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Effects of arginine-containing total parenteral nutrition on N balance and phagocytic activity in rats undergoing a partial gastrectomy

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The present study investigated the effect of arginine (Arg)-containing parenteral nutrition on phagocytic activity to elucidate the possible roles of Arg in the secretion of anabolic hormones and N balance in rats undergoing gastrectomy. Rats were divided into two experimental groups and received total parenteral nutrition (TPN). The TPN solutions were isonitrogenous and identical in nutrient compositions except for differences in amino acid content. One group received conventional TPN, the other group replaced 2 % of the total energy as Arg. After receiving TPN for 3 d, one-third of the rats in each experimental group were killed as the baseline group. The remaining rats underwent a partial gastrectomy and were killed 1 or 3 d after surgery. The results showed that there were no differences in N balance, plasma growth hormone and insulin-like growth factor-1 levels between the two groups before or after surgery. The phagocytic activity of peritoneal macrophages was higher in the Arg group than in the control group 1 d after surgery. There were no differences in the phagocytic activities of blood polymorphonuclear neutrophils between the two groups at various time points. TNF-α levels in peritoneal lavage fluid were lower in the Arg group than in the control group on post-operative day 3. These results suggest that parenterally infused Arg enhances phagocytic activity and reduces the production of inflammatory mediators at the site of injury. However, Arg supplementation did not influence the secretion of anabolic hormones nor N balance in rats with a partial gastrectomy.

Arginine: Gastrectomy: Phagocytosis: Nitrogen balance

A gastrectomy constitutes major abdominal surgery, which usually produces a moderate degree of metabolic stress to patients. It is known that visceral organ exposure and manipulation result in an intestinal hypodynamic state and dyssynchrony of intestinal enzyme secretion. These conditions may delay the resumption of oral alimentation (Finley *et al.* 1986; Tashiro *et al.* 1996). For most gastrectomized patients with gastric diseases, preoperative protein-energy malnutrition is often present and artificial nutritional support is essential for these patients. Although studies have shown that early enteral feeding was well tolerated and may be a suitable alterative to total parenteral nutrition (TPN; Braga *et al.* 1998*a*,*b*), most surgeons use the parenteral route to administer nutrients before and after a gastrectomy.

Arginine (Arg) is a semi-essential amino acid. Previous reports have shown that Arg stimulates anabolic hormone release and improves N balance (Daly *et al.* 1988; Kirk & Barbul, 1990). Studies have also revealed that Arg enhances T lymphocyte responses of surgical patients, accelerates wound healing and improves survival (Daly *et al.* 1988; Barbul *et al.* 1990; Gianotti *et al.* 1993; Cui *et al.* 2000). Previous work in our laboratory demonstrated that Arg supplementation attenuates oxidative stress, and a better *in vitro* macrophage response was observed in burned mice (Tsai *et al.* 2002). Also, enteral Arg supplementation before sepsis significantly enhances peritoneal macrophage phagocytic activity and intestinal immunoglobin A secretion in septic rats (Wang *et al.* 2003; Shang *et al.* 2004). Although meta-analysis of several studies focusing on immunonutrition indicated that Arg supplementation has no effect on infectious complications and may increase mortality in critically ill patients, immunonutrition with Arg is associated with a reduction in infectious complication rates and a shorter length of hospital stay with no adverse effect on mortality in patients undergoing elective surgery (Heyland *et al.* 2001). Arg is considered an essential amino acid for patients with catabolic conditions (De-Souza & Greene, 1998; Evoy *et al.* 1998). To our knowledge, there has been no study to date investigating the effect of Arg supplementation on gastrectomy patients. Therefore, in the present study, we infused Arg-containing parenteral nutrition in rats before and after a partial gastrectomy to investigate the effect of Arg on phagocytic activity at the site of injury and in the systemic circulation. We analysed plasma growth hormone (GH) and insulin-like growth factor (IGF)-1 levels to elucidate whether Arg supplementation enhances the secretion of anabolic hormones and thus attenuates N losses after a gastrectomy.

