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Effect of Vitamin C Administration on Neutrophil Apoptosis in Septic Patients After Abdominal Surgery

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Objective. **To investigate the effect of parenteral administration of vitamin C on neutrophil apoptosis by determining Fas receptor expression and caspase-3, poly (ADP-ribose) polymerase (PARP), and Bcl-2 levels in neutrophils from septic abdominal surgery patients.**

Study design. **Twenty septic abdominal surgery patients were studied in a prospective, randomized, double-blinded clinical trial. A group of healthy volunteers (n = 10) constituted a reference group for baseline parameter values. The patients were randomly assigned to a vitamin C-treated (n = 10) or placebo-treated (n = 10) group. For a 6-d period from 12 h post-surgery, the vitamin C group received 450 mg/d of the vitamin in 3 doses and the placebo group an identical administration of 5% dextrose. Early-morning peripheral blood samples were obtained daily from 24 h after vitamin C administration until d 6 post-surgery (T1d-T6d).**

Results. **Vitamin C group showed a nonsignificant reduction in Fas (CD95) expression on CD15-positive peripheral blood neutrophils, significantly decreased caspase-3, and PARP levels (caspase-3: T4d: $P < 0.05$, T5d: $P < 0.05$, T6d $P < 0.01$; and PARP: T3d: $P < 0.05$, T4d: $P < 0.05$, T6d: $P < 0.05$), and significantly increased Bcl-2 levels (T3d: $P = 0.001$) *versus* placebo group.**

Conclusions. **Postoperative vitamin C treatment of septic abdominal surgery patients exerts an antiapoptotic effect on peripheral blood neutrophils, reducing caspase-3 and PARP levels, and increasing Bcl-2 levels. However, these antiapoptotic effects are not maintained at all time points.**

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Key Words: **abdominal surgery patients; apoptosis; Bcl-2; caspase-3; Fas; neutrophils; PARP; sepsis; vitamin C.**

INTRODUCTION

During systemic inflammatory responses, e.g., in septic patients undergoing major surgery, the release of inflammatory mediators into the circulation contributes to cell apoptosis alterations. Apoptosis is an important mechanism of cell death in lymphocytes and parenchymal cells during sepsis, and occurs systemically in many organs [1]. Apoptosis can occur via at least 2 distinct pathways: a perforin-mediated pathway and a pathway triggered by the surface receptor Fas, also called APO-1 or CD95 [2]. Fas (CD95) is a leading member of the family of apoptotic death receptors [3], which includes receptors for tumor necrosis factor and tumor necrosis factor-related apoptosis-inducing ligand. These receptors are Type 1 transmembrane proteins characterized by the presence of 1 to 5 cysteine-rich repeats in their extracellular domain and of the so-called "death domain" in their cytoplasmic tail. Fas is expressed almost ubiquitously, but its stimulation does not necessarily lead to apoptosis. Fas is activated by the binding of its ligand, the Fas-ligand (FasL) or CD95L [4]. Fas activation is followed by aggregation of the receptor death domain, thereby forming the death-inducing signaling complex, which consists of the adaptor Fas-associated death domain protein and the protease procaspase-8. The latter is cleaved into active caspase-8, which cleaves downstream substrates such as caspase-3, completing the death program. Caspase activation is controlled by members of the Bcl-2 family, which includes both pro-

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apoptotic (e.g., Bad, Bax) and antiapoptotic (e.g., Bcl-2) molecules. The cell death regulating activity of Bcl-2 family members appears to depend on their ability to modulate mitochondrial function. Bcl-2 family members are major regulators of the apoptotic process, whereas caspases are executioners. Another characteristic event of apoptosis is the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. Caspases 3 and 7 cleave PARP, and this cleavage appears to be a marker of the apoptotic process.

Bcl-2 inhibits mitochondrial membrane rupture and the subsequent release of cytochrome *c* and caspase-activating proteins [5]. The release of cytochrome *c* from the mitochondria is sufficient to induce apoptosis. There is evidence that during sepsis, apoptosis is increased in parenchymal tissues, e.g., the lung, although increased apoptosis is most frequently observed in the lymphoid-rich organs, e.g., spleen, thymus, and small intestine [1, 6]. Apoptosis of peripheral blood lymphocytes is also higher during sepsis and surgical stress, reducing organ functionality and hindering recovery from inflammatory processes [7, 8]. The exaggerated inflammatory response produced during the systemic inflammatory response syndrome (SIRS) in septic and surgical patients produces a reduction in lymphocyte counts and a considerable enhancement in the number of circulatory neutrophils due, at least in part, to an alteration in cell apoptosis [7, 9].

