

## Is gastric ulceration different in normal and malnourished rats?

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Protein malnutrition can adversely affect all tissues. The aim of the present study was to test the hypothesis that protein deprivation influences gastric ulcer formation, as well as metabolism and organ growth, in rats. In the present study, there was a significant reduction in the body and organ weight of rats fed a low-protein diet ( $P < 0.001$ ). Malnourished rats were less susceptible to ulceration of the gastric mucosa in ethanol and indomethacin models of acute gastric ulcers when compared with rats fed a normoproteic diet (17% protein). Mucus production and prostaglandin E<sub>2</sub> formation increased in malnourished rats, possibly explaining the lower number of acute ulcers in these animals. Pylorus ligation altered gastric juice composition (increased pH and gastric volume, and decreased total acid concentration) in the animal group fed a low-protein diet compared with the group fed a diet containing 17% protein ( $P < 0.05$ ). The gastric mucosa was more damaged in malnourished rats than in normal rats evaluated for 14 d after acetic acid injection ( $P < 0.001$ ). Malnourished rats exhibited resistance to acute gastric lesions, owing to an increase in prostaglandin GE<sub>2</sub> release and mucus secretion, which protected their gastric mucosa. This phenomenon was not seen in subchronic gastric ulceration.

**Anti-ulcer activity: Malnourishment: Cytoprotection: Gastric ulcers**

Malnutrition induces a variety of metabolic disturbances in man and other mammals, some of which may be mediated by endocrine gland dysfunction. Hormonal changes may play a major role in adaptation to acute or chronic stress due to protein deficiency (Das *et al.* 1998). Malnutrition is associated with impaired insulin secretion in response to glucose and other secretagogues, as well as alterations in carbohydrate metabolism (Okitolonda *et al.* 1987; Carneiro *et al.* 1995). Exposure to malnutrition during early life may have immediate and long-term consequences in later life. Studies in animals have shown that maternal protein restriction during pregnancy and lactation can adversely affect the offspring (Hales & Barker, 1992). Such malnutrition may affect the growth of various organ systems differently. In early intra-uterine life, malnutrition tends to produce small but normally proportioned offspring, whereas at later stages of development, it causes a selective restriction of organ growth and function (Weaver *et al.* 1998). In general, the earlier malnutrition occurs in the life of mammals, the more likely it is to exert permanent effects on mammalian body weight and organ growth. A restriction of nutrient supply affects rapidly dividing cells and can lead to an irreversible limitation to organ growth and function. The sensitivity of different organs and tissues to the effects of malnutrition at different stages of growth and

differentiation has led to the concept of ‘programming’, whereby a stimulus or insult occurring in a critical period may have lasting or lifelong effects (Lucas, 1990).

Maternal dietary restriction during pregnancy and lactation causes diminished growth of the gastrointestinal tract organs and reduces enzyme activity in the small intestinal mucosa (Young *et al.* 1987). There have, however, been no studies of the long-term effects of perinatal malnutrition on the structure and function of the digestive system in adulthood. The purpose of the present study was to test the hypothesis that protein deprivation influences gastric ulcer formation as well as metabolism and organ growth in rats.

### Materials and methods

#### Drugs

Ethanol, acetic acid, NaHCO<sub>3</sub>, MgCl<sub>2</sub>, sodium acetate, NaOH, alcian blue, sucrose and Na<sub>2</sub>PO<sub>4</sub> were acquired from Allkimia (Campinas, Brazil), and indomethacin was obtained from Sigma Chemical Co. (St Louis, MO, USA). Indomethacin was prepared in sodium bicarbonate (5%). All reagents were of the highest purity commercially available. Appropriate dilutions of these substances were prepared just before use.

**Abbreviation:** PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

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### Animals

Female Wistar rats (90 d old) from the Central Animal House of the State University of Campinas (CEMIB-UNICAMP; São Paulo, Brazil) were used. After mating, the pregnant animals were randomly assigned to one of two groups. One group was maintained on a diet containing 60 g protein/kg (lower-protein diet), and the other group was fed 170 g protein/kg (normal protein diet) from the first day of pregnancy until the end of lactation (Reeves *et al.* 1993; Table 1). The lower-protein and normal protein diets (6 and 17% protein, respectively) were based on the AIN-93 report (see Reeves *et al.* 1993), which establishes dietary standards for nutritional studies on laboratory rodents.

