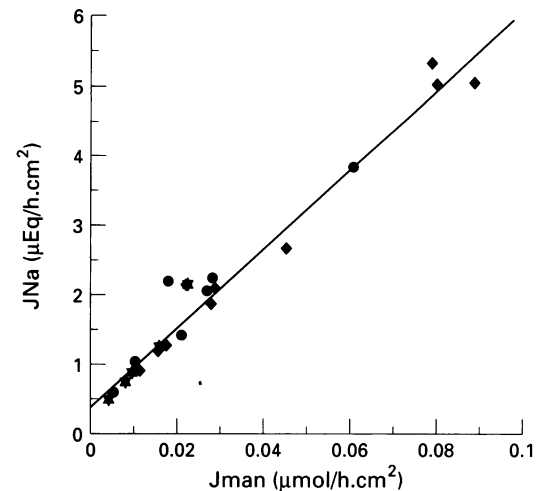

Figure 2: Relation between fluxes of mannitol (Jman) and Na (JNa) measured in Ussing chambers across jejunal segments of guinea pigs. Each point represents one animal. Circle=normal protein diet (NP); square=low protein diet (LP); star=low protein diet with 1800 ppm zinc (LPZn). The correlation between Na and mannitol fluxes is significant (r=0·981, p<0·0001) and the slope of this linear relation is 58. This is close to the theoretical value of 54 for the free diffusion of mannitol and Na, indicating that both markers use the same paracellular pathway.
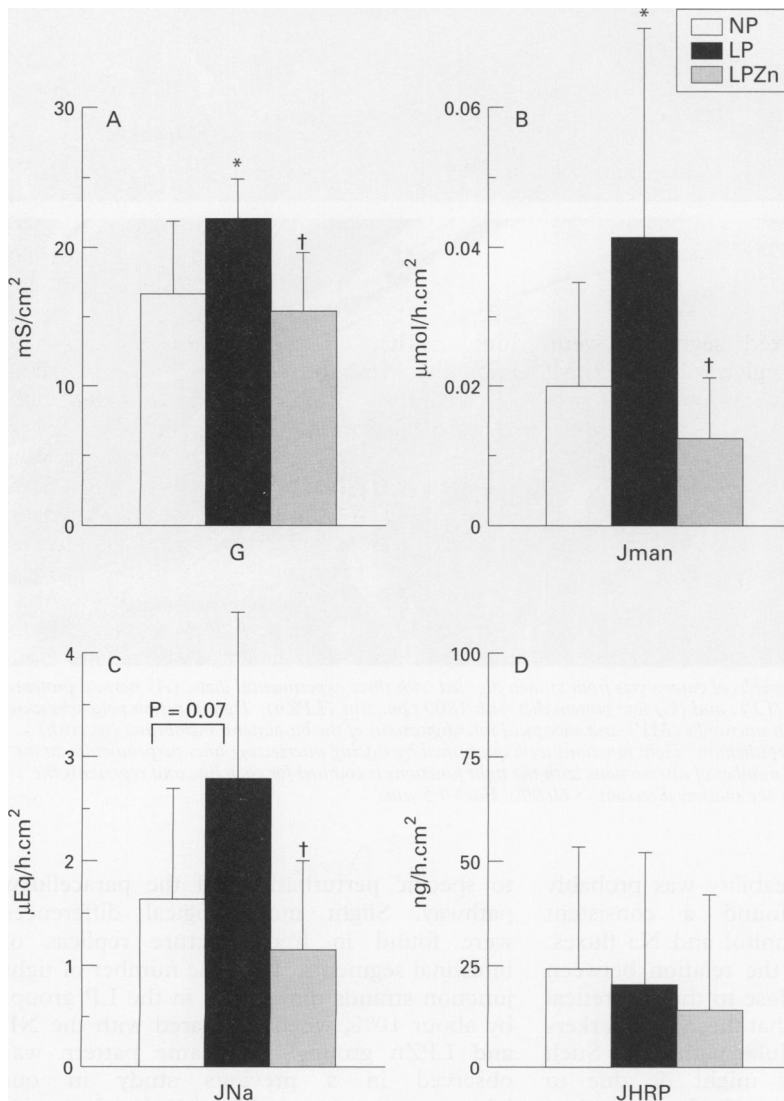