Abbreviations: Arg, arginine; GH, growth hormone; HBSS, Hank's buffered saline; IGF, insulin-like growth factor; PLF, peritoneal lavage fluid; PMN, polymorphonuclear neutrophil; TPN, total parenteral nutrition.

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Materials and methods

Animals

Male 7-week-old Wistar rats weighing 170–210 g at the beginning of the experiment were used. All rats were housed in temperature- and humidity-controlled rooms and were allowed free access to standard rat chow for 7 d prior to the experiment. The care of the animals followed standard experimental animal care procedures. This study was approved by the Taipei Medical University Animal Care Committee.

Study protocol and operative procedures

Rats were randomly assigned to two experimental groups, with each group containing thirty rats. The average weights between the groups were adjusted so as to be as similar as possible. After overnight fasting, rats were anaesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and the right internal jugular vein was cannulated with a Silastic catheter (Dow Corning, Midland, MI, USA) under sterile conditions. The catheter was tunnelled subcutaneously to the back of neck and exited through a coiled spring that was attached to a swivel, allowing free mobility of animals inside individual metabolic cages. All animals were allowed to drink water during the experimental period. The TPN provided 1128 kJ (270 kcal)/kg body weight per d, and the energy kJ (kcal): N (g) ratio was 606 (145):1. The calorie density was almost 4·18 kJ (1 kcal)/ml. The TPN solutions were isonitrogenous (6·84 mg/ml) and identical in nutrient composition except for the difference in amino acid content. One group received conventional TPN (control), and in the other group, 23 % of the total amino acid N was provided by Arg. Arg provided 2 % of the total energy of the TPN solution. The energy distribution of the TPN solutions in the experimental groups was 72 % from glucose, 18 % from protein and 10 % from fat (Table 1). The TPN solution was refilled daily and infused for 24 h at room temperature. On the first day, 2 ml/h were administered and then the rats received 200–238 kJ (48–57 kcal)/d according to individual body weights. The infusion rate was maintained with a Terufusion pump (model STC-503, Terumo, Tokyo, Japan). The TPN solution without fat was prepared every other day in a laminar flow hood and the fat emulsion was added daily just before use. After receiving TPN for 3 d, one-third of the rats (*n* 10) in each experimental group were killed as the baseline group. The remaining rats underwent a partial gastrectomy on the fourth day of TPN and were killed 1 or 3 d after surgery. A partial gastrectomy was performed using the same method as in our previous study (Lin *et al.* 2002). TPN was maintained for 3, 5 or 7 d according to the killing schedule of the rats.

Measurements and analytical procedures

Rats in the respective groups were killed before or 1 or 3 d after surgery. Animals were anaesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body weight). A middle abdominal incision was made and 10 ml PBS were intraperitoneally injected to elute the peritoneal cells. After harvesting the peritoneal lavage fluid (PLF), rats were exsanguinated by drawing arterial blood from the aorta. Blood samples were collected in tubes containing heparin and were immediately centrifuged. Plasma GH (Cayman Chemical, Ann Arbor, MI, USA) and IGF-1 (Diagnostic Systems, Webster, TX, USA) were determined using commercially available ELISA kits. IL-1β, IL-6 and TNF-α levels in plasma and PLF were measured using commercial ELISA microtitre plates, with antibodies specific for rat IL-1β, IL-6 and TNF-α having been coated on to wells of the microtitre strips provided (Amersham Pharmacia Biotech, Amersham, UK). NO is highly unstable in solution and cannot be readily assayed. However, NO is converted to stable nitrite and nitrate ions in aqueous solution. After conversion of nitrate to nitrite using nitrate reductase, nitrite concentrations were measured using the Griess reagent. The concentrations of NO_2^-/NO_3^- in plasma and PLF were determined with a commercial kit (Assay Designs, Ann Arbor, MI, USA). Procedures followed the manufacturer's instructions.