During the experiment, the mother rats were fed *ad libitum* their respective diets and allowed free access to water. The rats were housed in cages and maintained on a 12 h light–dark cycle at 24°C. Animal food intake was monitored daily. At 25 d old, the pups were weaned and maintained on the maternal diet. After 90 d, normal and malnourished rats were used in experiments to assess anti-ulcerogenic activity and mechanism. All the rats were weighed weekly for 1 month to obtain a growth curve. The experimental protocols were approved by the Institutional Animal Care and Use Committee and were conducted following the recommendations of the Canadian Council on Animal Care (Olfert *et al.* 1993).

### Anti-ulcerogenic activity

**Ethanol-induced ulcers.** The ethanol-induced ulcer assay was performed according to the method of Morimoto *et al.* (1991). Normal and malnourished male rats (weighing 150–200 g and 90–120 g, respectively) were fasted for 24 h before the experiment but were allowed free access to water. A volume of 1 ml 99.5% ethanol was given orally to each rat. Thirty minutes after receiving ethanol, the rats were killed by cervical dislocation, their stomachs were removed and the ulcerative index was determined.

**Acetic acid-induced gastric ulcers.** The experiments were conducted as described by Takagi *et al.* (1969). Normal and malnourished rats were deprived of food for 24 h. A laparotomy was performed under anaesthesia through a midline epigastric incision. After stomach exposure, acetic acid (0.05 ml 30% v/v) was carefully injected into the subserosal layer on the glandular part of the anterior wall of the stomach, avoiding damage to the

blood vessels. Their abdomens were then closed, and the rats were fed a standard diet for 14 d. The animals were subsequently killed by cervical dislocation to assess ulcer healing. Their stomachs were removed, and gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope (MA-107; Marconi, Piracicaba, SA, Brazil). The ulcer area (mm<sup>2</sup>) and healing ratio (%) were determined.

**Indomethacin-induced ulcers.** Normal and malnourished rats were fasted for 24 h before the experiment; 12% Tween 80 was then administered orally 1 h before the induction of gastric lesions. Indomethacin (30 mg/kg, dissolved in 5% bicarbonate) was subcutaneously administered to unanaesthetised rats of each group according to the method of Hayden *et al.* (1978). The rats were killed 4 h later by cervical dislocation, and their stomachs removed and opened. The ulcerative index was determined as described before.

**Shay ulcer.** The experiment was performed as described by Shay *et al.* (1945). Normal and malnourished rats were fasted for 24 h, with free access to water. After pylorus ligation, the vehicle (12% Tween 80 solution) was administered by the intra-duodenal route to evaluate the biochemical parameters of the gastric juice of these animals. The rats were killed 4 h later by cervical dislocation, their abdomens were opened, and another ligation was placed around the oesophagus close to the diaphragm. The rats' stomachs were removed and inspected internally, their contents being drained into a graduated centrifuge tube and centrifuged at 12 100 g for 10 min (MA-182; Marconi, SA). The supernatant volume and pH were recorded using a digital pH meter (PA 200; Marconi, SA). The total acid content of gastric secretion was also determined by titration to pH 7.0 with 0.05 N NaOH using a digital burette (E.M. Hirschmann Technicolor, Eberstadt, Germany). Gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope (MA-107; Marconi, SA). Mucosal lesions were counted and the ulcerative index was determined according to the method of Sertie *et al.* (1990).

**Determination of gastric wall mucus.** Gastric wall mucus was determined according to Rafatullah *et al.* (1994). Normal and malnourished rats were fasted for 48 h with free access to water, after which pylorus ligation was performed as described earlier. The rats were subsequently killed by cervical dislocation, their stomachs then being removed and opened along the greater curvature. Gastric glandular segments were removed and weighed. Each segment was immersed for 2 h in 10 ml 0.1% (w/v) alcian blue dissolved in 0.16 M-sucrose solution, buffered with 0.05 M-sodium acetate, pH 5.8. Excess dye was removed by washing the segments twice with 0.25 M-sucrose solution during a period of 15 and 45 min, respectively. Mucus–dye complex was extracted by immersing the gastric walls in 10 ml 0.5 M-MgCl and shaking this solution intermittently for 1 min at 30 min intervals for 2 h. A volume of 4 ml blue extract was mixed with an equal volume of diethyl ether, shaking the mixture vigorously for 2 min. The emulsion obtained was centrifuged for 10 min at 12 100 g (MA-182; Marconi SA), and the absorbance of the aqueous layer was recorded at 580 nm using a light spectrophotometer (U/2000; Hitachi, Tokyo, Japan). The free mucus in gastric content was calculated from the amount of alcian blue binding (mg/wet tissue (g)).

