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Burns 29 (2003) 43–48

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Effects of dietary arginine supplementation on antibody production and antioxidant enzyme activity in burned mice

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Accepted 20 August 2002

Abstract

This study investigated the effect of arginine (Arg) supplementation on specific antibody production and antioxidant enzyme activities in burned mice vaccinated with detoxified *Pseudomonas* exotoxin A linked with the outer membrane proteins I and F, named PEIF. Also, the survival rate of burned mice complicated with *Pseudomonas aeruginosa* was evaluated. Experiment 1: Thirty BALB/c mice were assigned to two groups. One group was fed a control diet with casein as the protein source, while the other group was supplemented with 2% Arg in addition to casein. The two groups were isonitrogenous. The mice were immunized twice with PEIF, and the production of specific antibodies against PEIF was measured every week. After 8 weeks, all mice received a 30% body surface area burn injury. Mice were sacrificed 24 h after the burn. The antioxidant enzyme activities and lipid peroxides in the tissues as well as the specific antibody production were analyzed. Experiment 2: Twenty-eight mice were divided into two groups and vaccinated as described in experiment 1. After the burn the mice were infected with *P. aeruginosa*, and the survival rate was observed for 8 days. The results demonstrated that antioxidant enzyme activities and lipid peroxides in tissues were significantly lower in the Arg group than in the control group after the burn. The production of specific antibodies against *P. aeruginosa* significantly increased in the Arg group at 4 and 7 weeks after immunization, and 24 h after the burn. The survival rates of vaccinated burned mice after bacterial infection did not significantly differ between the two groups. These results suggest that vaccinating mice with Arg supplementation may enhance humoral immunity and attenuate the oxidative stress induced by burn injury. However, Arg supplementation did not improve survival in vaccinated mice complicated with *P. aeruginosa* infection.
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Keywords: Burns; Arginine; Antioxidant enzyme activity; Antibody; Vaccination; *P. aeruginosa*

1. Introduction

A burn injury can give rise to a post-traumatic inflammatory disease. Major burns are often associated with secondary damage to tissues distant from the injured skin [1–4]. Nishigaki et al. [5] reported that lipid peroxide levels increase in burned rat skin, and that lipid peroxide generated in the burn wound accumulates in the liver, lung, kidney, and gut of injured animals. In addition, a characteristic and critical feature of burn injury is a decrease in host resistance to infection. This complication has been related to a depression of both humoral and cellular components of the host defense system [6–9].

Arginine (Arg) is a semi-essential amino acid. Previous reports have shown that Arg stimulates anabolic hormone release and improves nitrogen balance [10,11]. Studies have also revealed that Arg enhances T lymphocyte responses for surgical patients [10], accelerates wound healing, and improves survival when Arg is supplemented in the diet of humans and injured animals [12–15]. A report by Stinnett et al. [16] showed that after severe burn injury, plasma Arg declined 30–40%. Dietary Arg supplementation replenishes the Arg level in plasma [17]. A study by Cui et al. [18] showed that dietary Arg supplementation promotes protein anabolism and attenuates muscle protein catabolism after thermal injury. Previous work in our laboratory demonstrated that Arg supplementation attenuates oxidative stress at the hypercatabolic stage after burn injury. Also, a better in vitro macrophage response was observed [19]. Arg is considered a conditionally essential amino acid in burn patients [17,20].

