Vitamin A Deficiency Exacerbates Methotrexate-Induced Jejunal Injury in Rats\textsuperscript{1,2,3}

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ABSTRACT Two studies were conducted to investigate whether vitamin A–deficient rats were more susceptible to intestinal injury caused by methotrexate (MTX), since vitamin A deficiency alone causes only mild changes to jejunal structure and function. Weanling male rats were fed a vitamin A–deficient diet (–VA) for 40–42 d and compared to rats either pair-fed (PF) or with free access (+VA) to the same diet. Drinking water of PF and +VA rats was supplemented with 37.5 \(\mu\)g (Study 1) or 75 \(\mu\)g (Study 2) vitamin A (Rovimix A 500W)/d. Rats in each group received MTX (–VAMTX, PFMTX, +VAMTX) or vehicle. MTX administration reduced intestinal mucosal wet weight, protein and DNA concentrations, and sucrase and maltase activities in –VA and PF rats (\(P < 0.005\)). In Study 1, –VAMTX rats developed a severe jejunal enteropathy and had a higher incidence of diarrhea (\(P < 0.005\)), greater weight loss (\(P < 0.005\)), more disruption of villus architecture (\(P < 0.0001\)) and lower disaccharidase activity (\(P < 0.007\)) than PFMTX rats. Similar results were observed in Study 2. Liver retinol concentration (but no other variable) was greater in rats receiving 75 \(\mu\)g vitamin A/d (\(P < 0.001\)) than in those receiving 37.5 \(\mu\)g/d. The interaction of vitamin A deficiency and small intestinal injury may explain the efficacy of vitamin A supplementation in preventing childhood diarrheal disease mortality in developing countries, and highlights the need for ensuring adequate vitamin A status in people worldwide with diseases and/or treatments which may injure the gastrointestinal tract. J. Nutr. 127: 770–776, 1997.

KEY WORDS: vitamin A deficiency · diarrhea · rats · jejunal injury · methotrexate

Vitamin A status appears to be an important factor in childhood mortality in areas where infectious diseases are responsible for substantial childhood morbidity and mortality, such as in developing countries. Supplementation with vitamin A reduces overall mortality and that attributable to diarrhea in young children in localities where vitamin A deficiency is a major public health problem (Ghana VAST Team 1993, Rahmanullah et al. 1990, West et al. 1991). The effects on childhood morbidity are less certain, although two studies (Barreto et al. 1994, Ghana VAST Team 1993) have found a decrease in the severity of diarrhea in children who are supplemented with vitamin A.

The mechanism by which vitamin A supplementation reduces mortality is not clear. We previously determined that clinical vitamin A deficiency in rats reduced villus height and disaccharidase activity as compared to pair-fed controls, whereas subclinical vitamin A deficiency caused no changes in rat jejunal structure and function (Warden et al. 1996). Other investigators have found variable changes in intestinal structure and function in vitamin A deficient rodents (Ahmed et al. 1990, Chauhan and Kansal 1989, Majumdar and Ghosh 1987, Rojanapo et al. 1980, Singh and Krishnakantha 1987, Zile et al. 1977). Diarrhea was not observed in our vitamin A deficient rats (Warden et al. 1996), nor in other studies of intestinal structure and function in rodents with vitamin A deficiency alone (Ahmed et al. 1990, Chauhan and Kansal 1989, Majumdar and Ghosh 1987, Rojanapo et al. 1980, Singh and Krishnakantha 1987, Zile et al. 1977).

Vitamin A deficiency often occurs in environments in which gastrointestinal tracts are regularly insulted with pathogens and exogenous antigens. In light of both animal and epidemiological findings, we hypothesized that vitamin A deficiency may exacerbate an insult to the gut, such as that caused by an infectious agent (Warden et al. 1996). In the

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MATERIALS AND METHODS

Two studies were conducted to investigate the interrelationship of vitamin A status and methotrexate-induced jejunal injury. For each study, three groups of rats were used: vitamin A−deficient (−VA), pair-fed (PF) and vitamin A−sufficient (+VA). The PF and +VA rats were used to differentiate between the effect of vitamin A deficiency and malnourishment per se because rats fed a vitamin A−deficient diet consume less than those supplemented with vitamin A (Warden et al. 1996). In Study 2, the level of vitamin A supplementation was twice that of Study 1.