**Assessment of prostaglandin synthesis.** Assessment was made using the PGE<sub>2</sub>-BIOTRAK (EIA, Freiburg, Germany). The rats were deprived of food for 24 h prior to the experiment, which was

**Table 1.** A low-protein (6% protein) diet was compared with AIN-93\* formulated for rodents in growth, gestation and lactation conditions

Ingredient	Normal protein (AIN-93) (170 g protein/kg)	Low protein (60 g protein/kg)
Casein (840 g protein/kg)	202.0	71.5
Maize starch	397.0	480.0
Dextrinised maize starch	130.5	159.0
Sucrose	100.0	121.0
Soyabean oil	70.0	70.0
Fibre	50.0	50.0
Mineral mix (AIN-93) <sup>†</sup>	35.0	35.0
Vitamin mix (AIN-93) <sup>†</sup>	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorhydrate	2.5	2.5

\* For a detailed composition, see Reeves *et al.* (1993).

performed between 09.00 and 11.00 hours. Groups of at least seven rats received indomethacin (20 mg/kg subcutaneously) dissolved in 5 % NaHCO<sub>3</sub>, pH 8.3. Thirty minutes after treatment, the animals were killed by cervical dislocation, and their abdomens were opened. Samples of stomach corpus (full thickness) were excised, weighed and suspended in 1 ml 10 mM sodium phosphate buffer, pH 7.4. The stomach glandular tissue was finely minced with scissors and then incubated at 37°C for 20 min. The prostaglandin content of the buffer was measured using an enzyme immunoassay (RPN222; Amersham, Little Chalfont, Bucks). This experiment was conducted according to the method of Curtis *et al.* (1995).

#### Blood collection

Heparinised blood samples were collected from the retro-orbital plexus of normal and malnourished rats (Krous, 1980) and stored in test tubes at 0°C for 4 h. The tubes were subsequently centrifuged at 3000 rpm, at 6°C for 10 min, and the sera obtained were stored at 20°C until used.

#### Determination of corticosterone and somatostatin

Concentrations of corticosterone (Mattingly, 1962) and somatostatin (Yago *et al.* 1998) were determined in serum collected from normal and malnourished rats. These sera were assessed using radioimmunoassay (ICN Biomedicals, Costa Mesa, CA, USA and EURO-DIAGNOSTICA, Malmö Sweden, respectively). Results were expressed as µg/100 plasma of corticosterone and pmol/l of somatostatin, respectively.

#### Autopsy and organ weighing

After killing, the organs of normal and malnourished rats were weighed to assess differences in growth.

#### Statistical analysis

The results were expressed as means with their standard errors. Statistical significance was determined by the Student's *t* test, and the significance level was set at  $P < 0.05$ . All statistical analyses were carried out using the Statistica software, version 5.1 (Statsoft Inc. Tulsa, OK, USA).

## Results

#### Body weight

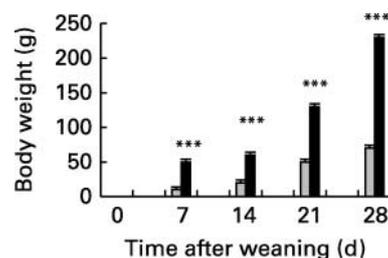
After weaning, normal and malnourished rats were weighed weekly for 1 month. The growth curve obtained indicated that normal rats had a significant increase in body weight compared with malnourished rats ( $P < 0.001$ ; Fig. 1).

#### Organ weight in normal and malnourished rats

Malnourished rats had a significant decrease ( $P < 0.001$ ) in organ weight compared with normal animals (Table 2).

#### Dosage of corticosterone and somatostatin concentrations

Before ulcer induction, corticosterone levels were significantly higher in malnourished rats (44.0 (SEM 1.22) µg/100 ml plasma)



**Fig. 1.** Body weights (g) of normal (■) and malnourished (▒) animals during 30 d after weaning (day 0). For detail of diets and procedures, see p. 2 of proofs. values are means with their standard errors represented by vertical bars for eight animals per treatment group. Mean values were significantly different from those for the malnourished rats: \*\*\* $P < 0.001$  (Student's *t* test).