Apoptosis of polymorphonuclear neutrophil leukocytes (PMNs) is deregulated in inflammatory and infectious processes [10–12]. Excessive apoptosis of endothelial cells and parenchymal cells of organs alongside changes in PMN apoptosis may contribute to multiple organ dysfunction syndrome (MODS), among other syndromes and diseases [8, 13].

Reactive oxygen species (ROS) constitute an internal mechanism of apoptosis, which they can induce in different ways [14] such as by the up-regulation of cell-death receptors after redox balance alterations [15, 16]. It has also been reported that ROS can affect the function of caspases [17, 18]. Vitamin C is a hydrosoluble antioxidant molecule that acts as ROS scavenger and vitamin E regenerator [19]. Administration of this vitamin was found to improve the respiratory function of elderly hospitalized patients with acute respiratory infections [20]. Low vitamin C levels have been detected in critical care, septic, and acute respiratory distress syndrome patients [21, 22], and may predict MODS in risk populations [23]. Vitamin C may have an inhibitory effect on monocyte apoptosis, since it has been associated with reductions in Fas expression and in caspase-3, caspase-8, and caspase-10 activities, and with the preservation of mitochondrial integrity [24]. However, although ROS have been observed to have a proapoptotic effect [25, 26], vitamin C has been associated with high levels of PMN apoptosis [27–29]. The objective of the present investigation was to study the antiapoptotic effect of the parenteral administration of vitamin C on neutrophil apoptosis by determining Fas receptor expression and caspase-3, PARP, and Bcl-2 levels in neutrophils from septic abdominal surgery patients.

In the present report, we show data that indicate an antiapoptotic effect of vitamin C in peripheral blood neutrophils from septic patients who underwent abdominal surgery. An antiapoptotic effect was defined by a reduction in caspase-3 and PARP levels and an increase in Bcl-2 levels.

PATIENTS AND METHODS

Study Design and Patients

A prospective, randomized, double-blinded clinical trial was performed in 20 patients undergoing abdominal surgery in our Digestive Surgery Department who had a physiological and operative severity score for the enumeration of mortality and morbidity (POSSUM) score indicating a postoperative mortality risk of >30% [30]. Patient characteristics are shown in Table 1. Ten healthy volunteers of similar age range to these patients were recruited as a control group. Written informed consent was obtained from all patients or their relatives, and the study was approved by the local Clinical Research Ethics Committee.

Treatments

Patients were allocated to vitamin C ($n = 10$) or placebo-treated ($n = 10$) group by envelope randomization (designed by a statistician), and treatments were started at 12 h post-surgery and administered daily on 6 consecutive postoperative d. The vitamin C group received 450 mg/d of the vitamin administered in 5% dextrose in 3 divided doses and the placebo group an identical administration of 5% dextrose, as previously described [31]. Placebo-treated patients received no supplemental vitamins or nutrition.

Samples

Early-morning, peripheral blood samples were obtained by venipuncture from all patients on every treatment day using a 3-mL sterile tube containing ethylenediamine tetraacetic acid (EDTA) as anticoagulant. Sample collection started at 24 h after vitamin C administration (T1d) and finished on d 6 post-surgery (T6d).

Measurements

Analysis of Fas (CD95) Expression

PMNs from the peripheral blood samples in EDTA tubes were immediately stained by a direct immunofluorescence technique with monoclonal antibodies (anti-CD15-fluorescein isothiocyanate and anti-CD95-PE; PharMingen, San Diego, CA) and acquired and analyzed in a FACScan flow cytometer using CellQuest software (BD Biosciences, Immunocytometry Systems, San Jose, CA). Fas (CD95) expression was specifically measured on CD15-positive neutrophil leukocytes. PMNs from peripheral blood samples were incubated for 15 min with saturating concentrations (20 μL/10^6 cells) of anti-CD15-fluorescein isothiocyanate-conjugated and anti-CD95-

TABLE 1

General Characteristics, Diagnoses and Surgical Procedures of Abdominal Surgery Patients Studied