Animal models. Vitamin A deficiency. Subclinical vitamin A deficiency was induced in 3-wk-old, male Wistar specific-pathogen-free rats (bred from or obtained directly from the Animal Resources Centre, Perth, Australia) by feeding a pelleted vitamin A−deficient diet [catalog no. 960220, ICN Biomedicals, Sydney, Australia (Warden et al. 1996)] for 40−42 d. These rats (−VA) were fed every second day and were allowed free access to vitamin A−deficient diet and water. PF and +VA groups were also fed the same vitamin A deficient diet every second day. The PF group were pair-fed the amount consumed by the −VA group on the previous two days, whereas the +VA group were allowed free access to the vitamin A deficient diet. Both PF and +VA groups were supplemented with vitamin A (Rovimix A 500W [70% retinyl acetate and 30% retinyl palmitate], gift of Roche Australia, Sydney, Australia) added to their drinking water every second day to deliver 37.5 μg/d (Study 1) or 75 μg/d (Study 2). The volume of water in which the vitamin A was provided was determined by that consumed over the previous two days. Additional water was provided if all water containing vitamin A was consumed.

All rats were housed in a single room in a conventional animal house, in wire bottom cages. A light:dark cycle of 12 h was maintained throughout the experiments, with the temperature kept at 20−22°C. Rats were monitored daily for signs of vitamin A deficiency (Warden et al. 1996) or diarrhea. These studies were approved by the Animal Care and Ethics Committee, The University of Newcastle.

Methotrexate induced jejunal injury. After 35−37 d of dietary treatment, rats in each diet group received three consecutive daily intraperitoneal doses (7 mg/kg body weight) of methotrexate (MTX, 500 mg in 20 mL vehicle, Delta West Pty Ltd, Western Australia) (−VAMTX, PFMTX, +VAMTX) or vehicle alone (98 mg NaCl, 88 mg NaOH in 20 mL total volume) (−VA, PF, +VA). Dosage and route of administration were determined from those described in the literature as causing consistent intestinal injury in normal rats (Kosai et al. 1991, Takeuchi et al. 1989, Taminiau et al. 1980, Tsurui et al. 1990, Vanderhoof et al. 1990). Weight was recorded on the first day of MTX administration. Rats were killed 48 h after the final dose of MTX or vehicle, since in a preliminary experiment 50% (36%) of −VAMTX rats died after this time.

Tissue procurement. Rats were weighed on the day of tissue procurement, and weight was calculated as the difference between two consecutive weighings. If death or moribund status was observed on the day of weighing, the rat was killed by cervical dislocation, and the weight was recorded as the final dose of MTX or vehicle, since in a preliminary experiment 50% (36%) of −VAMTX rats died after this time.

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Biochemical analyses. The length of the jejunal segment for biochemical analyses was measured using a noninfectious model of intestinal injury. The jejunal sections were fixed in 10% buffered formalin then embedded in paraffin, sectioned and stained with haematoxylin and eosin for examination by light microscopy. The appearance of deficiencies and MTX-treated jejunal sections was scored by a single observer (blinded to treatment) as 0: no villi present (epithelial desquamation), 1: moderate to severe villus atrophy (flattening of villus tips), 2: normal or mild villus atrophy.

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Statistical analysis. Values are means ± SEM. Within each study, two-way analysis of variance was used to determine whether there was a difference in effect of MTX across dietary treatment groups. Groups receiving MTX or vehicle within each study were compared by one-way analysis of variance or Kruskal-Wallis tests to enable explicit comparison of these groups. Where differences were detected, group means were compared using a Tukey-Kramer multiple comparisons test, or for nonparametric data, medians were compared using a Mann-Whitney test (Sokal and Rohlf 1981). Chi-square analyses were performed on noncontinuous data. Differences between the studies were examined using two-way analysis of variance and chi-square analyses.