A flow cytometric phagocytosis test was used to evaluate the phagocytic activity of blood polymorphonuclear neutrophils (PMN; Böhmer *et al.* 1992; Schiffrin *et al.* 1995). Heparinized whole blood (100 μl) was aliquoted on the bottom of 12 × 75 mm Falcon polystyrene tubes (Becton Dickinson, Fullerton, CA, USA) and placed in an ice-water bath. Precooled opsonized fluorescein isothiocyanate-labelled *Escherichia coli* (20 μl; Molecular Probes, Eugene, OR, USA) was added to each tube. Control tubes remained on ice, and assay samples were incubated for precisely 10 min at 37°C in a shaking water-bath. After incubation, samples were immediately placed in ice water and 100 μl precooled trypan blue (Sigma, St. Louis, MO, USA) solution (0·25 mg/ml in citrate salt buffer; pH 4·4) were added to quench the fluorescence of the bacteria merely adhering to the surface of the phagocytizing cells. Cells were washed twice in

Table 1. Formulation of the total parenteral nutrition solution (ml/l)

	Arg-supplemented	Control
50 % glucose	420	420
Lipofudin 20 %	50	50
Moriamine* 10 %	345	450
$NaCl_3$ 3 %	35	35
K_3PO_4 8·7 %	10	10
KCl 7 %	10	10
Calcium gluconate 10 %	10	10
$MgSO_4$ 10 %	4	4
$ZnSO_4$ 0·6 %	2	2
Infuvita†	8	8
Choline chloride (g)	1	1
Arg (g)	5	–
H_2O	105	–
Total volume	999	999
Total kcal	986	994

* From Chinese Pharmaceuticals, Taipei, Taiwan. Each 100 ml contains (mg): Leu 1250; Ile 560; Lys acetate 1240; Met 350; Phe 935; Thr 650; Trp 130; Val 450; Ala 620; Arg 790; Asp 380; Cys 100; Glu 650; His 600; Pro 330; Ser 220; Tyr 35; aminoacetic acid (Gly) 1570.

† From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Each ml contains: ascorbic acid 20 mg; retinol 200 μg; ergocalciferol 1 μg; thiamine HCl 0.6 mg; riboflavin 0.72 mg; niacinamide 8 mg; pyridoxine HCl 0.8 mg; *d*-panthenol 3 mg; *dl*-α-tocopheryl acetate 2 mg.

Hank's buffered saline (HBSS), and erythrocytes were lysed by the addition of a fluorescence-activated cell sorter lysing solution (Becton Dickinson). After an additional wash in HBSS, 100 μl propidium iodide solution (1 μg/ml in HBSS) were added to stain the nuclear DNA 10 min before the flow cytometric analysis. Flow cytometry was performed on a fluorescence-activated cell sorter Calibur™ flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. A live gate was set on the red (propidium iodide) fluorescence histogram during acquisition to include only those cells with a DNA content at least equal to human diploid cells. The number of cells with phagocytic activity did not exceed 6 % at 0°C.

Because isolated peritoneal macrophages tend to aggregate and adhere to the culture plates and adherent macrophages have stronger phagocytic activity than those suspended in solution, we used a Vybrant™ phagocytosis assay kit (Molecular Probes) instead of the flow cytometric method to evaluate the phagocytic activity of peritoneal macrophages. After washing the peritoneal macrophages three times with HBSS, the cell concentration was determined, and the cell number was adjusted to 10^6 cells/ml with RPMI-1640 supplemented with 5 % fetal bovine serum and an adequate amount of antibiotics. After distributing 100 μl diluted solutions into each well of 96-well microplates, it was transferred to a 37°C CO_2 incubator for 1 h to allow the cells to adhere to the microplate surface. The RPMI solution was removed from all microplate wells by vacuum aspiration, and then 100 μl prepared fluorescein isothiocyanate-labelled *E. coli* were added to each well for 2 h. Labelled bacteria were removed by vacuum aspiration, and 100 μl trypan blue suspension were added to all wells within 1 min. The excess trypan blue was immediately aspirated, and the experimental and control wells (without peritoneal macrophages) were read in the fluorescence plate reader using approximately 480 nm for excitation and approximately 520 nm for emission.