*Figure 1: Transepithelial ionic conductance and mucosal to serosal fluxes of mannitol, Na, and horseradish peroxidase measured in Ussing chambers across jejunal segments from guinea pigs. Results are means (SD). The number of animals analysed was 9, 10, and 5 in the NP, LP, and LPZn groups respectively. *p<0·05 v NP; †p<0·05 v LP. NP=normal protein diet; LP=low protein diet; LPZn=low protein diet with 1800 ppm zinc. (A) transepithelial ionic conductance (G); (B) mannitol fluxes (Jman); (C) sodium fluxes (JNa); (D) horseradish peroxidase fluxes (JHRP).*

junctional network in the three groups studied (Fig 3), jejunal epithelia from animals in the LP group, but not from those in the LPZn group, exhibited 10% less tight junctional strands than epithelia from animals in the NP group (Table III). We concede that application of ordinary hypothesis testing to these results would be flawed on account of non-independence of different sites in the same animals; the design of our study followed others[11][23] that also failed to allow for this. Another study in our laboratory,[24] using malnourished animals fed 4% milk proteins (instead of 4% soya proteins in this study) also showed that the number of strands decreased by 15% in malnourished animals (strands number=4·50) compared with control animals (strands number=5·28).

Discussion

These results confirm that intestinal ionic conductance[6-25] and unidirectional fluxes of small solutes[7][8] increase during experimental malnutrition, indicating that paracellular permeability is altered. They further suggest that this functional abnormality is associated with a reduction in the number of strands constituting the tight junctions. Our results also show that treatment with a pharmacological dose of zinc prevents intestinal changes found in malnourished guinea pigs.