**Table 2.** Organ weight of normal and malnourished rats after gastric ulcer induction with a 30% acetic acid solution

(Values are means with their standard errors for the number of rats shown)

Organs	Organ weight (g/kg body weight) normoproteic (control) rats (n 14)		Organ weight (g/kg body weight) malnourished rats (n 15)	
	Mean	SEM	Mean	SEM
Stomach	11.0	0.8	6.0	0.6 ***
Liver	33.0	9.8	18.7	0.9 ***
Heart	5.8	0.3	3.6	0.1 ***
Pancreas	4.5	0.8	1.8	0.1 ***
Mesentery	8.5	0.5	4.5	0.2 *
Kidney	8.7	0.2	3.2	0.3 ***

Mean values were significantly different from those for the normoproteic (control) group: \* $P < 0.05$ ; \*\*\* $P < 0.001$  (Student's *t* test).

than in normal rats (11.0 (SEM 1.9) µg/100 ml plasma;  $P < 0.001$ ). After ulcer induction, corticosterone levels, after two-tailed statistical analysis, were unchanged in both groups (42.3 (SEM 1.4) µg and 10.3 (SEM 1.83) µg/100 ml plasma ( $P < 0.001$ ) in malnourished and normal rats, respectively). Somatostatin levels before and after ethanol-induced damage to the gastric mucosa were the same in both groups of rats (10.0 (SEM 1.89) pmol/l and 10.2 (SEM 2.0) pmol/l, respectively).

#### Anti-ulcerogenic activity

**Ethanol and indomethacin-induced ulcers.** The oral administration of ethanol to normal rats produced the expected and characteristic zonal necrotising mucosal lesions, whereas no such lesions were seen in malnourished rats. Therefore, in normal rats, gastric lesions caused by ethanol were more severe than malnourished rats ( $P < 0.001$ ).

In normal rats, indomethacin-induced gastric ulcers showed punctuated necrotic lesions in the glandular portion of the stomach. No such lesions were seen in malnourished rats. In this experimental model, malnourished rats had a smaller number of gastric ulcers than normal rats ( $P < 0.05$ ). These last results suggest that prostaglandin production had a major role in protecting the mucosa of malnourished rats (Table 5).

**Acetic acid-induced gastric ulcers.** The administration of 30% acetic acid produced large, deep ulcers in the glandular portion of the stomach in normal and malnourished rats. Table 3 shows that gastric lesions caused by acetic acid were more

**Table 3.** Acetic acid-induced gastric ulcer in normal and malnourished rats treated for 14 d with 12% Tween 80

(Values are means with their standard errors for the number of rats shown)

Parameter	Normoproteic (control) rats (n 11)		Malnourished rats (n 13)	
	Mean	SEM	Mean	SEM
Lesion area (mm <sup>2</sup> )	26.3	1.58	51.9	1.20***
pH	3.0	0.25	2.0	0.80

Mean values were significantly different from those for the normal-protein group: \* $P < 0.05$ ; \*\*\* $P < 0.001$  (Student's *t* test).

severe in malnourished animals than in normal rats, but no difference was found in tissue pH ( $P < 0.001$ ).

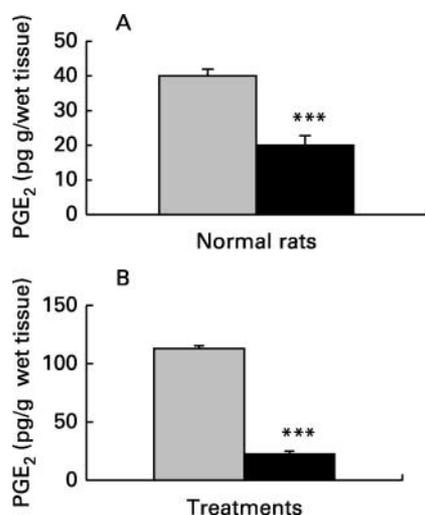
On the fifteenth day after acetic acid administration, the rats showed signs of less ulceration. These lesions, which had a punched-out appearance with steeped walls and loss of the floor, were more severe in malnourished rats. The external surface of the ulcerated region strongly adhered to the liver, which formed part of the ulcer base. Ulcer margins were poorly defined and slightly elevated as a result of submucosal oedema.