Differences were considered significant at P < 0.05. Data were analyzed using MiniTab Release 10 Xtra (Ministat, New York, NY). Rats were excluded from analyses if designated −VA but with serum or liver retinol concentrations greater than three standard deviations from the group mean (n = 2), or died (n = 3); their pair-fed controls...
not shown). PF rats had significantly higher sucrase activity days than both PF and significantly less weight was gained by –VA rats over the final four days than both PF and +VA rats (P < 0.02, Figure 1). Jejunal morphology of –VA rats did not appear different from that of PF and +VA rats, and there was no significant difference in villus injury scores among groups receiving vehicle (data not shown). PF rats had significantly higher sucrase activity than +VA rats when expressed per cm jejunum (P < 0.02, Figure 2), but not per g protein or per mg DNA (data not shown).

Methotrexate effects. Mortality. Three –VA rats died following MTX treatment, and those, with their paired controls, were excluded from analyses.

Clinical features, diarrhea and weight loss. Food intake of –VA rats declined, they became dishevelled, and in some instances, grossly emaciated following MTX administration. Diarrhea developed in 65% (11/17) of –VAMTX rats compared to 12% (2/17) of PFMTX and 22% (2/9) of +VAMTX rats (P < 0.005). Rats that received MTX lost significantly more (or gained significantly less) weight than rats that received vehicle (P < 0.005, Figure 1), and there was a significant difference in effect of MTX across dietary treatment groups (P = 0.006). Significantly more weight was lost by –VAMTX rats than PFMTX and +VAMTX rats (P < 0.02, Figure 1); PFMTX rats also lost significantly more weight than +VAMTX rats, which gained weight (P < 0.02, Figure 1).

Retinol concentrations. –VAMTX rats had negligible serum and liver retinol concentrations (Table 1). No differences in serum or liver retinol concentrations were detected between PFMTX and +VAMTX rats (Table 1).

Histology. Administration of MTX resulted in gross damage to the jejunal architecture of the –VAMTX rats, which exhibited almost total destruction of villi, with lymphocytic and red blood cell infiltration and crypt disruption (Figure 3a, b). The damage was less severe in PFMTX (Figure 3c) and +VAMTX (Figure 3d) rats, with a variable degree of villus atrophy and inflammatory infiltrate in the lamina propria. Seventy-six percent (13/17) of –VAMTX rats had a histology score of 0, compared to 12% (2/17) PFMTX and 11% (1/9) +VAMTX; in contrast, 55% (5/9) +VAMTX and 88% (15/17) PFMTX had a score of 2, compared to 17% (3/17) of –VAMTX (P < 0.0001).

Jejunal mucosal wet weight, protein and DNA. Mucosal wet weight, protein and DNA were significantly less in –VAMTX and PFMTX rats than in –VA and PF rats (P < 0.005, Figure 4). VA-MTX rats had a significantly lower mucosal wet weight than PFMTX rats (P = 0.009, Figure 4A). No differences were detected in protein or DNA content between groups treated with MTX (Figure 4B, C).

Jejunal disaccharidases. Sucrase and maltase activities were significantly less in –VAMTX and PFMTX rats than in –VA and PF rats (Figure 2), whether expressed as U/cm jejunum, U/g protein or U/mg DNA (P < 0.005), and there was a significant difference in effect of MTX across dietary treatment groups for sucrase (P = 0.007), with a trend for maltase (P = 0.058). Both sucrase and maltase activities were significantly

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<td>Serum and liver retinol concentrations of vitamin A deficient (–VA) pair-fed (PF), and vitamin A sufficient (+VA) rats treated with vehicle or methotrexate (MTX)</td>
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Notes: Values are means ± SEM. For each study, and within each MTX treatment group (vehicle/MTX), values in a column with a different superscript letter are significantly different (P < 0.02). ND = not detected. * Significantly different from same diet treatment (–MTX) in Study 2 (P = 0.005). # Significantly different from same treatment (diet, ±MTX) group in Study 1 (P < 0.02).

RESULTS

Study 1: Supplementation with 37.5 µg vitamin A/d

Induction of vitamin A deficiency. No rat exhibited clinical features of vitamin A deficiency, nor did any develop diarrhea prior to MTX administration. Weight increased in all three dietary treatment groups from commencement of diets until MTX administration. There was no difference in weight between any group of rats on the first day of MTX or vehicle administration.

