Transpl Int (2003) 16: 866–871
DOI 10.1007/s00147-003-0645-8

ORIGINAL ARTICLE

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Glycyl-glutamine-supplemented long-term total parenteral nutrition selectively improves structure and function in heterotopic small-bowel autotransplantation in the pig

Received: 20 January 2003
Revised: 23 May 2003
Accepted: 3 June 2003
Published online: 12 August 2003

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Abstract Marked atrophy and impaired absorptive and barrier function occur in transplanted small intestinal graft during total parenteral nutrition (TPN), TPN is required by all the patients after small bowel transplantation (SBT). Glutamine (Gln) is a conditional indispensable amino acid that is not included in regimens for parenteral nutrition because of its chemical instability in aqueous solution. Glutamine-containing dipeptide, however, is heat-stable. With this study, we determine whether the glycyl-glutamine-supplemented long-term TPN improves mucosal structure and function in heterotopic transplanted small intestinal graft in the pig. Ten outbred pigs, randomly divided into two groups, underwent heterotopic small bowel autotransplantation. In the STPN group, the animals received standard TPN without glycyl-glutamine (Gly-Gln) and in the GTPN group, the animals received isonitrogenous (0.3g kg day^{-1}) and isocalories (nonprotein calories, 30 kcal kg day^{-1}) TPN with Gly-Gln (3% Gln) for 28 days. At the end of TPN, there was no significant difference in the body weight loss between two groups ($P > 0.05$). The mucosal contents of Gln and protein were significantly higher in the GTPN group than in the STPN group ($P < 0.05$). The mucosal disaccharidase activities in the homogenate of the graft mucosa of the GTPN group were significantly higher than that of the STPN group ($P < 0.05$). The villous height, surface area, mucosal thickness were significantly higher in the GTPN group than in the STPN group ($P < 0.05$). There was no significant difference in crypt depth between the two groups ($P > 0.05$). These results suggest that glycyl-glutamine-supplemented long-term TPN improves graft mucosal structure in heterotopic autotransplanted small bowel grafts in the pig. Long-term (4 weeks) TPN supplemented with Gln could alleviate small intestinal graft atrophy, but could not completely eliminate atrophy.

Keywords Glutaine · Small intestine transplantation · Total parenteral nutrition

Introduction

Improvement in immunosuppression and surgical technique has lead to the adoption of small bowel transplantation (SBT) for the patients with inadequate gastrointestinal absorptive capacity [1, 2]. Previous studies suggested that intestinal function was impaired following SBT, which included malabsorption of dietary nutrients, reduced uptake of glucose, and electrolytes from the isolated loops of small bowel and

increased permeability of loop following SBT [1, 2, 3, 4].

Total parenteral nutrition (TPN) is required for all patients in need of SBT. TPN, however, causes significant mucosal atrophy, hypofunction, decreases secretary IgA, and induces bacterial translocation [5, 6, 7]. Glutamine (Gln) is a necessary component for protein and nutrient synthesis. It is also a major substance for intestinal oxidative metabolism and essential for proliferation and differentiation of enterocytes [8, 9]. Despite its potential effects, Gln is not contained in standard commercially available amino acid solution because of its instability in aqueous solution. To overcome these drawbacks, Furst et al. proposed the dipeptides concept, which adopts stable and highly soluble synthetic Gln-containing dipeptides [9]. The stable dipeptides, such as alanyl-glutamine (Ala-Gln), or glycyl-glutamine (Gly-Gln), improves protein synthesis, increases lymphocyte count and subpopulations, maintains intestinal function and gut permeability and reduces sepsis frequency, morbidity, and mortality [3]. Gln-enriched TPN was found to improve mucosal structure and function after SBT in a rat model [10]. In this model, Yagi and his colleagues demonstrated that a Gln-enriched element diet promotes the regeneration and differentiation of the small bowel allograft during immunosuppression therapy. [11]. There are, however, many physiological differences between the digestive tract of the rat and that of the human. TPN-induced intestinal atrophy was observed within 3 days in the rat [5]. In humans, intestinal atrophy was not found in patients receiving TPN for over 2 months [6, 7]. In this study, we investigated the effect of Gly-Gln-supplemented long-term (4 weeks) TPN on the function and structure of heterotopic autotransplantation small intestine in the pig, the physiology of which is similar to that of humans [12].