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PII: S0305-4179(02)00243-7

Pseudomonas aeruginosa is an opportunistic pathogen that often infects burn patients [21,22]. Therapy for *P. aeruginosa* infection is hindered by its well-known antibiotic resistance [23]. Production of specific antibodies is important for resolving bacterial infections, because antibodies neutralize the bacterial toxins and attract phagocytic cells to ingest and kill the bacteria. Saito et al. [24] demonstrated that Arg supplementation improved survival rates in a non-infected burned animal model. To our knowledge, there is no study, so far, investigating the effect of Arg supplementation on the production of specific antibodies and the potential benefit of Arg on survival rates in burned animals complicated with infection. We have designed a novel vaccine, PEIF, against *P. aeruginosa*, which can effectively block *P. aeruginosa* challenge in burned mice [25]. The chimeric protein is composed of the receptor binding and membrane translocation domains of *Pseudomonas* exotoxin A (PE) linked with the outer membrane proteins I and F, together designated as PEIF [25]. In this study, we immunized mice with this novel vaccine against *P. aeruginosa* before burn injury to investigate whether Arg supplementation has beneficial effects on antioxidant enzyme activity, T lymphocyte subpopulations, and specific antibody production against PEIF. In addition, the survival rate in vaccinated burned mice complicated with a lethal dose of *P. aeruginosa* was also evaluated.

2. Materials and methods

2.1. Animals

Male BALB/c mice weighing 10–15 g (4 weeks of age) were used in this study. All mice were housed in temperature- and humidity-controlled rooms, and allowed free access to standard chow for 1 week prior to the experiment. Animals included in this study were kept under standard experimental animal care protocols.

2.2. Study protocol

2.2.1. Experiment 1

Thirty mice were randomly assigned to two groups, 15 mice to a group. One group was fed a control diet (control), in which all amino acids were provided by casein. The other group was fed arginine (Arg), by which 2% of total kcal was Arg in addition to casein. Both diets were isonitrogenous (Table 1). Mice were anesthetized with ether, and blood was taken from the retrobulbar vessels before immunizing with the novel PEIF vaccine against *P. aeruginosa*. The production and purification of the recombinant PEIF protein followed procedures described previously [25]. The emulsified vaccine was prepared by mixing the purified recombinant PEIF protein with an equal volume of complete Freund's adjuvant, and then each mouse was vaccinated subcutaneously at a dose of 2 μg per mouse on day 1. A booster injection was given at a dose of 4 μg per mouse of PEIF emulsified with an equal volume of incomplete Freund's adjuvant on day 28. Before the burn, immunized mice were bled (50 μl) from the retrobulbar vessels on days 21, 28, 35, 42, 49 and 56. The respective sera were isolated and stored at −70 °C until assay. After 8 weeks, a modification of the burned mouse procedure was used [26,27]. Mice were anesthetized with sodium pentobarbitol (0.71 μl/g body weight) and shaved dorsally prior to burning. A Teflon template with a precisely cut window (2.5 cm × 3 cm) was pressed firmly against the shaved back. Ethanol (95% (v/v), 0.5 ml) was evenly spread over the area of the back outlined by the window, ignited, and allowed to burn for 15 s [25,26]. Animals were immediately resuscitated by an intraperito6neal injection of sterile 0.9% saline (10 ml/100 g body weight) [28]. This procedure produced a full-thickness burn injury on approximately 30% of the total body surface area. They were deprived of food for 24 h with only free access to water, in order to induce a hypermetabolic state in the burned mice [29]. These experimental conditions simulate metabolic disorders observed in burn patients [29,30]. Mice were anesthetized and sacrificed by cardiac puncture 24 h after the burn. Blood samples for analysis of T lymphocyte subpopulations were collected in tubes containing heparin, and other blood samples were centrifuged to isolate the sera. Other tissues including liver, lungs, and kidney were rapidly excised. All samples were stored at −70 °C until being assayed.

Table 1
Composition of the experimental diets (g/kg)

Component	Arg	Control
Casein	200	248
Arginine	24	–
Protein N	39.7	39.7
Soybean oil	50	50
Corn starch	470	446
Salt mixture[a]	35	35
Vitamin mixture[b]	10	10
Methylcellulose	30	30
Choline chloride	1	1
DL-Methionine	3	3
Sucrose	200	200

[a] Salt mixture contains the following (mg/g): calcium phosphate diabasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 220; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; curpric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate, 0.55.

[b] Vitamin mixture contains the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.02; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; menaquinone, 0.005.