#### Prostaglandin synthesis and gastric wall mucus

Fig. 2 shows that malnourished rats had a larger prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production than normal rats, while indomethacin inhibited PGE<sub>2</sub> formation in both groups. Twice the amount of mucus was produced by the gastric mucosa of malnourished rats compared with normal rats (1.8 (SEM 1.2) g and 0.8 (SEM 1.34) g, respectively;  $n$  8;  $P < 0.001$ ).

#### Shay ulcer

The administration of 12% Tween 80 to pylorus-ligated animals significantly altered gastric volume, pH and gastric acid content in



**Fig. 2.** Gastric prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in normal (A) and malnourished (B) rats in the absence (sham; □) and presence of indomethacin (Indo; ■). For details of diets and procedures, see p.2 of proofs. Values are means with their standard errors represented by vertical bars for eight animals per treatment group. Mean values were significantly different from those for the sham group: \*\*\* $P < 0.001$  (Student's *t* test).

**Table 4.** Biochemical parameters of gastric juice (volume, total gastric acid and pH) obtained from pylorus-ligated rats

(Values are means with their standard errors for seven rats per group)

Rats	pH		Gastric juice (ml)		Total gastric acid (mEq/ml)	
	Mean	SEM	Mean	SEM	Mean	SEM
Normoproteic (control)	3.17	0.20	1.18	0.27	7.57	0.27
Malnourished	5.86	0.17*	4.05	0.31*	4.00	0.31*

Mean values were significantly different from those for the group: \* $P < 0.05$  (Student's *t* test).

malnourished animals compared with normal animals ( $P < 0.05$ ), respectively (Table 4).

#### Discussion

Malnourishment initially affects tissues with a rapid cell turnover such as the intestinal mucosa, whereas the nervous system, stomach and pancreas are often the last organs to be affected (Sant'ana *et al.* 1997). A low-protein diet administered to the mother rat up to the end of the suckling period results in pups with a decreased body weight at birth and during weaning (Latorraca *et al.* 1999). In addition, there is a permanent reduction in the size of tissues and organs, including the pancreas and stomach (Desai *et al.* 1996).

In the present study, we examined the effects of perinatal protein restriction on the gastrointestinal tract. After weaning, body weight gain was significantly higher in normal rats than in malnourished rats, indicating that maternal protein restriction during pregnancy and lactation adversely affected the offspring (Fig. 1). Malnourishment affects the digestive system in early life and may exert long-term effects on body weight. As shown in Table 2, rats subjected to protein restriction experienced marked effects on organ weight in early life, in comparison to normoproteic (control) rats.

Malnourished rats had elevated plasma corticosterone levels compared with normal rats, probably as a result of stress during pregnancy and lactation. Stone *et al.* (1986) observed that stress reduced the sensitivity of the rat cerebral cortex to noradrenaline, suggesting that an increase in the plasma levels of adrenocortical hormones could mediate the effects of stress. Corticosterone, the major adrenal steroid secreted by the rat, is released via catecholamine action during repeated stress related to malnutrition.

The gastrointestinal hormone somatostatin had a protective effect in various experimental models of gastric mucosal injury, i.e. ethanol and non-steroidal anti-inflammatory drugs (Karmeli *et al.* 1994). As shown in our work, the oral administration of ethanol (Table 5) produced characteristic zonal necrotising mucosal lesions, probably by promoting acid secretion and histamine release. In both groups of rats, histological examination also revealed damage to the oxyntic mucosa. Normal and malnourished rats showed low somatostatin levels, which failed to protect their gastric tissue from damage.