Materials and methods

Animals

Outbred white pigs of both sexes, weighting 18.5–27.0 kg, were used. The animals were housed in individual cages and fed pig chow and water ad libitum for at least 5 days before the study. The cages were maintained in a light-and temperature-controlled environment. Operation was performed following an overnight fast. Anesthesia was induced with ketamine (20 mg/kg) and atropine (0.05 mg/kg) intramuscularly and was maintained with sodium pentobarbital (2%) intravenously. All animals received a venous catheter in the external jugular vein before SBT. All procedures were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985).

Surgical protocol

Small bowel autotransplantation was performed with a technique modified by Kimura [13]. A 600 cm jejunal segment was isolated on a vascular pedicel that included the jejunal artery and vein. After harvesting the segment and clamping the proximal vessel for reanastomosis, the remaining intestinal continuity was restored by end-to-end anastomosis. The isolated jejunal graft was kept in Ringer's lactated solution at 4°C until the venous effect was cleared. The graft lumen was perfused with 100 ml of 5% metronidazole solution at 4°C. The segmental jejunal graft was placed heterotopically. Revascularization was performed between the donor jejunal artery and vein and the recipient superior artery and vein. This was accomplished in end-to-end fashion with 6–0 monofilament nylon sutures. Both of ends were brought out as stomas (Thiry-Vella loop).

Grouping and regiment of TPN

After operation, ten animals were randomly divided into two groups. In the STPN group ($n=5$), the animals received standard TPN devoid of Gly-Gln; in the GTPN group ($n=5$), the animals received isonitrogenous and isocalories TPN supplemented with Gly-Gln (Glamin, Pharmacia, 3% glutamine) for 28 days. TPN provided 0.3 g of nitrogen and 33 kcal of nonprotein calories per kg body weight per day.

Study variables

Study variables included measurement of histological indices and mucosal composition. On postoperative day (POD) 29, TPN infusion was stopped, and the animals were re-anesthetized and re-laparotomized. A 2-cm length of intestinal graft (from the proximal stoma) was taken for histological examination and measurement of mucosal deoxyribonucleic acid (DNA), ribonucleic acid (RNA), Gln, protein and disaccharidase activity.

Body weight

The postoperative observation period was 28 days, animals were weighed every morning.

Mucosal contents of glutamine and protein

The mucosa of graft was scraped off the muscle layer and the scrapings were weighed, homogenized and stored at −80°C until analyzed, The mucosal protein was measured according to the method of Lowry [14], and mucosal Gln was measured with high performance liquid chromatography prescribed previously [15].

Mucosal disaccharidase activity

The specific activity of sucrase, maltase, and lactase were measured according to the method of Dalqvist [16] and expressed in micromoles of disaccharidase hydrolyzed per minute per milligram of protein, and mucosal protein was measured according to Lowry's method [14].

Histological examination

A small, full-thick segment of the graft was obtained from a standardized site and fixed in 10% buffered formaldehyde, embedded in paraffin, cut 3–5 μm thick, and then stained with hemotoxylin and eosin. The mucosal villous height, width and crypt depth were determined under light microscopy by an investigator, Dr. Wu, who was blinded to the study groups. Twenty crypts and villi were examined per animal.

Mucosal contents of DNA and RNA

The mucosal tissue was fixed and embedded in paraffin, cut into sections 3–5 μm thick, and stained with the methyl green-pyronine. Nuclear objective matching the present "area" and "shape" limits were accepted for further analysis. The integrate optical density nuclear area and contour was then recorded. The finial served as the nuclear mask definition for immunochemical staining measurement.

D-xylose absorption test

Five grams of D-xylose was instilled directly into the proximal stoma through a Foley catheter, and venous blood samples were obtained at 30, 60, 120, and 180-minute intervals. Serum xylose was determined by colorimetrically [17]. Based on these results, the absorption cure for the entire 180-minute study period was developed, and the area under the curve (AUC) was integrated.

Statistical analysis

Data are expressed mean ± SD and analyzed by variance analysis (ANOVA) with a statistical software package (SPSS 8.0). Statistical significance was accepted at the $P < 0.05$ levels.

Results

Body weight

There was no significant difference in body weight loss between the two groups. At the end of TPN, the body weight loss was 7.7% in STPN group and 5.9% in the GTPN group, respectively.

Mucosal contents of glutamine and protein

Mucosal Gln content dropped to level of about 68.5% in STPN group, whereas Gln was unsaturated in the GTPN group on POD 28, compared with pretransplantation levels. Mucosal Gln content was significantly lower in the STPN group than in the GTPN group at the end of TPN ($P < 0.05$). Mucosal protein content dropped to about 43.3% in the STPN group and 26.2% in the GTPN group at the end of TPN, respectively. No difference was observed on POD 28 compared with pretransplantation (normal values) in the GTPN group (Table 1).