2.2.2. Experiment 2

Twenty-eight mice were divided into two experimental groups, with each group containing 14 mice. All mice were immunized twice with the novel PEIF vaccine against *P.*

aeruginosa and were fed control or Arg diets for 8 weeks as described in experiment 1. After 8 weeks, burn injury was induced and *P. aeruginosa* strain PAO1 (ATCC 15692; in 0.2 ml PBS with about 2× LD_{50} of 3.2×10^5 CFU) was immediately subcutaneously injected into the burned area. The mice were also deprived of food for 24 h with only free access to water as mentioned above. Survival of the burned mice was noted every 6 h in the first 3 days, and then every 12 h until the end of 8 days.

2.3. Measurements of antioxidant enzymes and TBARS

A 15% tissue homogenate was prepared at 4 °C in 0.01 M phosphate buffer (pH 7.4) with 1.15% KCl, using a homogenizer [31]. Homogenates were centrifuged at $12000 \times g$ for 20 min to remove cell debris and mitochondria. The supernatant was used for analysis of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities (enzyme kits of Randox, Antrim, Ireland) as described previously [32]. Protein concentrations of supernatants were measured using Lowry et al.'s method [33]. The production of thiobarbituric acid-reactive substances (TBARS, assumed to be mainly malondialdehyde and its precursors) in mouse liver, lung, and kidney homogenates was determined by the method of Uchiyama and Mihara [34]. The molar extinction coefficient of malondialdehyde was assumed to be 156,000 [31].

2.4. Analysis of specific antibody production against PEIF

The specific antibody production of vaccinated mice was measured by ELISA as described previously [35]. Briefly, purified recombinant PEIF protein was coated on polyvinylchloride, flat-bottom, 96-well Falcon microtiter plates overnight at 4 °C with a protein concentration of 3 μg/ml in coating buffer (pH 9.6 carbonate buffer). The coated plates were then blocked with 0.5% BSA–PBS. Mouse sera from each group were diluted 1000-fold with 0.5% BSA–PBS, and 50 μl of diluted sera was added to the coated well and incubated for 1 h at 37 °C. Bound specific antibodies were detected using peroxidase-conjugated goat anti-mouse secondary antibody (Sigma). After three washings, 100 μl of substrate solution (0.54 mg/ml 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 0.03% H_2O_2 in 0.1 M citric acid) was added to each well, and the absorbance was read after 15 min using a microplate reader at 405 nm. Normal mouse serum was used as the negative control.

2.5. Analysis of T lymphocyte subpopulations

Flow cytometry was used to determine the proportions of $CD4^+$ and $CD8^+$ T lymphocytes in fresh blood. One hundred microliters of blood was incubated for 15 min at 4 °C with 10 μl of fluorescein-conjugated (FITC) mouse monoclonal anti-mouse $CD4^+$ (0.1 mg/ml) and phycoerythrin-conjugated (PE) mouse anti-mouse $CD8^+$ (1 mg/ml) (Serotec, Oxford, UK). After this, red blood cells were lysed with lysing buffer (Serotec). Fluorescence data were collected on 5×10^4 viable cells and analyzed by flow cytometry (Coulter, Miami, FL, USA).

2.6. Statistics

Data are expressed as the mean ± S.D. Differences among groups were analyzed by analysis of variance using Duncan's test. Survival rate was measured by Kaplan–Meier survival analysis. A P-value <0.05 was considered statistically significant.

3. Results

There were no differences in initial body weights and weights after experimental diets for 8 weeks between the two experimental groups in either experiment 1 or 2 (data not shown). There were no differences in the percentages of CD4, CD8, and CD3 T cells or the CD4/CD8 T cell ratio between the Arg and control groups after the burn (Table 2). Antibody production increased logarithmically after the second booster and reached a plateau after 7 weeks (data not shown). The specific antibody production in the Arg group was significantly higher than in the control group at various times (Fig. 1). The SOD and GSHPx activities in liver, lung, and kidney homogenates were significantly lower in the Arg group than in the control group after the burn (Figs. 2 and 3). Also, lipid peroxidation products in liver and kidney homogenates were significantly lower in the Arg group after the burn than in the control group (Fig. 4).