During the three infusion days after surgery, 24 h urine specimens were collected for determination of the N balance. Non-protein N in the urine was measured by a colorimetric method (Randox, Antrim, Northern Ireland).

Statistical analysis

Data are expressed as the mean value with the standard deviation. Differences among groups were analysed by ANOVA using Duncan's test. A value of $P < 0{\cdot}05$ was considered statistically significant.

Results

There were no differences in initial body weights between the two experimental groups. All rats gained weight after the TPN infusion, and the weight was maintained post-operatively (data not shown).

A negative N balance was observed after surgery. There was no difference in the N balance between the two groups on various post-operative days (Fig. 1). Compared with levels before surgery, plasma GH concentrations were significantly lower after surgery in the experimental groups on both post-operative days 1 and 3; however, there were no differences in GH or IGF-1 levels between the two groups before or after surgery (Fig. 2(A, B)). Concentrations of NO_2^-/NO_3^- in plasma and PLF were significantly higher after surgery than pre-operative day. No differences were observed between the two groups at different time points (Fig. 3(A, B)). The phagocytic activity of peritoneal macrophages was higher in the Arg group than the control group on post-operative day 1 (Fig. 4(A)). The phagocytic activity of blood PMN was significantly higher after surgery than at the baseline, regardless of whether or not Arg was given. There were no differences in the phagocytic activity of blood PMN between the two groups before or after surgery (Fig. 4(B)). Plasma IL-1β, IL-6 and TNF-α levels were undetectable. TNF-α levels in PLF were significantly lower in the Arg group than the control group on post-operative day 3 (Table 2).

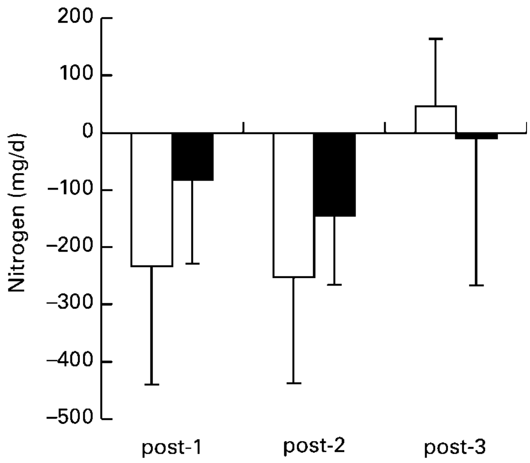

Fig. 1. Nitrogen balance between the control (□) and arginine-supplemented (■) groups after the operation. No significant differences were observed between the two groups on various post-operative days. post-1, post-operative day 1; post-2, post-operative day 2; post-3, post-operative day 3.

Discussion

In the present study, 2 % total energy was supplied by Arg. This amount of Arg was previously found to enhance the immune response in rodents (Saito *et al.* 1987; Gianotti *et al.* 1993). We administered TPN before and after a gastrectomy, this model mimics the usual treatment for patients who are scheduled to undergo a gastrectomy. Human studies may encompass wide variations owing to the ages of patients, severity of the diseases, areas of the stomach involved and complications of other diseases; these variables may make interpretation of the data difficult. We used an animal model with a partial gastrectomy to investigate the effect of Arg on the catabolic and immune responses after abdominal surgery.

After an abdominal operation and trauma, a negative N balance with progressive loss of body protein is observed, possibly resulting from hormonal changes and cytokine secretion (Fong *et al.* 1990; Baigrie *et al.* 1992). A report by Oka *et al.* (1993) showed that TPN with Arg improved the host N balance in tumour-bearing rats.

