Original Article

In vitro efficacy of hyperbaric oxygen therapy against *Leishmania tropica* promastigotes and amastigotes

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Aim: To assess the efficiency of hyperbaric oxygen (HBO) therapy on *L. tropica*, which is the major causative agent of cutaneous leishmaniasis in Turkey.

Materials and methods: The effects of HBO on *Leishmania tropica* promastigote and amastigote forms were studied at a pressure of 2.5 atmosphere absolute (ata). We examined the cytotoxic effect of HBO on promastigotes using the trypan blue exclusion test and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye-reduction assay. To investigate the effects of HBO treatment on the growth of the promastigotes, HBO-treated and non-HBO-treated cell culture flasks were incubated at 26 °C for 3 days, while the effects of HBO on *L. tropica* amastigotes were detected using the acridine orange staining method.

Results: We observed that the cytotoxic effect emerged after 2 h and reached a maximum at 6 h with both methods. The number of live promastigotes was monitored for 24 h after treatment and there was a slight elevation at 2 h of treatment, but significant suppression was seen after 4 and 6 h of treatment (P < 0.001). We detected that the effect of HBO on *L. tropica* amastigote forms increased by 3.3%, 17.2%, and 38.5% at 2, 4, and 6 h, respectively.

Conclusion: The results presented here indicate that HBO treatment could be used as supportive therapy in association with chemotherapeutic agents against *L. tropica* infection.

Key words: Hyperbaric oxygen, *Leishmania tropica*, cytotoxicity

Leishmania tropica amastigot ve promastigot formlarına karşı hiperbarik oksijen tedavisinin in vitro etkinliği

Amaç: Bu çalışmanın amacı, Türkiye'de kutanöz leishmaniasisin en önemli etkeni *L. tropica* üzerinde HBO tedavisinin etkinliğini değerlendirmektir.

Yöntem ve gereç: *Leishmania tropica* promastigot ve amastigot formları üzerinde hiperbarik oksijenin etkileri 2,5 atmosfer basıncı (ata) altında çalışıldı. HBO'nun promastigot formları üzerindeki sitotoksik etkileri, tripan mavisi testi ve MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) yöntemiyle incelendi. HBO tedavisinin *L. tropica* promastigot formları üzerinde etkilerini araştırmak amacıyla HBO uygulamasına maruz bırakılmış ve bırakılmamış hücre kültür plakları 26oC'de üç gün boyunca inkübe edildi. Diğer taraftan HBO'nun *L. tropica* amastigot formları üzerine olan etkileri akridin oranj boyama yöntemiyle tespit edildi.

Bulgular: Her iki yöntem ile sitotoksik etkinin iki saat sonra başladığı ve altı saat sonra en üst seviyeye ulaştığı gözlemlendi. Canlı promastigotların sayısı tedaviden sonra 24 saat boyunca izlendi ve tedavinin ikinci saatinin sonunda promastigot sayılarında hafif bir artış olduğu, dört ve altı saat sonrasında ise promastigot sayılarının artışında önemli bir azalma olduğu tespit edildi (P < 0,001). Diğer taraftan HBO tedavisinin *L. tropica* amastigot formları üzerinde etkisinin 2, 4 ve 6. saatler için sırasıyla % 3,3, % 17,2 ve % 38,5 olduğu gözlemlendi.

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Sonuç: Bu çalışmada elde edilen sonuçlar, HBO tedavisinin *L. tropica* formlarına karşı kemoterapi ile birlikte destekleyici tedavi olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: Hiperbarik oksijen, *Leishmania tropica*, sitotoksitite

Introduction

The leishmaniases are a group of parasitic diseases caused by different species of the genus *Leishmania* (1). More than 1.5 million people are affected by these diseases each year in tropical, subtropical, and Mediterranean regions of the world (2). There are about 22 *Leishmania* species that cause various clinical manifestations. Leishmaniasis can result in visceral, cutaneous, or mucocutaneous infections according to the species (2-5). Visceral leishmaniasis (VL) is most commonly caused by the *Leishmania donovani* complex, but *L. amazonensis* in Latin America and *L. tropica* in the Middle East, Mediterranean, and Central Anatolia regions can be causative agents for VL. On the other hand, cutaneous leishmaniasis caused by the *L. tropica* complex is mainly seen in the Middle East, the Mediterranean, Africa, India, and Asia (1-3,6,7). In Turkey, *L. infantum*, transmitted by *Phlebotomus* (*Larroussius*) *tobbi*, *P.* (*Larroussius*) *syriacus*, *P.* (*Larroussius*) *neglectus*, and *P.* (*Larroussius*) *perfiliewi*, produces VL as reported sporadically in the Aegean, Mediterranean, and Central Anatolia regions. Although cutaneous leishmaniasis is caused by *L. tropica* in southeastern Anatolia and the Mediterranean regions by its suspected vectors, *P.* (*Paraphlebotomus*) *sergenti* or *P.* (*Phlebotomus*) *papatasi*, it has been also reported from the Central Anatolia and Aegean regions (7-9).

The main treatment choice for leishmaniasis is chemotherapy, mainly based on pentavalent antimonials (10). However, these are extremely toxic and cause serious side effects, and there is a worldwide increasing frequency of chemoresistance to antimonials (11). Recently, liposomal amphotericin B (AmBisome) has been used as the first drug; it was approved by the FDA for VL in immunocompetent patients. However, data on this drug are limited. Sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) are 2 pentavalent antimony drugs that are recommended for use in the treatment of both VL and cutaneous leishmaniasis. Similarly, these compounds have dose-dependent side effects and toxicities including the elevation of amylase and

lipase and electrocardiographic changes. In addition, multiple painful injections are undesirable and especially difficult to administer to children (12-14). Furthermore, using a single drug or a formulation for all forms of leishmaniasis is impossible at the present time (15,16). These issues emphasize the urgent need for affordable alternative drugs or implementation against leishmaniasis.