In experiment 2, there were 13 survivors among the 14 mice in the Arg group, and 10 survivors in the control group after challenge with 2× LD_{50} of *P. aeruginosa* to vaccinated burned mice and observing them for 8 days. The survival rate of vaccinated burned mice in the Arg group tended to be higher than that of the control group after bacterial infection, however, no statistically significant difference was observed between the two groups (Fig. 5).

Table 2
Blood CD4, CD8, CD3 cells and the CD4/CD8 ratio between the two groups after the burn

	CD4 (%)	CD8 (%)	CD4/CD8	CD3 (%)
Arg	29.4 ± 6.1	12.1 ± 1.5	2.4 ± 0.3	46.9 ± 10.5
Control	29.3 ± 4.1	11.2 ± 2.0	2.6 ± 0.5	49.3 ± 6.6

There were no significant differences in the CD4, CD8, or CD3 populations or the CD4/CD8 ratio between the two groups.

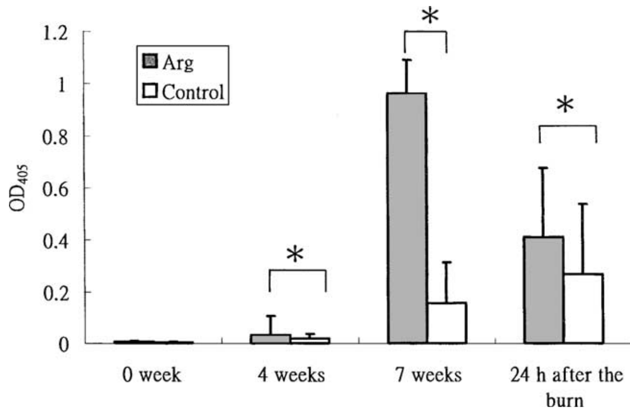

Fig. 1. Production of PEIF-specific antibodies in the Arg and control groups. Mice were immunized twice with recombinant PEIF protein on days 1 and 28, and sera antibody titers were measured by ELISA at weeks 0, 3, 4, 5, 6, 7 and 8. The dilution of mice antiserum was 1:1000. Significant difference between the two groups ($^*P < 0.05$).

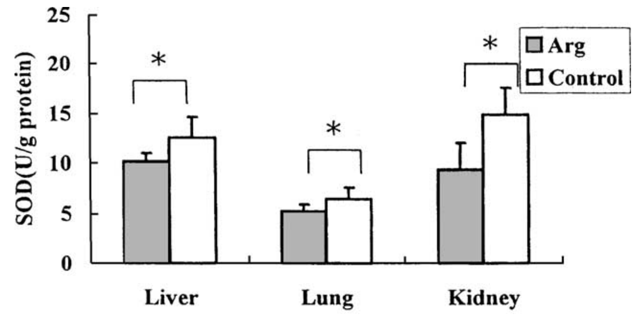

Fig. 3. Superoxide dismutase (SOD) activities in tissue homogenates between the two groups after the burn. Significant difference between the two groups ($^*P < 0.05$).

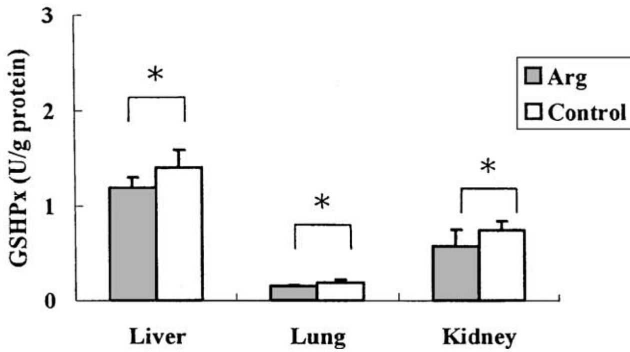

Fig. 2. Glutathione peroxidase (GSHPx) activities in tissue homogenates between the two groups after the burn. Significant difference between the two groups ($^*P < 0.05$).