In this study, malnutrition caused functional changes in the intestine. Firstly, the total electrogenic transport of ions, short circuit current, increased, thereby confirming previous findings[6][8] and suggesting that malnutrition induces a hypersecretory state in the intestine. This increase in short circuit current is not due to increased Na$^+$-glucose cotransport,[26] because no glucose was added in our experiments. Secondly, ionic conductance as well as mannitol and Na permeabilities also
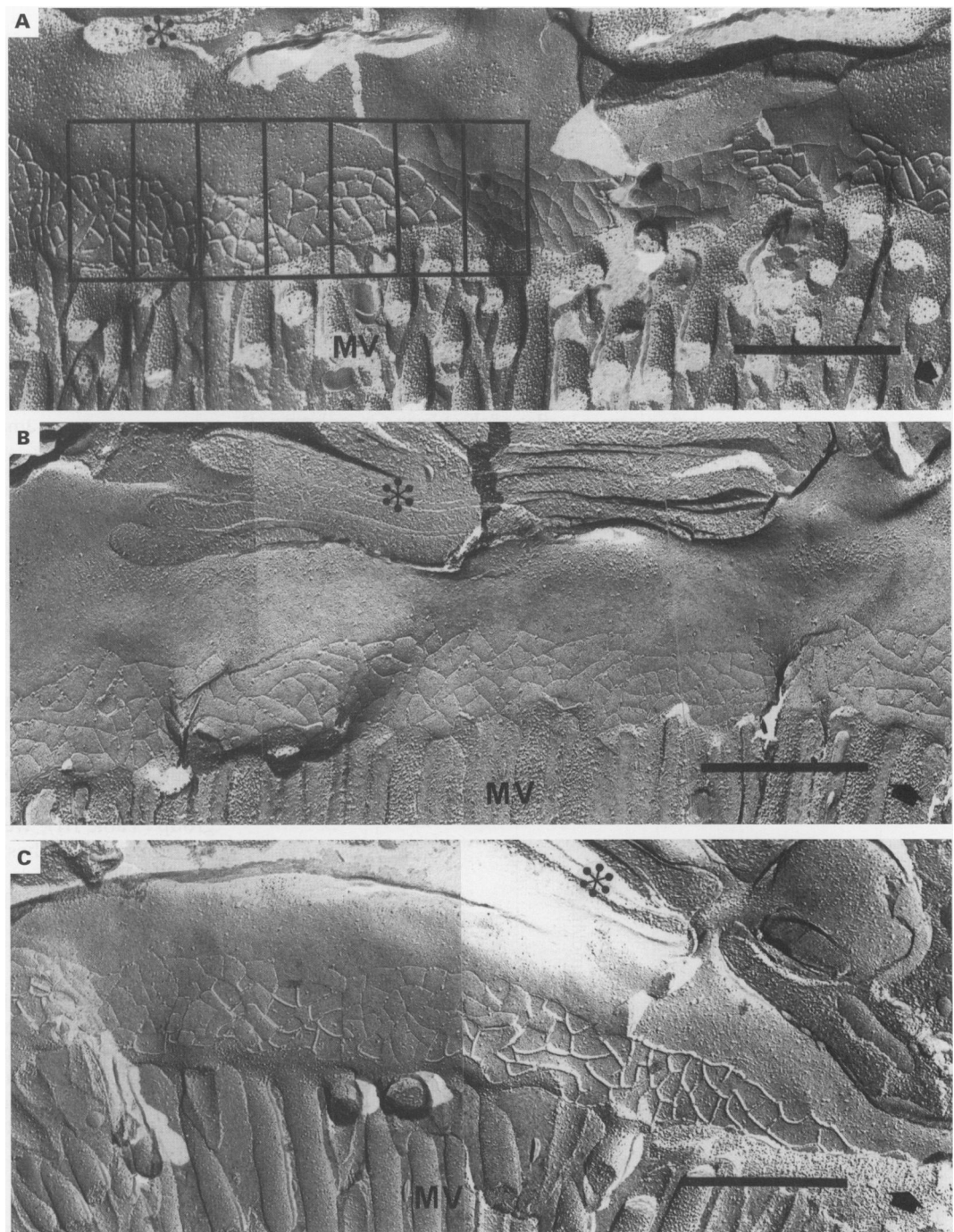
Figure 3: Freeze-fracture micrographs of enterocytes from guinea pigs fed with three experimental diets. (A) normal protein diet (NP); (B) low protein diet (LP); and (C) low protein diet with 1800 ppm zinc (LPZn). Tight junction networks were similar in appearance. Abundant microvilli (MV) and subapical interdigitations of the basolateral membrane (asterisk) characterised the jejunal villous epithelium. Tight junctions were quantified by tracing intersecting lines perpendicular to the axis every 0·2 μm (see A). The number of intersections with the tight junctions is counted for each line and represents the strands number. Arrows indicate the shadow direction. ×60 000. Bar=0·5 μm.

rose. This rise in permeability was probably paracellular, as we found a consistent correlation between mannitol and Na fluxes. Moreover, the slope of the relation between them was 58 – that is, close to the theoretical value of 54 – suggesting that these two markers shared the same paracellular pathway.[22] Such functional perturbations might be due to morphological alteration of the intestine, because malnutrition is generally reported to alter intestinal histology at both the light and electron microscopy levels.[27] [28] In our study, the absence of epithelial necrosis indicated that the increase in permeability was probably due

to specific perturbations of the paracellular pathway. Slight morphological differences were found in freeze-fracture replicas of intestinal segments. Thus the number of tight junction strands diminished in the LP group, by about 10%, when compared with the NP and LPZn groups. The same pattern was observed in a previous study in our laboratory,[24] using malnourished guinea pigs fed a low protein diet (4% milk proteins instead of 4% soya proteins in this study), in which the strands number decreased by 15% in malnourished animals compared with control animals. It has previously been suggested that

TABLE III *Quantification of tight junctions in freeze-fracture replicas of jejunum from the three dietary groups of guinea pigs*

	Strands number
NP (n=20)	5·21 (0·50)
LP (n=15)	4·79 (0·41)
LPZn (n=15)	5·07 (0·62)