Control rats. No diarrhea, nor clinical features of vitamin A deficiency occurred in any rat that received vehicle. Serum and liver retinol concentrations were significantly lower in –VA rats than PF and +VA rats (P < 0.02, Table 1). Significantly less weight was gained by –VA rats over the final four days than both PF and +VA rats (P < 0.02, Figure 1). Jejunal morphology of –VA rats did not appear different from that of PF and +VA rats, and there was no significant difference in villus injury scores among groups receiving vehicle (data not shown). PF rats had significantly higher sucrase activity than +VA rats when expressed per cm jejunum (P < 0.02, Figure 2), but not per g protein or per mg DNA (data not shown).

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VITAMIN A DEFICIENCY AND JEJUNAL INJURY

Figure 2: Intestinal disaccharidase activity of vitamin A deficient (−VA), pair-fed (PF) and vitamin A sufficient (+VA) rats treated with methotrexate (MTX) or vehicle, and expressed as U/cm jejunum for Study 1 and Study 2. Values are means ± SEM in vitamin A sufficient (+VA); pair-fed, (PF) and vitamin A deficient (−VA) rats; n = 6 (vehicle), n = 9 (MTX) per group in Study 1, and n = 5 (vehicle), n = 6 (MTX) per group in Study 2. For each panel, and within each treatment (vehicle or MTX), bars with a different superscript letter are significantly different (Study 1, sucrase, vehicle: P < 0.02, MTX: P < 0.006; maltase, MTX: P < 0.007; Study 2, maltase, MTX: P < 0.04). Within each panel, ** indicates mean is significantly different (P < 0.005), and * (P < 0.02), than the same dietary group receiving vehicle. There was no difference between Study 1 and Study 2 for any group receiving the same treatment (diet, MTX).

Results obtained in Study 2 were similar to those obtained in Study 1. The 100% greater dose of vitamin A did not result in a difference in any intestinal variable for similarly treated (diet, +/− MTX) groups of rats. Study 2 rats that received vehicle also had no difference between diet groups (−VA, PF, +VA) in any intestinal variable measured.

Methotrexate effects. Clinical features, diarrhea and weight loss. Rats treated with MTX showed similar changes in appearance as observed in Study 1. More −VAMTX rats (62%, 5/8) developed diarrhea than did PFMTX (38%, 3/8) or +VAMTX (33%, 2/6) rats (P < 0.04) in Study 2. There was also a significant difference (P = 0.006) in effect of MTX on weight loss across dietary treatment groups in Study 2. More weight was lost by −VAMTX rats (33.9 ± 2.2 g) than either PFMTX rats (8.3 ± 3.5 g) or +VAMTX rats (14.1 ± 3.7 g, P < 0.004).

Retinol concentrations. Liver retinol concentration was significantly higher in rats receiving 75 μg retinol/d than in those receiving 37.5 μg/d (P < 0.001, Table 1). However, there was no difference in serum retinol concentration between similarly treated groups of rats in Study 1 and Study 2. There was a significant difference in effect of MTX on serum retinol concentration across dietary treatment groups (P = 0.029) in Study 2. Rats +VAMTX had significantly lower serum retinol (but not liver retinol) than +VA rats (P < 0.005).

Histology. Gross damage to jejunal architecture was also observed in MTX-treated rats in Study 2, with 71% (5/7) −VAMTX rats having an injury score of 0, compared to 14% (1/7) PFMTX and 0% (0/4) +VAMTX rats (P < 0.05). Mucosal wet weight, protein and DNA concentrations were significantly less in all three groups treated with MTX than in those administered vehicle (data not shown, P < 0.05), but no differences were detected among the three MTX-treated groups for any mucosal variables.

Jejunal disaccharidases. Sucrase and maltase activities were significantly less in all groups of rats treated with MTX compared to those that received vehicle (P < 0.05, Figure 2). Maltase activity was also significantly less in the −VAMTX group.
Vitamin A–deficient rats to the toxic effects of MTX at this dose—indeed, only vitamin A–deficient rats died after methotrexate treatment.

Malnourished rats exhibit signs of host toxicity at lower doses of MTX (Torosian et al. 1988) and have greater intestinal injury (Grossie et al. 1982, Mihranian et al. 1984, Torosian et al. 1988). It was essential to use pair-fed controls since we have previously found that consumption of the vitamin A–deficient diet results in reduced food consumption by vitamin A–deficient rats compared to vitamin A–sufficient rats from the first week of consuming such a diet (Warden et al. 1996).