Mucosal disaccharidase activity

Table 1 shows the activity of mucosal disaccharidase. The sucrase, lactase, and maltase levels were significantly lower at the end of TPN than before transplantation. The mucosal sucrase, lactase, and maltase activities were significantly higher in the GTPN group than in the STPN group at the end of TPN ($P < 0.05$).

Mucosal morphology

Atrophy of small bowel grafts was observed in both groups of grafts at the end of TPN, especially in STPN group marked atrophy was observed. Mucosal villous height and surface area were significantly decreased in the STPN group compared with those of the GTPN group and pretransplantation (normal values). There was no difference in Crypt depth between two groups on POD 28 (Table 2).

Mucosal contents of DNA and RNA

Figure 1 shows the mucosal contents of DNA and RNA. Villous contents of DNA and RNA were significantly higher in the GTPN group than in the STPN group at the end of TPN (Fig. 1, A, B, $P < 0.05$). However, no difference in crypt contents of DNA and RNA was observed (Fig. 1, C, D, $P > 0.05$).

D-xylems absorption test

Figure 2 shows the results of D-xylose absorption test. The AUC was significantly higher in the GTPN group than in the STPN group ($P < 0.05$).

Table 1 Mucosal contents of glutamine and protein and disaccharidase activity. [a] $P < 0.05$ Pretransplant vs. 28th POD, [b] $P < 0.05$ GTPN group vs. STPN group

	STPN		GTPN	
	Pretransplant	POD 28	Pretransplant	POD 28
Gln (μmol/g wet weight)	1.17 ± 020	0.40 ± 0.18[a]	1.03 ± 0.22	1.14 ± 0.20[a, b]
Protein (mg/g wet weight)	127.84 ± 27.14	72.40 ± 18.41[a]	137.50 ± 55.46	101.49 ± 22.72[a, b]
Lactase (μmol/g protein· min)	10.08 ± 1.61	5.72 ± 2.08[a]	11.06 ± 2.96	8.40 ± 1.59[b]
Sucrase (μmol/g protein· min)	12.72 ± 2.48	6.94 ± 0.99[a]	12.12 ± 2.84	10.69 ± 2.71[b]
Maltase (μmol/g protein· min)	103.02 ± 14.92	67.92 ± 13.89[a]	114.10 ± 17.41	92.76 ± 10.05[b]

Table 2 Mucosal morphometric evaluations. [a]$P < 0.05$ pretransplantation vs. POD 28, [b]$P < 0.05$ GTPN group vs. STPN group

	Pretransplantation		POD 28	
	STPN	GTPN	STPN	GTPN
Villous height (μm)	309.90 ± 33.53	325.74 ± 23.57	254.79 ± 22.18[a]	308.27 ± 52.52[b]
Villous surface (mm^2)	0.159 ± 0.030	0.166 ± 0.017	0.120 ± 0.042[a]	0.154 ± 0.019[b]
Crypt depth (μm)	179.39 ± 45.69	191.38 ± 29.19	179.06 ± 58.98	161.45 ± 21.12