	PLACEBO (n = 10)	Vitamin C (n = 10)
Age (y)	65.1 ± 3.6	67.8 ± 4.5
Mortality risk: %	52.0 ± 4.1	60.5 ± 8.7
POSSUM: Score	50.4 ± 1.4	55.0 ± 3.3
Sex (Men/Women)	6/4	5/5
Diagnoses		
Peritonitis (perforations or inflammations of gallbladder, colon, biliary or cholecystitis and ulcers)	6	7
Complicated intestinal ischemia	1	1
Intra-abdominal abscesses	1	1
Anastomotic leakage after gastrectomy	1	0
Cholangitis	1	1
Surgical procedure		
Intestinal resection with or without anastomosis	5	3
Perforation suture	3	3
Collection drainage or anastomosis dehiscence	1	1
Cholecystectomy with or without drainage of biliary tract	1	3
Pressors	2/10	3/10
Ventilatory support	2/10	3/10
Oxygenation parameters PO_2 (mmHg)	144.7 ± 47.3	113.9 ± 50.5
Oxygenation parameters PCO_2 (mmHg)	47.2 ± 7.4	45.7 ± 7.7
Creatinine (mg/dL)	1.99 ± 0.21	2.15 ± 0.12
Lactate (meq/L)	1.97 ± 0.55	3.22 ± 1.25
Volume status (based on the blood sodium level in mmol/L)	137.42 ± 7.19	138.07 ± 6.11

Note. Data are presented as mean values ± standard error of mean.

PE-conjugated antibodies, then washed once with phosphate buffer saline (PBS) and lysed with ammonium chloride 1X solution (PharmLyse; BD Biosciences). Simultaneous negative control staining was performed with a saturating concentration of murine-PE-conjugated IgG. Mean fluorescence intensity (MFI) was used as a relative molecular-density measurement for Fas (CD95) expression on CD15-positive neutrophils.

Apoptosis Measurement

Immediately after the blood extraction, neutrophils were isolated by centrifugation (300 × g) at room temperature in a Ficoll Hypaque density gradient (Monopoly Resolving Medium; ICN-Biomedicals, Aurora, OH), recovering the upper layer with lymphocyte population. Isolated neutrophils were washed twice with PBS and RPMI 1640 medium (Sigma Chemicals, St. Louis, MO). Lymphocytes were discriminated from neutrophils and monocytes by their characteristic forward/sideward light scatter.

Caspase-3, PARP, and Bcl-2 Levels

Concentrations of these proteins were determined by flow cytometry in a FACScan flow cytometer (BD Biosciences) using the BD Cytometric Bead Array (CBA) technique (BD Biosciences). This CBA technique employs a series of particles with discrete fluorescence intensities to simultaneously detect soluble analytes. The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a BD CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an enzyme-linked immunosorbent assay plate. The assay sensitivity range was 4.9–6000 units/mL for caspase-3, 18.5–6000 units/mL for PARP, and 28.8–6000 units/mL for Bcl-2.

Caspase-3, PARP, and Bcl-2 concentrations were determined in the precipitate obtained after centrifugation (1800 rpm/16 min) of blood samples from EDTA tubes after its reconstitution in PBS.

Leukocyte Counts

Total leukocyte counts and neutrophil percentage were quantified daily in the blood samples in EDTA tubes before their centrifugation, using a Sysmex KX 21N automatic counter (Roche, Barcelona, Spain).

Hemoglobin Levels

These were determined daily in the blood samples in EDTA tubes using a Sysmex KX 21N automatic counter (Roche). Data were also gathered on the number of blood transfusion units received by each patient.

Statistical Analysis

A 2-variant analysis of variance was performed between quantitative independent variables (Fas expression, caspase-3, PARP, Bcl-2 values, and neutrophil counts) and qualitative dependent variables (placebo and vitamin C-treated groups). Because of the small number of patients in each treatment group, groups were compared using a nonparametric test (Mann-Whitney t-test). $P < 0.05$ was considered significant. Data are presented as means ± standard errors.

RESULTS

Fas receptor expression on CD15-positive neutrophils was significantly lower in vitamin C-treated patients (MFI, 9.5 ± 1.3) *versus* healthy volunteers (MFI, 15.3 ± 0.9) but not *versus* placebo-treated patients (MFI, 10.4 ± 1.1) (Fig. 1), while it did not significantly differ between the placebo-treated and healthy volunteer groups.

Neutrophil count and percentage were higher ($P <$ 0.05) in both treatment groups *versus* healthy controls and showed a tendency to decrease over time (Table 2).

Plasma hemoglobin levels were lower in both treatment groups *versus* healthy controls but did not significantly differ between treatment groups (Table 2). Two patients (20%) in the placebo group received blood transfusions (6–10 blood units), and 3 patients (30%) in the vitamin C group (1–6 blood units).

