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Original article

Glutamine supplementation, but not combined glutamine and arginine supplementation, improves gut barrier function during chemotherapy-induced intestinal mucositis in rats

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SUMMARY

Background & aims: Increased intestinal permeability occurs during chemotherapy-induced intestinal mucositis. Previous data suggest that glutamine and arginine may have additive or synergic effects to limit intestinal damage. The present study aimed to evaluate the effects of glutamine and arginine, each alone or in combination, on gut barrier function during methotrexate (MTX)-induced mucositis in rats.
Methods: Eighty Sprague Dawley rats received during 7 days (d) standard chow supplemented with protein powder (PP), glutamine (G, 2%), arginine (A, 1.2%) or glutamine plus arginine (GA). All diets were isonitrogenous. Rats received subcutaneous injections of MTX (2.5 mg/kg) from d0 to d2. The intestinal permeability and tight junction proteins were assessed at d4 and d9 in the jejunum by FITC-dextran and by western blot and immunohistochemistry, respectively.
Results: At d4, intestinal permeability was increased in MTX-PP, MTX-A and MTX-GA rats compared with controls but not in MTX-G rats. The expression of claudin-1, occludin and ZO-1 was decreased in MTX-PP group compared with controls but was restored in MTX-G and MTX-A rats. In MTX-GA rats, occludin expression remained decreased. These effects could be explained by an increase of erk phosphorylation and a decrease of IκBα expression in MTX-PP and MTX-GA rats. At d9, Intestinal permeability remained higher only in MTX-GA rats. This was associated with a persistent decrease of occludin expression.
Conclusions: Glutamine prevents MTX-induced gut barrier disruption by regulating occludin and claudin-1 probably through erk and NF-κB pathways. In contrast, combined glutamine and arginine has no protective effect in this model.

1. Introduction

Mucositis is a common side-effect of cancer chemotherapy for which there is no current treatment. Mucositis occurs approximately in 40% of patients after standard doses of chemotherapy and in 100% of patients undergoing high-dose chemotherapy.[1,2] In addition, mucositis is a risk factor for infection that could be related to gut barrier disruption. Methotrexate (MTX), an inhibitor of dihydrofolate reductase and DNA synthesis, is widely used in cancer chemotherapy, with documented activity against leukemia, lymphoma, breast cancer, head and neck cancer. MTX treatment is associated with an increase of intestinal permeability[1,3] that is partly related to alteration of tight junction (TJ) proteins. Indeed, we recently reported that cellular distribution and expression of zonula occludens (ZO)-1, occludin and claudin-1 are altered in MTX-treated rats.[4] Hamada et al. also reported that ZO-1 phosphorylation and cellular localization are modified after MTX treatment.[5]

Abbreviations: MTX, methotrexate; TJs, tight junctions; ZO-1, Zonula Occludens-1; PP, protein powder; G, glutamine; A, arginine; GA, glutamine plus arginine.

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Nutritional interventions may be beneficial to limit chemotherapy-induced intestinal mucositis. Previous studies described intestinal protective effects of glutamine or arginine supplementation in chemotherapy-induced intestinal mucositis.[6–8] Glutamine, the preferential substrate for enterocytes, has beneficial effects in intensive care patients by limiting infectious complications and intestinal permeability.[9] In addition, glutamine is able to modulate TJ proteins in different models[10] but its effects on TJ proteins during chemotherapy-induced mucositis remains unknown. Arginine modulates immune response and promotes wound healing. To our knowledge, the effects of arginine on intestinal TJ proteins have not yet been studied *in vivo*, even if arginine restored intestinal permeability in infected pigs.[11] In addition, several studies focused on the combination of glutamine and arginine and reported either additive/synergic[12–14] or inhibitory effects.[15] We have recently assessed the effects of combined glutamine and arginine supplementation on TJs barrier function in MTX-treated Caco-2 cells,[16] showing that the combination glutamine/arginine prevented the alterations of TJ proteins and the increase of paracellular permeability but did not show additive effects compared with glutamine alone. However, as arginine improves protein synthesis and wound healing,[11] the combination glutamine/arginine should be evaluated *in vivo*.

Thus, the aim of the present study was to evaluate the effects of glutamine and arginine supplementation, each alone or in combination, on the occurrence of intestinal mucositis and in particularly on gut barrier disruption after MTX treatment in rats.

2. Materials and methods

2.1. Ethics

Animal care and experimentation complied with both French regulation and European Community regulation (Official Journal of the European Community L 358, 18/12/1986) and M.C is authorized by the French government to use animal models (authorization no 76-107).