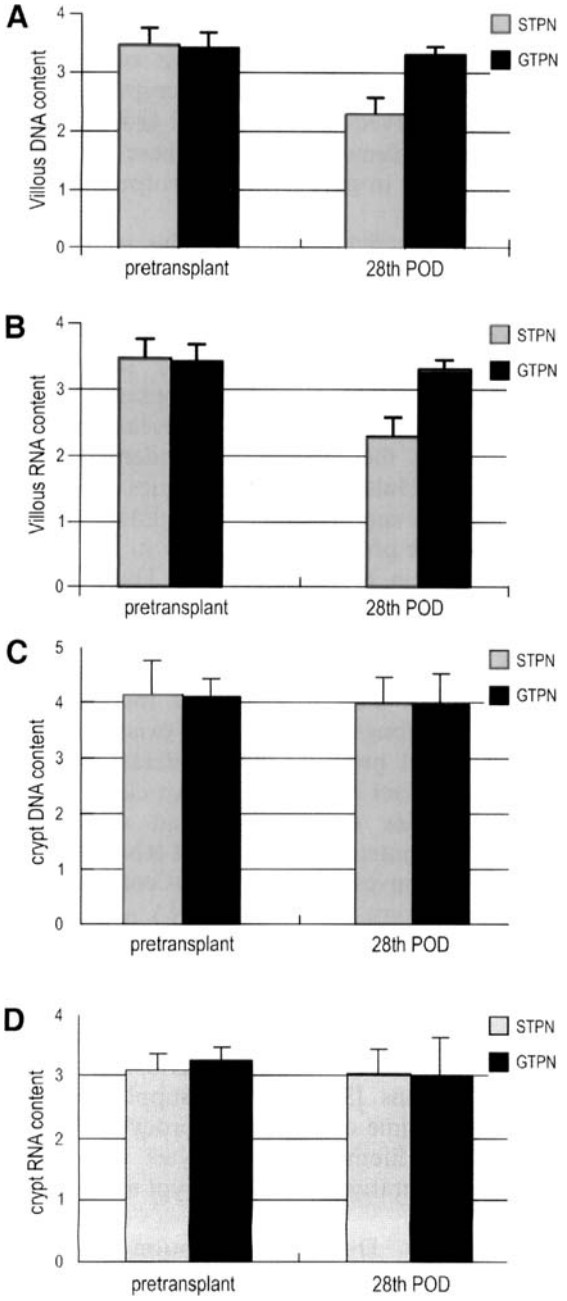

Fig. 1 Mucosal contents of DNA and RNA On POD28.Villous contents of DNA and RNA was significantly higher in the GTPN group compared with that of the GTPN group on POD 28 (**A**, **B**, $P < 0.05$). No difference in crypt contents of DNA and RNA was observed on POD 28 (**C**, **D** $P > 0.05$)

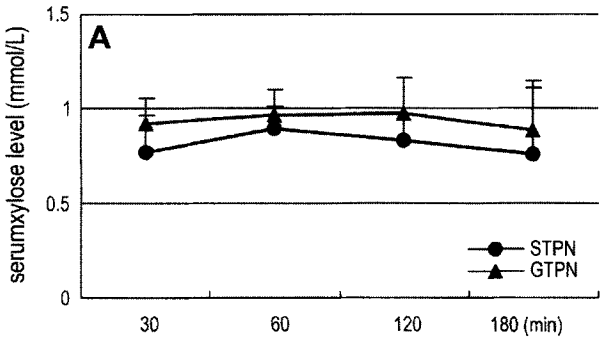

Fig. 2 D-xylose absorption test. **A** serum D-xylose level was significantly higher in the GTPN group (●) compared with that of the STPN group (▲) at 120 min after D-xylose was instilled ($P < 0.05$); **B** the AUC of D-xylose absorption test was significantly higher in the GTPN group compared with that of the STPN group ($P < 0.05$)

Discussion

This study demonstrates that Gly-Gln-supplemented long-term (4 weeks) TPN significantly improves the mucosal structure and absorptional function of heterotopically transplanted small intestinal autografts in the pig model. The improvement was characterized by a significant increase in villous height, villous surface, mucosal contents of Gln, protein, and disaccharidase activity and villous contents of DNA, RNA. This study does not show any advantage of TPN supplemented with Gly-Gln in terms of increased crypt depth and crypt contents of DNA and RNA.

Glutamine is a necessary component for protein and nucleotide synthesis, but also an important energy substrate for rapidly dividing cells including enterocytes and

lymphocytes. Furthermore, it serves homeostasis as a nitrogen and ammonium transporter to the liver and the kidney [18, 19]. It has been convincingly demonstrated that supplementation with Gln improves nitrogen balance, enhances the rate of protein synthesis [20, 21], supports immune cells [22, 23], and maintains integrity of intestinal mucosa [4, 23], decreases incidence of infection and average hospital stay [20]. It has been shown that in various stress situations, such as prolonged fasting, sepsis, trauma, and critical illness, Gln consumption by intestine is significantly increased. Nemoto and his colleagues have demonstrated that the demand for Gln increased in the preserved small bowel graft in the early postoperative period (3 days after transplantation), while the intestinal mucosa was actively regenerated. Gln is essential for proliferation and differentiation of intestinal enterocytes. Exogenous supplementation should be beneficial for the recovery of intestinal graft [24].

Ischemia/reperfusion injury is an event immediately following SBT, which causes damage to the graft mucosa. Postoperative nutritional support, such as parenteral nutrition and/or enteral nutrition is often required in the early postoperative period, until mucosal function impaired by ischemia/reperfusion injury recovers [1, 3]. Many investigations have shown the trophic effects of the glutamine dipeptide (glycine or alanine). Recently it has been shown that the composition of the glutamine dipeptide may have different physiologic effects [25]. Several publications show that glycine has anti-inflammatory and anticarcinogenic properties and shortens the surgery-induced period of immunosuppression [26].