Four patients (40%) in each treatment group developed MODS during the 6-d study period. At the end of the study, the mortality rate was 40% in the placebo group and 60% in the vitamin C-treated group.

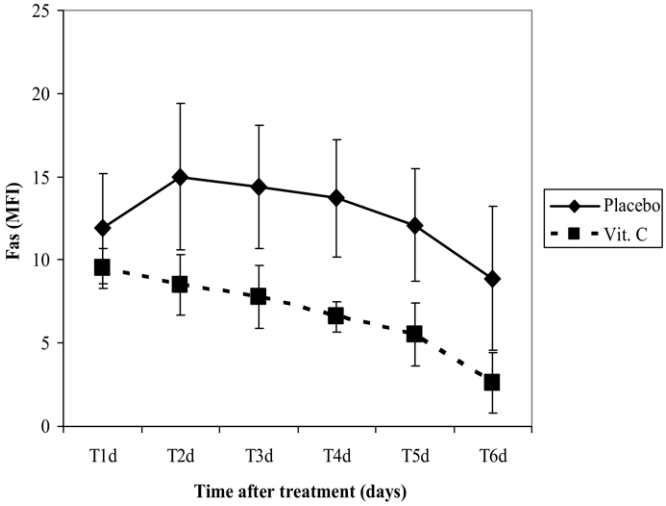

FIG. 1. Time course of Fas expression, expressed as MFI, on CD15-positive neutrophils from vitamin C- and placebo-treated surgical patients.

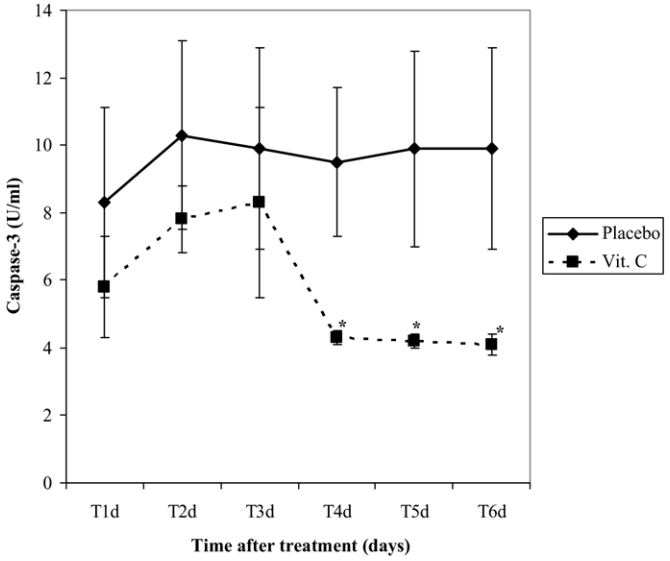

FIG. 2. Time course of caspase-3 levels (U/mL) in vitamin C and placebo-treated surgical patients.

Glucose levels in the vitamin C and placebo groups were not affected by the administration in 5% dextrose of either vitamin or placebo.

Caspase-3, PARP, and Bcl-2 levels were significantly lower in healthy volunteers than in the placebo and vitamin C groups throughout the study period ($P < 0.033$ and $P = 0.001$, respectively). Caspase-3, PARP, and Bcl-2 levels in healthy volunteers were 1.2 ± 0.1 U/mL, 1.4 ± 0.2 U/mL, and 3.9 ± 1.2 U/mL, respectively. Caspase-3 and PARP levels were lower in vitamin C-treated than in placebo-treated patients (caspase-3: T4d: $P = 0.001$, T5d: $P = 0.001$, T6d: $P = 0.001$; PARP: T2d: $P = 0.01$, T3d: $P = 0.038$, T4d: $P = 0.001$, T6d: $P = 0.005$) (Figs. 2 and 3). Bcl-2 levels were higher in vitamin C-treated than in placebo-treated patients (T3d: $P = 0.001$) (Fig. 4).

DISCUSSION

Polymorphonuclear neutrophils are an essential element of the inflammatory response in septic surgical patients, when senescent or nonfunctional cells must be eliminated [10, 32]. This study reports a nonsignificantly lower Fas expression level on PMNs from vitamin C-treated *versus* placebo-treated septic abdominal surgery patients. Hence, PMNs in vitamin C-treated patients may be less likely to undergo apoptosis by Fas receptors in response to proapoptotic death receptor-mediated stimuli, e.g., FasL [33]. A soluble form of FasL was previously demonstrated to be enhanced in critical patients who developed SIRS and MODS [12, 34–36]. Therefore, a reduction in Fas receptor expression may exert a protective effect on circulatory neutrophils in septic patients who have undergone abdominal surgery.

