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# Thalidomide prolongs survival after experimental musculoskeletal injury, through an effect on mononuclear apoptosis

*Konstantinos Panousis, MD, PhD,*[a] *Vassilios S. Nikolaou, MD, PhD, MSc,*[a,*] *Thomas Tsaganos, MD, PhD,*[b] *Stergios Lallos, MD,*[a] *Evangelos J. Giamarellos-Bourboulis, MD, PhD,*[b] *and Nicolas Efstathopoulos, MD, PhD*[a]

[a] *2nd Department of Orthopaedics, University of Athens, Medical School, Greece*
[b] *4th Department of Internal Medicine, University of Athens, Medical School, Greece*

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ABSTRACT

*Background:* This study was conducted to investigate the effects of intravenous thalidomide administration in an experimental model of musculoskeletal trauma. We hypothesized that because thalidomide inhibits secretion of tumor necrosis factor alpha (TNF-α), survival of animals that received thalidomide would be significantly prolonged.

*Material and methods:* After an open fracture of the right femur, 24 rabbits were randomly assigned to control and thalidomide groups. Intravenous therapy with thalidomide was started 30 min after fracture. Hemodynamic monitoring of all animals was performed for 4 h. Survival was recorded and bacterial growth in blood and organs was measured after animal death or sacrifice. Blood was sampled for TNF-α measurement and for isolation of peripheral blood mononuclear cells (PBMCs). Apoptosis of PBMCs was measured by flow cytometry.

*Results:* Survival was significantly prolonged in the thalidomide group. Apoptosis of PBMCs was increased in the control group compared with the thalidomide group at 24 h. There were no differences in vital signs, blood and tissue cultures, and serum TNF-α concentration between the two groups.

*Conclusions:* Intravenous thalidomide prolonged survival in an experimental model of severe musculoskeletal injury in rabbits. Its mechanism of action did not involve TNF-α suppression but prevention of mononuclear apoptosis. In view of these promising results, further research is needed to clarify the immunomodulatory mechanism of action of thalidomide and its potential use for the management of severe trauma.



## 1. Introduction

The systemic inflammatory response syndrome (SIRS) is a common pathway to multiple organ dysfunction and death after severe trauma. SIRS is induced by the secretion of a variety of proinflammatory cytokines [1] and it is known that the release of mitochondrial DNA following trauma can trigger the production of proinflammatory cytokines [2].

Conflicts of Interest: None of the authors has nothing to declare related with this submission.

* *Corresponding author*. 2nd Department of Orthopaedics, University of Athens, Dimitriou Ralli 21 St, Maroussi, Athens 15124, Greece. Tel.: +30 693 254 3400; fax +30 210 802 2142.
E-mail address: vassilios.nikolaou@gmail.com (V.S. Nikolaou).

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Tumor necrosis factor (TNF) alpha is a central mediator of the immune response after trauma. It appears early in serum after the traumatic event, stimulates the production of other proinflammatory cytokines, primes loss of integrity of vascular endothelium, and drives apoptosis of lymphocytes [3–5]. Therefore, it may be considered a major target for therapies aiming to modulate the immune response of the host. Developed immunomodulatory strategies so far comprise monoclonal antibodies targeting TNF-α and soluble TNF receptors. These agents have been tested in phase III randomized studies of patients at severe sepsis and have failed to show survival benefit [6–10]. These results probably indicate that strategies aiming to modulate excess production of TNF-α require another approach.

Thalidomide (a-N-phthalimidoglutarimide) was initially used as a sedative and antiemetic during pregnancy, but it was withdrawn from the market due to its teratogenic effects. It is an immunomodulatory compound that selectively inhibits TNF-α synthesis [11] by reducing the half-life of messenger RNA of TNF in human monocytes [12]. Thalidomide is currently used for its anti-angiogenic and anti-TNF-α properties in the treatment of a variety of diseases like erythema nodosum leprosum, Crohn disease, cutaneous lupus erythematosus, cutaneous sarcoidosis, Behçet syndrome, graft-versus-host disease, multiple myeloma, and human immunodeficiency virus–related aphthous ulcers and wasting syndrome [13].

In a previous study by our group, thalidomide was administered orally to rats as pretreatment for experimental sepsis by *Escherichia coli* and by *Pseudomonas aeruginosa* [14,15]. Administration of thalidomide prolonged survival and reduced circulating levels of TNF-α.

The aim of this study was to investigate the effects of intravenous thalidomide administration in an experimental model of severe musculoskeletal trauma in an animal (rabbit) model. We hypothesized that because thalidomide inhibits secretion of TNF-α, survival of animals that received thalidomide would be significantly prolonged.