During parenteral nutrition and /or enteral nutrition, the heterotopically transplanted small bowel graft lacks not only enteral nutrient, but also biliary and pancreatic secretion, which causes graft atrophy. All the patients in need of SBT require TPN, which causes intestinal atrophy, mucosal hypofunction and disturbed intestinal absorption function, which has been demonstrated almost uniformly in the early recovery period after SBT [27]. Gln-enriched TPN reduces bacterial translocation and improved glucose absorption and mucosal structure in orthotopic and heterotopic grafts in the rat model [10, 28]. Frankel and his colleagues [10, 28] also reports that enteral or parenteral Gln supplementation improves mucosal structure and absorptive function. All these studies were conducted in rat models. Moreover, there are species differences in TPN-induced atrophy and increased mucosal permeability, both phenomena being much more significant in rats than in humans or other animals [5]. In humans, only prolonged starvation is associated with marked changes in intestinal mucosa [6]. In adult patients, the use of TPN for up to 2 weeks did not cause mucosal atrophy. Short-term TPN in normal volunteers caused only a mild decrease in mucosal thickness which was not related to alter cell numbers [7]. Similarly, prolonged TPN in children has been shown to cause only mild focal villous atrophy [29]. In rat models, graft villous height, villous surface area and crypt depth were significantly higher in the Gln-enriched TPN group than those of the group without Gln after 14 days of TPN. After 14 days of Gln-supplemented TPN, graft villous height, villous surface area and crypt depth were the same as before transplantation.

Gln-supplemented TPN could maintain mucosal structure and function [28]. In this study, we, however, did not demonstrate any advantage of Gln-supplemented TPN in terms of increasing crypt depth. Graft atrophy was observed in both animal groups, with and without Gln-supplemented TPN. These deficits were thought to be due in part to the heterotopic location of the graft.

One of the significant results of this study was that long-term (4 weeks) TPN supplemented with Gln decreased graft villous atrophy, but not completely eliminated atrophy. This is in agreement with a study in humans. Patients who had received standard TPN without Gln for 10–14 days were biopsied at the duodenum. The endoscopies did not reveal macroscopic abnormalities of the mucosa of duodenum [28]. Recently, van der Hulst and his colleagues demonstrated that 10-day TPN supplemented with glutamine (5.9 g/l) did not affect the proliferative activity in the crypt area of the intestine in humans [30, 31]. These differences between this and other studies in rat models were due to a difference in species. The gut anatomy and physiology of the pig are more similar to those of humans than to those of the rat. This may suggest that Gln-supplemented TPN prolongs the life-time of existing cells in the gut, but does not promote the proliferative of enterocytes [32]. The exact mechanism is not clear.

Various studies demonstrate that Gln improves intestinal graft contents of DNA and RNA [10, 11, 28]. In this study, we investigate the villous contents of DNA and RNA and crypt contents of DNA and RNA. The results indicate that the villous contents of DNA and RNA is significantly higher in the GTPN group than those of the STPN group at the end of TPN. There is no difference in the crypt contents of DNA and RNA. This study is in agreement with Van der Hulst's and Platell's studies in humans [30, 32]. Gln-supplemented TPN prolongs the lifetime of existing enterocytes, but it does not promote the lifetime of enterocytes. Gln has no effect on the proliferative activity of crypt after 2 weeks of TPN [30, 32].

In this study, D-xylose absorption and mucosal disaccharidase activity were investigated. The results show that Gln improves graft D-xylos absorption and disaccharidase activity. Frankel and his colleagues [29] had demonstrated that Gln maintains the ultrastructure of graft enterocytes. Comparison of animals not receiving Gln with such receiving Gln shows that the

enterocytes are better preserved by taller and less degenerative microvilli; graft microvilli and organelles (i.e., mitochondria, Golgi apparatus, and endoplasmic reticulum) were damaged less.

In conclusion, this study demonstrates that Gly-Gln-supplemented long-term (4 weeks) TPN significantly improves the structure and absorptive function of transplanted small intestine in the pig model. The improvement is characterized by a significant increase in villous height, villous surface area, mucosal contents of Gln, protein, disaccharidase activity and villous contents of DNA and RNA. This study does not show any advantage of Gly-Gln-supplemented TPN in terms of increasing crypt depth and crypt contents of DNA and RNA. Long-term TPN supplemented with Gln could alleviate, but not completely eliminate graft atrophy.

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