Values are means (SD); NP=normal protein diet; LP=low protein diet; LPZn=low protein diet with 1800 ppm zinc. n is the number of electron micrographs analysed, taken from two sites per animal and from two animals per group.

due to the nature of the transepithelial resistance, relatively minor alterations in junctional permeability produce substantial alterations in electrical resistance,[23] [29] and that the presence of a small subpopulation of epithelial cells with permeable junctions may quickly decrease transepithelial resistance before obvious permeability changes are detected.[22]

In our model, only the permeability to small molecules was modified, without any changes in macromolecular horseradish peroxidase transport. It has generally been reported that under conditions of protein malnutrition, the intestine becomes more permeable to macromolecules. However, in most animal studies in which macromolecular absorption increased during malnutrition, animals were fed a diet containing milk proteins.[4–6] In this connection, we showed in a previous investigation that in guinea pigs, the increase in macromolecular (horseradish peroxidase) permeability is linked to the presence of cows' milk proteins in the diet and to subsequent milk sensitisation.[30] This is consistent with the absence of modification in protein permeability reported in mice given a low protein milk free soya based formula.[31]

Although the present low protein diet did not in itself induce hypozincaemia or intestinal zinc depletion, feeding malnourished guinea pigs with a large amount of dietary zinc prevented the increases in short circuit current, ionic conductance, and Na and mannitol fluxes found in the LP group without additional zinc (Fig 1). Zinc has been found to be beneficial in clinical trials of zinc supplementation in developing countries where the prevalence of zinc deficiency is high.[32] In many of these trials, supplementation consisted of physiological doses of zinc, given during the nutritional rehabilitation phase. Under such clinical conditions, the efficacy of zinc may mostly be due to the covering of the high zinc requirement known to be associated with catch up growth.[33] By contrast, in the present experiments, zinc was given at high doses throughout the period of malnutrition. Under these conditions, its efficacy in improving intestinal permeability suggests a specific role in maintaining intestinal structure and function. Ultrastructural lesions of the intestinal mucosa, as well as changes in the net transport of water, electrolytes, and glucose have been reported in zinc deficient rats.[34] [35] Zinc is an essential micronutrient present in biomembranes. It stabilises membrane

structure and is involved in membrane function by interacting, especially at SH group level, with membrane enzymes, transporters, receptors, and channels.[36] Zinc also interacts with intestinal phospholipids, protecting them from degradation.[37] This might explain why zinc prevented the activation of short circuit current in our malnourished guinea pigs, as experimental malnutrition was shown to be associated with an alteration in lipids in the microvillus membrane,[26] which may be involved in intestinal dysfunction, especially in alterations affecting active electrolyte transport. The beneficial role of zinc in function of the epithelial barrier has been shown both in vitro[15] [16] and in vivo.[1] [38] In vitro studies showed that zinc deficiency induced partial disruption of the endothelial barrier[15] and that adding zinc to the normal culture medium totally prevented the disruption of this barrier induced by TNFα.[16] In vivo studies showed that the increased intestinal permeability in children with diarrhoea was significantly reduced by zinc supplementation.[1] [38] Previous findings regarding the effects of severe zinc deficiency on intestinal permeability in rats have shown that the integrity of junctional complexes, as assessed by lanthanum hydroxide diffusion, did not alter.[39] However, this study indicates that a high zinc diet maintains a normal intestinal permeability in malnourished guinea pigs. Our morphological findings further suggest that zinc may prevent the loss of barrier function induced by malnutrition at the tight junctional level. Such hypothetical interaction between zinc and tight junctions may be related to the regulation of zinc sensitive genes in the intestine[40] or to the binding of zinc to cytoskeletal proteins,[41] [42] the role of which in regulating intestinal tight junctions is well established.[10] [22]

In conclusion, our findings show that experimental malnutrition increases intestinal paracellular permeability to small molecules and that this increase is fully prevented by pharmacological doses of zinc. They further suggest that such intestinal dysfunction might take place in the tight junctions, the stabilisation of which may be improved by zinc. The exact mechanism involved in the effects of zinc remains to be determined, as well as its clinical relevance to human malnutrition.

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