## 2. Materials and methods

### 2.1. Animal care

A total of 24 white New Zealand male rabbits, mean (± standard deviation [SD]) weight 3.37 ± 0.35 kg, were used for the study. The study received permission from the Veterinary Directorate of the Prefecture of Athens, according to Hellenic legislation in conformance to the 160/91 Directive Council of the EU. The study was conducted in the Center of Experimental Medicine of the ATTIKON University Hospital.

All experiments and handling were conducted under the supervision of an expert veterinary surgeon. The animals were housed in single metal cages and had access to tap water and standard balanced rabbit chow *ad libitum*. Temperature ranged between 18°C–22°C, relative humidity between 55% and 65%, and the light/dark cycle was 6 AM/6 PM.

### 2.2. Thalidomide preparation

Thalidomide was obtained as a white amorphous powder (Sigma, St. Louis, MO). Thalidomide is sparingly soluble in water (0.05 mg/L) and undergoes rapid spontaneous hydrolysis in the physiological pH range. Therefore, solutions of thalidomide were produced by adding 30 mg of preweighted powder to 3 mL of a (2-hydroxypropyl)-β-cyclodextrin aqueous solution (Sigma-Aldrich), as previously described [16]. The solution was further diluted to a volume of 10 mL with the addition of water for injection in an ultrasound water bath. The final 1 mg/mL thalidomide solution was prepared with the addition of another 20 mL of 5% dextrose water. Thalidomide solutions of 0.2 and 0.5 mg/mL were also produced using the same protocol.

Preliminary pharmacokinetics were done to determine the minimum IV thalidomide dose that would provide serum concentrations of 4 μg/mL, as this was shown to inhibit TNF-α *in vitro* [11,12]. In addition, the safety of the prepared thalidomide solutions for intravenous administration was determined. More precisely, 30 mL of dextrose water solutions, each containing 0.2, 0.5, and 1 mg/mL of thalidomide, were administered over 10 min to six animals. Two milliliters of blood was sampled at the end of the infusion and at 60 min after infusion. Serum was immediately separated by centrifugation at 2800 rpm for 10 min. Animals were intensively followed up for 1 wk and no adverse effects were observed.

Thalidomide concentrations in prepared solutions and animal sera were determined by a high-performance liquid chromatography (HPLC) method after protein precipitation with trichloroacetic acid, as described previously [17]. Briefly, 200 μL aliquot of thalidomide solution or serum was mixed with an equal amount of trichloroacetic acid 20% (Merck, Darmstadt, Germany) and rigorously vortexed for 30 s. The mixture was then centrifuged for 15 min at 15,000 rpm at 4°C. An aliquot of 20 μL of supernatant was injected into the HPLC system using a Zorbax Eclipse SB-C18 (4.6 × 150 mm, 5 μm) reversed-phase column (Agilent 1100 Series; Agilent Technologies, Inc; Waldbronn, Germany). The mobile phase was 10% water, 10% methanol, and 80% potassium phosphate buffer 10 mM (pH 3.0) at a flow rate of 1.5 mL/h at 37°C. A water sample treated in the same way was applied as blank. The column effluent was monitored for ultraviolet absorbency at 220 nm. The retention time of the chromatographic peak corresponding to thalidomide was 7.7 min. Concentrations of thalidomide were measured applying a standard curve generated by known concentrations of purified substance (range 0.39–100 mg/L). All determinations were performed in duplicate and their mean was applied. The interday variation of the assay was 11.5%. Although this interday variation may appear excessive, it is a result of the limitations of the assay and is similar to what was previously reported [10,18].

HPLC analysis showed that all prepared solutions were stable. Animals receiving the 0.2 mg/mL solution had mean (±SD) serum concentration 1.44 ± 0.93 μg/mL at the end of infusion; it declined to 0.64 ± 0.89 μg/mL at 60-min post-infusion. Animals administering the 0.5 mg/mL solution had mean (±SD) serum concentration 1.80 ± 0.40 μg/mL at the end of infusion; it declined to 0.91 ± 1.13 μg/mL at 60-min post-infusion. Animals receiving the 1.0 mg/mL solution had mean (±SD) serum concentration 7.30 ± 1.50 μg/mL at the end of infusion; it declined to 5.82 ± 1.45 μg/mL at 60-min post-infusion. Based on these results, the 1 mg/mL solution was selected for administration in the rest of the study because target serum levels exceeding 4 μg/mL were achieved with it.