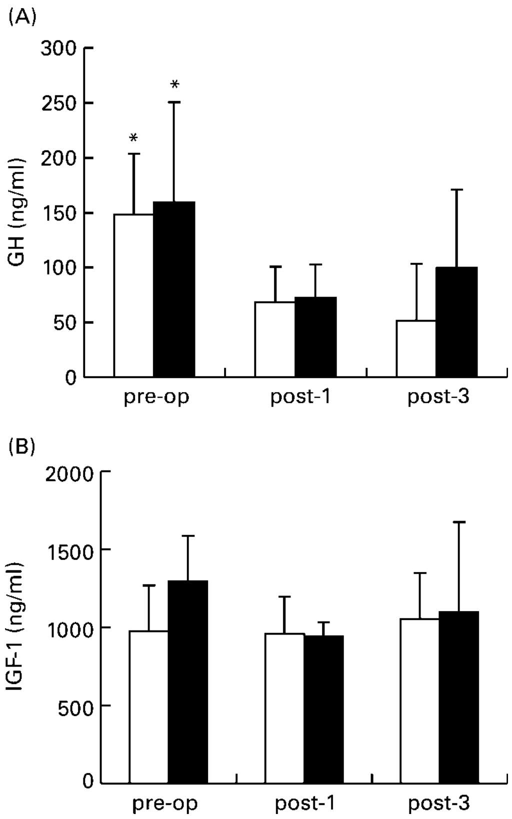

Fig. 2. Plasma growth hormone (GH; A) and insulin-like growth factor-1 (IGF-1; B) concentrations between the control (□) and arginine-supplemented (■) groups before and after the operation. Mean values were significantly different from the corresponding group post-operatively: $^{*}P<0{\cdot}05$. No significant differences in plasma GH and IGF-1 levels were observed between the two groups pre- or post-operatively ($P>0{\cdot}05$). pre-op, pre-operative; post-1, post-operative day 1; post-3, post-operative day 3.

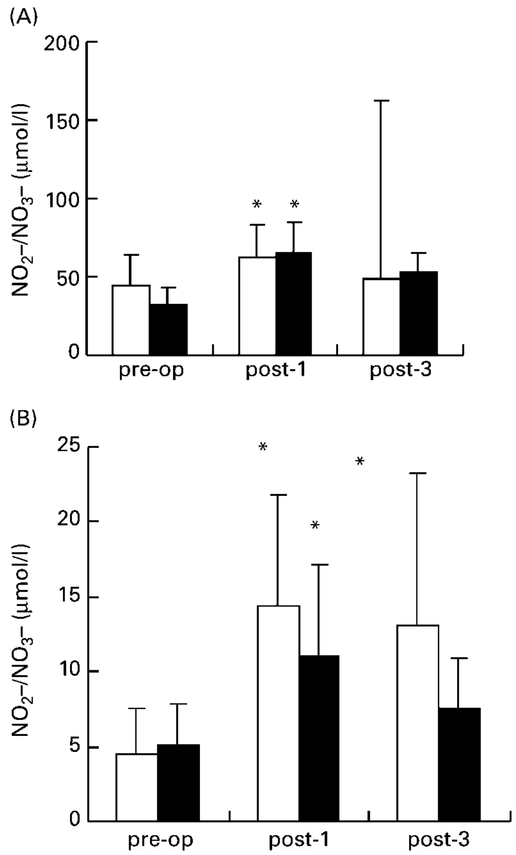

Fig. 3. Nitric oxide concentrations in plasma (A) and peritoneal lavage fluid (B) between two groups (□, control; ■, arginine-supplemented) before and after surgery. Mean values were significantly different from the corresponding group on pre-operative day: $^{*}P<0{\cdot}05$. No differences were observed between the two groups pre- or post-operatively ($P>0{\cdot}05$). pre-op, pre-operative; post-1, post-operative day 1; post-3, post-operative day 3.