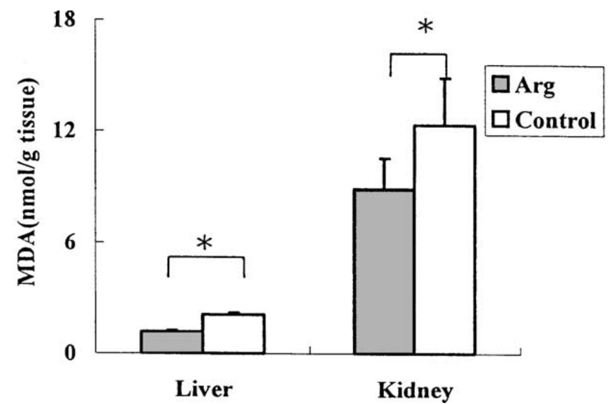

Fig. 4. Malondialdehyde (MDA) concentrations in liver and kidney between the two groups after the burn. Significant difference between the two groups ($^*P < 0.05$).

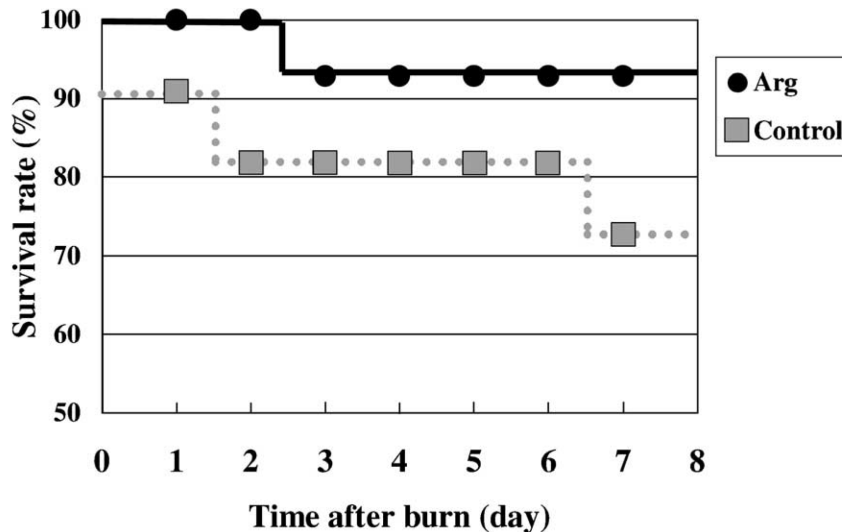

Fig. 5. Survival curves of vaccinated burned mice complicated with *P. aeruginosa* infection. There was no significant difference in the survival rate between the two groups.

4. Discussion

Supplemental Arg has been demonstrated to improve immunologic response in both in vivo and in vitro studies. Augmentation of cell-mediated immunity was seen by Barbul et al. [36] with Arg supplementation. Saito et al. [24] also confirmed this observation by demonstrating that dietary supplementation of Arg had a dose-response effect on a delayed hypersensitivity test. Since *P. aeruginosa* is a major cause of nosocomial infections in burned patients, an effective protective mechanism of burn patients against *P. aeruginosa* infection is based on the production of specific antibodies against bacterial virulent factors. Therefore, in this study, we investigated the effect of Arg supplementation on humoral and cellular immunity, in order to determine whether Arg together with PEIF vaccination might have a synergistic protective effect in burned mice with *P. aeruginosa* infection. In this study, 2% of total energy was supplied by Arg; this amount of Arg was found to reduce mortality in burned guinea pigs [24]. Also, a shortened hospital stay and reduced wound infection were observed in burn patients consuming this level of Arg when compared to those using other enteral formulations [37].