FIGURE 3  Jejunal morphology of rats from Study 1: a) vitamin A deficient, methotrexate treated (−VAMTX); b) −VAMTX, higher magnification, c) pair-fed control treated with methotrexate (PFMTX) and d) vitamin A sufficient, methotrexate treated (+VAMTX).

rats than in the PFMTX and +VAMTX rats (Fig. 2) when expressed as U/cm jejunum, U/g protein or U/mg DNA (P < 0.04), with a similar trend for sucrase activity when expressed as U/g protein (P = 0.06).

DISCUSSION
Vitamin A–deficient rats had severe jejunal damage when treated with methotrexate. More vitamin A–deficient rats developed diarrhea, they lost more weight, had greater disruption of villus architecture and lower disaccharidase activity than pair-fed, vitamin A–sufficient rats also treated with MTX. This effect was consistent irrespective of whether the rats were supplemented with 37.5 μg or 75 μg vitamin A/d.

Vitamin A deficiency alone did not cause any changes in intestinal variables in this study, in concordance with our previous findings in subclinically vitamin A–deficient rats (Warden et al. 1996). However, treatment with MTX resulted in consistent, major injury to the −VAMTX rats in all experiments. The same degree and consistency of injury was not observed in the vitamin A–sufficient groups that received MTX, which may be due to a greater susceptibility of the vitamin A–deficient rats compared to vitamin A–sufficient rats from the first week of consuming such a diet (Warden et al. 1996).

FIGURE 4  Mucosal variables of vitamin A deficient (−VA), pair-fed (PF), and vitamin A sufficient (+VA) rats treated with methotrexate (MTX) or vehicle in Study 1: wet weight (A), protein (B) and DNA (C). Values are means ± SEM; n = 6 (vehicle), n = 9 (MTX) per group. Mucosal wet weight was significantly less in −VAMTX (P < 0.009) than in PFMTX. Within each panel, ** indicates mean is significantly different (P < 0.005) than the same dietary group receiving vehicle.
Similarly, subclinically deficient rats (40–42 days of dietary treatment) were studied to avoid the malnutrition associated with a longer duration of vitamin A deficiency (Warden et al. 1996). However, there were no differences in intestinal variables between vitamin A–sufficient, MTX-treated rats irrespective of whether they were pair-fed or had free access to the diet, suggesting that the degree of reduced food intake observed in our vitamin A deficient rats was not enough to exacerbate the MTX injury.

Pair-fed rats were also included to control for intake of any specific nutrient such as folate, whose metabolism is effected by MTX (Jollivet et al. 1983). The necessity for controlling for both macro- and micronutrients in experiments of this nature is exemplified by the study of Kehoe et al. (1986) who concluded that rats fed an elemental diet had greater injury following MTX administration than did those fed a pelleted diet. Although energy intake was controlled for, rats fed the elemental diet consumed substantially less vitamin A, which could have contributed to the findings.

The 100% difference in vitamin A supplementation did not result in significant differences for the intestinal variables measured when similarly treated groups were compared from these two studies. As expected, the higher level of supplementation resulted in greater liver retinol concentrations in all vitamin A–sufficient groups. However, the vitamin A–sufficient rats’ liver retinol concentrations in Study I were less than that considered indicative of satisfactory vitamin A status (Battes and Olson 1987). Despite this, administration of MTX to these rats did not cause a more severe intestinal injury than in rats that received twice as much vitamin A. Although liver retinol concentration is considered a more satisfactory measure of vitamin A status than serum retinol (Underwood et al. 1979), liver retinol is mobilized more rapidly than extrahepatic retinol when insufficient vitamin A is available (Green and Green 1994), and models of retinol distribution in rats predict that relatively large pools of vitamin A may exist in extrahepatic and extravascular tissues when liver retinol is almost depleted (Green and Green 1994). An adequate store of intestinal mucosal retinol may explain why no differences were observed for intestinal variables in PF, PPMTX, +VA or +VAMTX rats irrespective of whether they received 37.5 or 75 μg vitamin A/d. Similarly it could also explain our previous findings of a significant difference in disaccharidase activity and intestinal morphology only in clinically vitamin A–deficient rats, despite both clinically and subclinically vitamin A–deficient rats having negligible serum and liver retinol concentrations (Warden et al. 1996).