Further, Barbul *et al.* (1984) reported that a high Arg infusion decreased N loss in rats with a femoral fracture. These reports were inconsistent with our results, which showed that Arg had no effect on reducing protein catabolism. However, the present findings are similar to a previous report by our laboratory that parenterally administered Arg had no effect on preventing N loss in septic rats (Yeh *et al.* 2002). GH is known to exert many metabolic effects. Among them are N retention and preservation of muscle protein mass (Ponting *et al.* 1988; Jiang *et al.* 1989). IGF-1 is one of the major effectors of GH's action. The effects of GH are mediated in part by IGF-1, which is produced in the liver and locally in GH target tissues (Isgaard *et al.* 1986). A study by Daly *et al.* (1988) showed that neither plasma GH and IGF-1 levels nor the N balance differed between Arg and control groups during post-operative days 1–5. However, plasma IGF-1 levels were significantly increased in the Arg-supplemented group on post-operative day 7, concomitant with a better N balance in surgical patients. In the present study, we observed no difference in GH and IGF-1 levels after Arg supplementation and this may partly explain the obscure difference in the N balance between groups. Since the N balance was only noted for 3 d, determining whether Arg supplementation changes the anabolic hormone secretion and thus improves the N balance over a longer period requires further investigation.

In this study, we found that the phagocytic activity of peritoneal macrophages was much higher in the Arg group after surgery compared to the control group, whereas no differences in the phagocytic activities of blood PMN between the two groups were found. These findings were similar to those of a report that found that enteral Arg supplementation enhanced peritoneal macrophage phagocytic activity in septic rats (Wang *et al.* 2003). PMN are potent inflammatory cells, and the total number and percentage of circulating PMN can be induced by acute infection and endotoxins (Ringer & Zimmermann, 1992). It is possible that a partial gastrectomy as performed in the present study resulted in moderate metabolic stress and the rats were free of infection that causes a systemic stress. Therefore, Arg augments phagocytic activity at the site of injury, but the effect of Arg on phagocytic cells in the systemic circulation was not obvious. Arg is a substrate for inducible NO synthase and a precursor of NO. The inflammatory cytokine may activate macrophage inducible NO synthase activity and improve bactericidal mechanisms via the Arg–NO pathway (Gianotti *et al.* 1993). Macrophages also secrete arginase. Induction of arginase

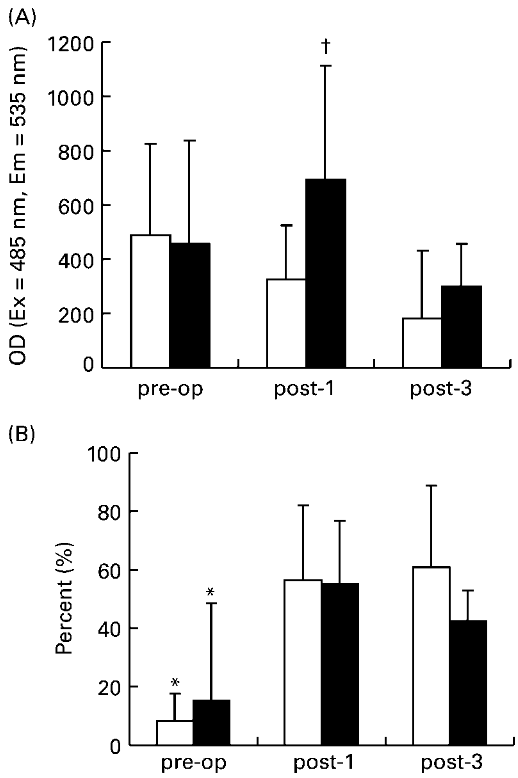

Fig. 4. (A), Phagocytic activity of peritoneal macrophages measured by a phagocytosis assay and read in a fluorescence plate reader using 480 nm for excitation and 520 nm for emission (□, control; ■, arginine-supplemented groups). Mean values were significantly different from the corresponding group on post-operative day 1: †$P<0·05$. (B), Peripheral blood neutrophils measured by flow cytometry. No significant differences were observed between the control (□) and arginine-supplemented (■) groups pre- or post-operatively ($P>0·05$). pre-op, pre-operative; post-1, post-operative day 1; post-3, post-operative day 3.