A previous study reported by Daly et al. [10] demonstrated that supplemental Arg increased the mean $CD4^+$ T lymphocytes in surgical patients. Reynolds et al. [38] also showed that Arg supplementation significantly enhanced cytotoxic T lymphocyte development and natural killer cell activity. In this study, $CD4^+$ helper, $CD8^+$ suppressor-type cells, and the $CD4^+/CD8^+$ ratio did not differ between the two groups. Also, there were no differences in CD3 populations between the two groups. Our finding was inconsistent with the reports mentioned above. It is possible that the metabolic stress in different disease conditions varies, which may lead to different immune response. However, we found that the production of specific antibodies against *P. aeruginosa* was significantly higher in the Arg group than in the control group at various times. This result suggests that burned mice supplemented with Arg had obviously enhanced humoral immunity, but the proliferation of $CD4^+$ T cells might not be responsible for the production of specific antibodies. Whether the stimulating effect of Arg on humoral immunity is due to the regulation of cytokines is currently being investigated.

After burn injury, generalized tissue inflammation is present in uninjured organs within hours [39]. Organ injury remote from the region of thermal injury has been shown to be due to intravascular action of complements, resulting in stimulation of intravascular neutrophils, leading to the formation of toxic oxygen products [40]. Lipid peroxide is thought to be one of the most harmful substance produced after burns [41]. Studies have shown that lipid peroxide in lung, liver, kidney and other tissues is seen early post-burn [1–5]. SOD and GSHPx are enzymes which protect tissues from the effects of free radicals and lipid peroxides, and the activities of both SOD and GSHPx increase after free-radical-mediated injury and lipid peroxidation [42]. Saitoh et al. [41] demonstrated that Mn-SOD activities in lung and kidney were significantly higher than in the control group after a burn. The results of this study reveal that SOD and GSHPx activities in liver, kidney, and lung were significantly lower in the Arg group when compared with the control group. Also, lipid peroxide concentrations in liver and kidney were lower in the Arg group than in the control group after the burn of the vaccinated mice. These results were similar to our previous report [19]. This finding may indicate that Arg supplementation attenuates the oxidative stress induced by burn injury. This was true whether the mice were vaccinated or not, and increasing humoral immunity may play a role in reducing oxygen radicals after the burn.

Arg is known to stimulate the local wound immune system, mainly lymphocyte activation, thereby modulating infection and healing. Saito et al. [24] demonstrated that Arg supplementation improved survival rates in a non-infected animal model. Our previous study showed that the survival rate of burned mice after $1\times$ LD_{50} of *P. aeruginosa* infection was 26.7% for both the Arg and control groups after observation for 8 days (unpublished data). In this study, we infected immunized burned mice with $2\times$ LD_{50} *P. aeruginosa* to increase the mortality. The survival rates of the Arg and control groups were 92 and 71%, respectively, at 8 days after the burn. This result indicates that PEIF vaccination effectively reduced the mortality of burned mice after *P. aeruginosa* infection. Although there was a tendency for mice with Arg supplementation to have higher survival rates than mice in the control group, no significant difference was observed between the two groups. It is possible that the vaccination effect of PEIF against *P. aeruginosa* infection is too strong to observe the beneficial effect of Arg supplementation on the survival rates in burned mice. Whether a higher challenge dose of *P. aeruginosa* to burned vaccinated mice is needed to observe the difference of survival rates between these two groups requires further investigation.

In conclusion, the findings of this study suggest that Arg supplementation may enhance humoral immunity in vaccinated mice. Also, oxidative stress induced by burn injury was attenuated. However, Arg demonstrated no appreciable benefit on enhancing cellular immunity, and survival rates were not improved when vaccinated burned mice were complicated with *P. aeruginosa* infection.

Acknowledgements

This study was supported by research grant NSC 90-2320-B-038-037 from the National Science Council, ROC.

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