The reduction in caspase-3 levels and activity (as measured by low PARP levels) in neutrophils from the vitamin C-treated patients indicates a low level of apoptosis by caspase-3. The higher levels of antiapoptotic Bcl-2 protein found in the vitamin C-treated patients would also contribute to a reduction in neutrophil apoptosis, suggesting an antiapoptotic effect for vitamin C in the mitochondria. These results are in agreement

TABLE 2

Neutrophil Count and Plasma Hemoglobin Levels in Healthy Volunteers (control, $n = 10$) and Septic Patients (placebo group: $n = 10$, vitamin C group: $n = 10$)

	Control	Group	T1d	T2d	T3d	T4d	T5d	T6d
Neutrophil count (×1000/μL)	4.34 ± 0.5	Placebo	10.05 ± 0.7	8.07 ± 0.9	8.71 ± 0.9	9.97 ± 1.0	9.36 ± 0.9	12.29 ± 1.3
		Vit. C	22.67 ± 0.9	11.72 ± 0.8	10.88 ± 0.8	9.28 ± 0.7	10.23 ± 0.7	11.46 ± 0.6
Neutrophil count (%)	65.1 ± 5.9	Placebo	82.2 ± 3.1	80.4 ± 4.1	77.5 ± 4.6	82.0 ± 3.1	74.1 ± 3.5	75.5 ± 4.1
		Vit. C	87.6 ± 2.4	79.2 ± 3.5	84.8 ± 2.0	81.9 ± 3.0	69.9 ± 2.4	72.4 ± 4.8
Hemoglobin (g/dL)	15.38 ± 0.5	Placebo	9.28 ± 0.9	9.49 ± 1.1	9.46 ± 1.0	9.30 ± 1.2	10.17 ± 1.9	10.84 ± 2.5
		Vit. C	9.17 ± 0.8	8.82 ± 0.9	10.17 ± 1.9	10.30 ± 1.5	10.25 ± 1.4	8.48 ± 1.1

Note. Data are presented as mean values ± standard error of mean.

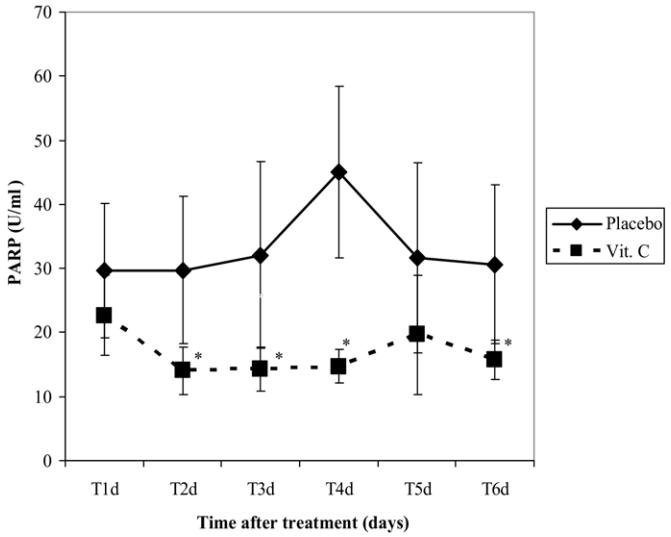

FIG. 3. Time course of PARP levels (U/mL) in vitamin C and placebo-treated surgical patients.

with previous findings of an accumulation of high vitamin C levels by neutrophils, protecting them from products released during the respiratory burst in an inflammatory setting [37]. It was also reported that ROS increase Fas-mediated apoptosis of neutrophils in mice, related to a reduction in cell levels of reduced glutathione [25]. Various authors have studied the effect of vitamin C on PMN apoptosis by using mice neutrophils [28] or culture cells [29], reporting that vitamin C has a proapoptotic effect by increasing ROS production [29]. However, these results would have been influenced by the experimental conditions, which were very different from those in the present investigation of peripheral blood samples.