In the healthy human stomach and duodenum, there is a balance between the potential of gastric acid and pepsin to damage gastric mucosal cells and the ability of these cells to protect themselves from injury (Bagchi *et al.* 1999). Disruption of the balance results from a breakdown of the normal mucosal defence mechanisms (Forsell, 1988). Several mechanisms are believed to be important

**Table 5.** Ethanol- and indomethacin-induced gastric ulcer in normal and malnourished rats treated for 1 h with 12% Tween 80 before the induction of gastric lesions. Ulceration was observed 1 h after ethanol and indomethacin ingestion

(Values are means with their standard errors for the number of rats shown)

Ulcer inducer	Ulcerative index (mm) normoproteic (control) rats (n 7)		Ulcerative index (mm) malnourished rats (n 8)	
	Mean	SEM	Mean	SEM
Ethanol	7.0	0.53	3.0	0.70**
Indomethacin	18.0	1.32	7.0	1.29*

Mean values were significantly different from those for the normal-protein group: \* $P < 0.05$ ; \*\*\* $P < 0.001$  (Student's *t* test).

in protecting the gastric mucosa from damage, including mucus formation, mucosal blood flow, cell renewal and bicarbonate production (D'Souza & Dhume, 1991).

The extent of ethanol-induced damage to the rat gastric mucosa correlated with degranulating mast cell content (Diel & Szabo 1986), a source of several neuropeptides and inflammatory mediators, including histamine and leukotrienes. Ethanol-induced ulcers were not prevented by antisecretory agents such as cimetidine but were inhibited by agents that enhanced mucosal defence factors such as PGE<sub>2</sub> (Robert *et al.* 1979). Malnourished rats usually had a lower number of ulcers than normal rats, owing to an increased production of protective factors, for example, mucus and PGE<sub>2</sub> (Fig. 2). Among the many factors that may contribute to the gastroprotective actions of prostaglandins are phospholipid stimulation, mucus and bicarbonate secretion, maintenance of gastric blood flow during exposure to an irritant factor, inhibition of inflammatory mediator release from mast cells and inhibition of free-radical production (Sun *et al.* 1991).

Adaptation of malnourished rats to stress caused by intra-uterine malnutrition may explain the lower number of ulcers observed in these rodents. Therefore, the increase in mucus and PGE<sub>2</sub> formation and higher plasma corticosterone levels are survival mechanisms against adverse factors (Weaver *et al.* 1991). Stress hormones contribute to a hypermetabolic state that may produce a significant and dangerous body weight loss in malnourished rats. The gastrointestinal tract thus has an extraordinary capacity for adaptation (Klein & McKenzie, 1993).

Several studies have shown a severe reduction in gastric mucosal blood flow after treatment with indomethacin, a typical non-steroidal anti-inflammatory drug that inhibits cyclo-oxygenase. As a result, both mucosal prostaglandin levels and mucosal blood circulation decrease (Trevethick *et al.* 1995).

We could observe that subchronic gastric ulcers were more pronounced in malnourished rats because their low protein concentration impaired ulcer healing or favoured gastric ulcer formation (Table 3). According to Szabo & Vincze (2000), growth factors stimulate cellular elements involved in ulcer healing, for example, angiogenesis, granular tissue formation and re-epithelialisation. Basic fibroblast growth factor accelerates the healing of experimental gastric ulcers, as well as recovery from chronic erosive gastritis (Motilva *et al.* 1996). In our study, epidermal growth factor mRNA was not detected in the gastric mucosa of normal and malnourished rats (data not shown), so this result may reflect the absence of gastric healing in these rats. Similarly, in their experiments, Konturek *et al.*

(1998) found no increase in the epidermal growth factor mRNA content of the gastric mucosa.

The gastrointestinal tract in protein-restricted rats is adaptive and responds to pathological and physiological challenges, with the gastric mucosa being constantly renewed (Klein & McKenzie, 1993). Intestinal length ensures that even when a considerable portion is lost, it still has an adequate surface area to meet the nutritional demands of the animal (Weaver *et al.* 1991).

Pylorus ligation caused a significant increase in the volume and pH of the gastric juice, decreasing gastric acidity in malnourished rats (Table 4). Prostanoids are probably involved in the accumulation of fluid in the gastric lumen. Mucus and PGE<sub>2</sub> can significantly increase gastric volume in malnourished rats, as discussed earlier.

We conclude that malnourished rats subjected to acute gastric ulceration showed less mucosal damage than normal rats, because the former had a larger luminal production of PGE<sub>2</sub> and mucus, thereby increasing the volume of gastric juice. This situation is related to adaptations that occur during intra-uterine malnutrition. In subchronic experiments, malnutrition complicates the prevention of gastric ulcer formation and delays recovery. In malnourished rats, there is severe ulcer formation because stress and low dietary protein intake make the mucosa more susceptible to damage.

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