The effect of vitamin A status on MTX-induced enteropathy appears to be due to an interaction between vitamin A and the action of MTX on the mucosa. However, it is unclear whether this is a direct interaction between these two agents, is mediated through other intestinal sequelae of vitamin A deficiency, or both. A reduction in crypt cell mitosis has been postulated to be the most likely cause for MTX-induced intestinal injury (Pinkerton and Milla 1984), although these authors suggest that there could also be a direct toxic effect of MTX on enterocyte protein and RNA synthesis. Whereas co-administration of vitamin A has been shown to prevent both the jejunal histological changes and the reduction in crypt cell de novo purine synthesis and pyrimidine salvage induced by MTX (Kosaki et al. 1991), we found no effect of vitamin A status on mucosal DNA concentration, whether or not MTX was administered. However, Zile et al. (1977), who also found no effect of mild vitamin A deficiency on mucosal jejunal DNA content, did find an increased jejunal crypt cell cycle time associated with a decreased rate of DNA synthesis, and Chauhan and Kansal (1989) reported a reduction in small intestinal DNA content. Additionally, although small intestinal RNA concentration has been found to be reduced (Johnson et al. 1969) or unchanged (De Luca et al. 1969) in vitamin A deficiency, addition of retinoids stimulates small intestinal RNA synthesis in vitamin A–deficient rats (Johnson et al. 1969, Zile and Deluca 1970) and sucrase-isomaltase and alkaline phosphatase mRNA synthesis in small intestinal epithelial (IEC-6) cells (Nikawa et al. 1995).

Although the effect of vitamin A deficiency on DNA and RNA synthesis could provide an explanation for a direct interaction as a mechanism for the jejunal injury observed in the −VAMTX rats, the agreement of our results with those of Ahmed et al. (1990), who infected vitamin A–deficient mice with rotavirus, suggests that other mechanisms should be considered. Indeed, vitamin A deficiency causes changes to mucosal barrier functions of the gastrointestinal tract which could predispose to or exacerbate an intestinal injury.

Vitamin A deficiency reduces mucosal immunity with decreased numbers of Peyer’s patches and immunoglobulin-bearing cells in the gut-associated lymphoid tissue (Majumder et al. 1987), Peyer’s patch T-cell proliferative response to mitogens (Majumder and Abdus Sattar 1987) and intestinal secretory IgA (Puengtonwanakul and Sirisinha 1986). A reduction in goblet cell numbers in vitamin A deficiency (Ahmed et al. 1990, Rojanapo et al. 1980, Zile et al. 1977) and mucus production (Ahmed et al. 1990) has also been detected, suggesting an impairment of nonspecific mucosal defense. Macromolecule permeability is also increased in vitamin A deficiency (Gmoshinskii et al. 1987), indicating a defect in epithelial integrity. Furthermore, vitamin A deficiency results in decreased expression of Transforming Growth Factor (TGF) β2 in lamina propria, surface epithelium and crypts of rat intestine, whereas administration of retinoic acid increases intestinal mucosal TGFβ2 and TGFβ3 expression (Glick et al. 1991). TGFβ2 have been implicated in the regulation of epithelial growth and differentiation (Gudas et al. 1994), and intestinal epithelial restitution (Dignass and Podolsky 1993), and indeed many of their actions on cells have been found to be similar to those of retinoids (Gudas et al. 1994).

The results of these studies may have not only clinical relevance for the prevention and minimization of diarrhea in children in developing countries in localities where both clinical and subclinical vitamin A deficiency are recognized as major public health problems, but also to areas where satisfactory vitamin A status is assumed, but is not necessarily present. In developed countries, diarrhea resulting from intestinal enteropathies either due to diseases such as human immunodeficiency virus (HIV) and inflammatory bowel disease, or as a result of treatments such as MTX and other chemotherapeutic agents that may injure the gastrointestinal tract, may be ameliorated by optimizing the patient’s vitamin A status in addition to conventional treatment.

In summary, vitamin A–deficient rats have a more severe jejunal injury following methotrexate administration than do pair-fed controls. A 100% increase in dose of vitamin A caused a difference in liver retinol concentration in those rats receiving vitamin A, but in no intestinal variable studied, suggesting that locally available vitamin A may be important. These results have clinical implications in the prevention and management of diarrhea in both developing and developed countries.

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LITERATURE CITED