We propose that the antioxidant capacity of vitamin C, acting as a ROS scavenger, may be responsible for the lower apoptosis of neutrophils observed in septic surgical patients treated with this vitamin. ROS produce an up-regulation of death receptors [25, 26, 38, 39], therefore the reduction in Fas expression would be explained by the ROS scavenging action of the vitamin. ROS also participate in receptor assembly and caspase-3 actions [17], hence the antioxidant capacity of vitamin C would explain the decrease in caspase-3 activity, which is mediated by PARP. ROS may also act via caspase-independent mechanisms, e.g., DNA degradation [17, 18], which would be inhibited by vitamin C. The higher Bcl-2 protein levels in the vitamin C-treated patients would exert antiapoptotic and antioxidant effects, preventing oxidative damage to cell components [17, 40].

An increased peripheral blood leukocyte count is observed in surgical patients, especially in those who develop SIRS or sepsis, with an increase in neutrophils and a reduction in lymphocytes [41]. Thus, these septic surgical patients showed a higher leukocyte count with a higher percentage of neutrophils in comparison with healthy volunteers (Table 2). It has been proposed that the presence of inflammatory mediators in septic patients produces an over-activation of neutrophils, which remain longer in the peripheral blood circulation and exert lesional effects on endothelium and tissues, thereby increasing the risk of organ failure [8]. Consequently, the antiapoptotic effect of vitamin C on neutrophils may be detrimental to the patient by increasing their presence in the circulation. Nevertheless, positive effects for vitamin C have been reported in inflammatory processes, and adverse outcomes have been related to deficits in this vitamin, supporting its beneficial properties [20–23]. Vitamin C also enhances many neutrophil functions, increasing chemotaxis, particle ingestion, and lysozyme activity for cell elimination and protecting against the toxic effects of superoxide radicals, among other actions [42]. Finally, Hotchkiss *et al.* [8] considered that a delay in neutrophil apoptosis may be a beneficial response because the administration of granulocyte colony-stimulating factor, which delays neutrophil apoptosis, improved the survival of non-immunosuppressed pneumonia patients [8].

It is well-known that neutrophils are enhanced in sepsis as part of the inflammatory response. The antiapoptotic effect of vitamin C on neutrophils suggested in this report implies that administration of this vitamin in septic patients after abdominal surgery would be detrimental, maintaining an elevated presence of neutrophils in their circulation. However, as noted previously, the enhancement of neutrophil functions by vitamin C increases chemotaxis, particle ingestion, and lysozyme activity for cell elimination, and protects against the toxic effects of superoxide radicals, among

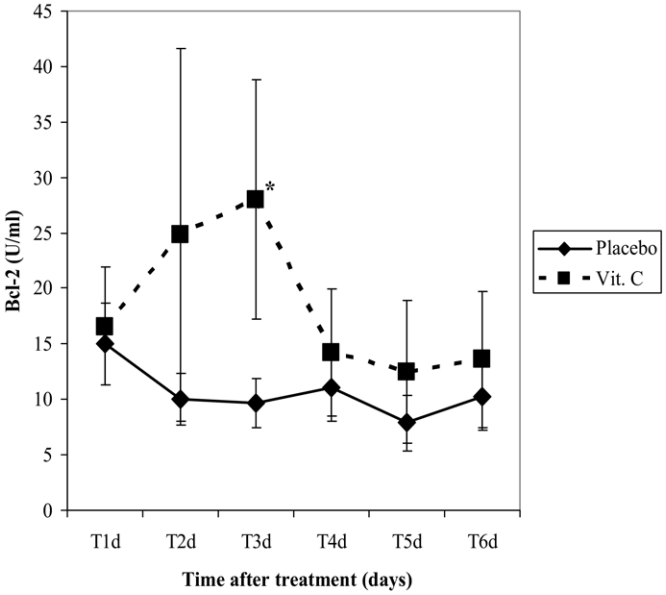

FIG. 4. Time course of Bcl-2 intracellular levels (U/mL) in vitamin C and placebo-treated surgical patients.

other actions. It should be taken into account that vitamin C was administered for 6 consecutive postoperative in this study, and a longer treatment period may reveal a variation in effects on neutrophil apoptosis.

Further research is required to determine whether the effects of vitamin C on neutrophil apoptosis are reflected in postsurgical recovery time for septic surgery patients, relating apoptotic parameters and neutrophil counts to postsurgical complications, the development of MODS, and the mortality rate.

CONCLUSIONS

Septic patients who were treated with vitamin C for 6 d after abdominal surgery showed a significant reduction in caspase-3 and PARP proteins and a significant increase in Bcl-2 levels in neutrophils. These results suggest that vitamin C has antiapoptotic effects on neutrophils in these patients.

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