Table 2. Peritoneal lavage fluid IL-1β, IL-6 and TNF-α concentrations between the control and arginine-supplemented groups before and after the operation

(Mean values in pg/ml with their standard errors)

	Pre-operative (*n* 10)		Post-operative day 1 (*n* 10)		Post-operative day 3 (*n* 10)	
	Mean	SE	Mean	SE	Mean	SE
IL-1β						
Control	10.1	6.8	13.6	13.6	17.9	22.6
Arg	6.8	5.9	10.6	7.5	10.7	6.0
IL-6						
Control	88.9	46.1	130.0	21.7	131.5	50.8
Arg	92.4	14.0	166.7	103.4	84.2	5.1
TNF-α						
Control	24.0	16.6	10.2	8.3	54.7	28.5*
Arg	12.7	5.3	13.5	8.9	15.0	14.6†

* Significant difference from the pre-operative and post-operative day 1 groups in the same row at $P<0·05$.
† Significant difference from the control group at $P<0·05$.

has been proposed as the effector in macrophage-mediated cytotoxicity (Currie, 1978). In this study, NO levels in plasma and PLF did not differ between two groups pre- or post-operatively. It is possible that NO synthesis in response to metabolic stress in this study was already at a peak (Lindblom *et al.* 2000). This finding may indicate that phagocytic activity was not influenced by NO. We speculated that arginase expressed by the peritoneal macrophage plays a role in enhancing phagocytic activity at the site of injury. We did not analyse arginase in PLF because the resident peritoneal macrophage contained essentially no arginase activity when assayed immediately after harvest (Albina *et al.* 1988). Whether arginase expression by the macrophage is responsible for enhancing peritoneal macrophage phagocytic activity requires further investigation.

Surgical injury and infection stimulate the production of a variety of endogenous mediators. TNF-α, IL-1β and IL-6 are major pro-inflammatory mediators in the acute-phase response (Ertel *et al.* 1991). Although these cytokines have beneficial effects after injury, exaggerated or prolonged secretion of these proteins is detrimental to the host (Fong *et al.* 1990; Baigrie *et al.* 1992). Studies have proposed that alterations in TNF-α and IL-6 can be used as biochemical markers of the stress response (Foex & Shelly, 1996). IL-6 is thought to be the most consistently identified cytokine mediator of post-injury infections (Biffl *et al.* 1996). High plasma concentrations of IL-1 and TNF-α are associated with increased severity of inflammatory diseases (Foex & Shelly, 1996). These cytokines in plasma were not detectable at the times we took measurements. However, cytokines in the PLF were measurable. Compared with the baseline, IL-1β and IL-6 levels did not change after surgery. This result may indicate that post-injury infection was not obvious in this study. We observed that TNF-α was lower in the Arg than in the control group on post-operative day 3. This might mean that TPN with Arg reduces the production of inflammatory mediators at the site of injury after surgery. Some investigators have suggested that the beneficial effect of oral Arg supplementation on the immune system is distinct from its effect on N metabolism (Daly *et al.* 1988). Under the present experimental conditions, the priority of Arg in alleviating the inflammatory reaction and promoting local phagocytic activity may have been superior to that for N metabolism. Further, the disease conditions in various reports differed, and so the effect of Arg on protein metabolism may vary.

In summary, the present study showed that parenterally infused Arg enhances phagocytic activity of peritoneal macrophage and reduces the production of inflammatory mediator TNF-α at the site of injury. However, other parameters including anabolic hormone secretion and N balance were not affected by the treatment.

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