2.2. Induction of mucositis

Rats were injected subcutaneously during the three consecutive days (d0, d1 and d2) with 2.5 mg/kg methotrexate (MTX, Teva Pharma, Courbevoie, France) or NaCl solution (0.9%) as control as previously described.[3,17]

2.3. Diets

During 1 week, eighty male Sprague–Dawley rats (225–250 g; Elevage Janvier, Le Genest St Isle, France) were acclimatized at 25 °C with a 12 h light–dark cycle. Rats were given free access to water and standard diet. At d-7, rats were randomly assigned into one of the 5 groups: 1/Control group had free access to the control diet and received subcutaneous (s.c) injections of saline; 2/MTX group (MTX-PP) had free access to the control diet and received s.c MTX injections; 3/Glutamine-supplemented MTX group (MTX-G) had free access to glutamine-supplemented diet (2%) and received s.c MTX injections; 4/Arginine-supplemented MTX group (MTX-A) had free access to Arginine-supplemented diet (1.2%) and received s.c MTX injections; 5/Glutamine and Arginine-supplemented MTX group (MTX-GA) had free access to Glutamine (2%) and Arginine (1.2%)-supplemented diet and received s.c MTX injections.

These dosages of Glutamine and Arginine have been chosen accordingly to previous studies evaluating the effects of Glutamine on intestinal inflammation[18] or the effects of Arginine on intestinal inflammation[11] and on amino acid metabolism in head-injured rats.[19]

Rats had free access to water and diets from d-7 to the euthanasia. All diets were isonitrogenous as shown in Table 1. Body weight and food intake were monitored at 24-h intervals.

2.4. Euthanasia and tissue sampling collection

In previous studies,[4,20] we showed that MTX-treated rats exhibited more marked intestinal damage at d4 that were restored at d9. Thus, in the present study, rats were euthanized at d4 or d9. Rats were deeply anesthetized with pentothal (400 mg/kg i.p.) and a laparotomy was performed. Jejunal segments were taken and rinsed with ice-cold PBS. For histological assessments, one sample of jejunum (1 cm long) was fixed in formalin (10%). Two consecutive pieces (1 cm long each) were then removed, and mucosa was scraped for the subsequent determination of proteins and mRNA expression and were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Then middle pieces (1 cm long) were removed for assessment of GSH content. Another sample of jejunum was used for the determination of intestinal permeability in Ussing chambers. For mRNA, TRIZOL® Reagent (Invitrogen, Cergy-Pontoise, France) was previously added to collecting tubes. After removing jejunal segments, rats were immediately transcardially perfused with 20 ml phosphate buffer saline (PBS, 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) followed by 30 ml 4% paraformaldehyde (PFA) in PBS as previously described.[4] The fixed segments of jejunum were collected, post-fixed for 12 h in PBS-4% PFA, dehydrated by soaking in 15% and 30% sucrose solutions, embedded in Tissue-Tek (OCT compound, Fischer Scientific, Sakura, USA) and immediately frozen at −80 °C for immunofluorescence analysis.

2.5. Villus height

For histological analysis, jejunal samples were fixed in formalin (10%), stained with hematoxylin and eosin, and examined by light microscopy. Briefly, sections were scored by the same pathologist blinded to the treatment allocation. Epithelial necrosis, inflammatory cells infiltration and villus atrophy were assessed using semi-quantitative scores which ranged from 0 (no damage) to 3 (severe damages) for each parameter as previously described.[17] Villus height was measured on 10 well-oriented villi from each rodent using the analysis software Leica QWin (Leica Microsystems).

2.6. Assessment of intestinal permeability

A 4-cm segment of jejunum was removed and placed in ice-cold Hank's Balanced Salt Solution (HBSS; 0.137 M NaCl; 5.4 mM KCl; 0.25 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 1.3 mM $CaCl_2$; 1.0 mM $MgSO_4$; 4.2 mM $NaHCO_3$; pH 7.4). The jejunum specimen was opened along the mesenteric side, and full thickness tissue preparations were mounted in Ussing chambers (Harvard Apparatus,

Table 1
Composition of nutritional supplements.[a]

Groups	Protein powder[b] (g/100 g diets)	Glutamine (g/100 g diets)	Arginine (g/100 g diets)
Control	5.53	–	–
MTX-PP	5.53	–	–
MTX-G	2.78	2	–
MTX-A	2.75	–	1.2
MTX-GA	–	2	1.2

[a] Composition of nutritional supplements added to the standard chow (RM1, Special Diets Services). Values are expressed as g for 100 g of diets. All supplements were isonitrogenous and supplied 0.76 g Nitrogen.
[b] As Protifar Plus (Nutricia Advanced Medical Nutrition, Rueil Malmaison, France).