Many studies have shown that hyperoxia and hyperbaric oxygen (HBO) inhibit microbial growth by inducing various microbial metabolic reactions in bacteria, fungi, and parasites through increased generation of reactive oxygen species or free radicals (17-19). Thus, it is used adjunctively with surgery and antibiotherapy. The antioxidant defense mechanisms of parasites for protection from free radicals have not developed very well. Therefore, these organisms are very susceptible to HBO. Inducing oxidative stress inhibits the growth of parasites. The actions of several antiparasitic drugs also have effects on reducing the levels of antioxidant enzymes in parasites (17,18,20-22). There are a few studies reporting the efficiency of HBO therapy for *Leishmania amazonensis* and *Leishmania braziliensis panamensis*. These studies concluded that HBO exposure was sufficient to reduce promastigote growth and had an inhibitory effect on the ability of transformation into amastigotes (23-25). In the present study, we aimed to assess the efficiency of HBO therapy on *L. tropica*, which is the major causative agent of cutaneous leishmaniasis in Turkey.

Materials and methods

Parasite and culture procedures

L. tropica (MHOM/TR/05/EP117) promastigotes were provided by the Department of Parasitology, Ege University Medical Faculty, and cultured at 24-26 °C in RPMI-1640 medium containing 10% fetal calf serum (FCS) (both products from Biological Industries, Israel). At regular intervals, the parasites were harvested in the log phase of growth. Amastigotes were obtained from promastigotes using primary mouse macrophage culture.

Macrophage cultures and infection of macrophages

BALB/c female mice, 6 weeks old and weighing 25-30 g, were used. The animals were supplied by the Gülhane Military Medical Academy Research Center. Macrophages were obtained from normal BALB/c mice by peritoneal lavage. The peritoneal macrophages were stimulated by injecting approximately 1 mL of RPMI-1640 medium into the peritoneum and then the macrophages were collected the following day. The peritoneal exudates were washed with 5 mL of RPMI-1640 and then centrifuged. The precipitated peritoneal exudate was suspended in RPMI + 10% FCS. The concentration was adjusted to provide 10^6 macrophages/mL. The macrophage-containing medium was then transferred to 25-cm² culture flasks and allowed to incubate for 24 h at 37 °C under 5% CO_2. Following incubation, the macrophages were infected with *L. tropica* promastigotes in the log phase at a concentration of 10 promastigotes/macrophage.

HBO treatment

Promastigotes (10^6 parasites/mL), suspended in 5 mL of phenol red-free RPMI + 10% FCS, were placed in 25-cm² culture flasks. The parasites in the flasks and infected macrophage monolayers in 24-well culture plates were placed in a horizontal position in the HBO chamber (ETC Monoplace Chamber, ETC Biomedical Systems, USA). The pressure (PO_2 253.3 kPa, 2.5 atmosphere absolute) was maintained for 2, 4, and 6 h, and then the chamber was decompressed over a period of 5 min. The HBO treatment protocol was planned at a level that could be tolerated by humans. The antioxidant N-acetylcysteine (NAC) (30 mM) was used as a protective agent for promastigotes exposed to HBO, and some cultures were treated for 90 min with NAC before the HBO treatment.

Evaluation of HBO effects on *L. tropica* promastigotes

The number of promastigotes and their morphology were checked using a Neubauer hemocytometer. The numbers of viable cells were counted using a trypan blue dye exclusion test. The growth inhibition was calculated using the following equation:

(Number of promastigotes in the control group – number of promastigotes in the treated group) / number of promastigotes in the control group = growth inhibition.

The cytotoxicity of the promastigotes was analyzed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye-reduction assay (Sigma, USA). Briefly, after 200 µL of each parasite suspension exposed to HBO was transferred to ELISA wells, the formation of formazan was measured by adding MTT (2 mg/mL in phenol red-free RPMI-1640, 100 µg/well) and incubating the wells for 4 h in the dark at 37 °C. The wells were centrifuged at 2000 rpm for 5 min and the supernatant was removed, the pellet was dissolved by isopropanol, and absorbances were measured at 490 nm (26). The negative control well was used for baseline zero absorbance (A). Results were presented as percentage of cytotoxicity, determined as: (1 – A of experimental well / A of positive control well) × 100. These experiments were repeated following days 1, 2, and 3.

HBO effects on *L. tropica* amastigotes

Amastigote viability was determined by a modification of the method of Channon et al. (27). Briefly, following the final HBO incubation, the macrophages were scraped out using a cell scraper (Sigma), and 50 µL of cell suspension was incubated with a mixture of acridine orange (5 µg/mL) and ethidium bromide (50 µg/mL) for 10 min at room temperature. The stained smears were examined via fluorescence microscopy (Zeiss 100 W/2, Carl Zeiss, Germany). To determine the ratio of affected amastigotes, the average of 100 macrophages that contained amastigotes, either live (green fluorescence) or dead (red-orange fluorescence), was calculated.

Statistics

The results were expressed as the percentage of dead cells. The Mann-Whitney U test was used to compare each experimental group with the control. The Friedman test was used for the comparison of cytotoxic effects on different days. $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 11.0 for Windows.

Results

Cytotoxicity and the growth inhibitory effect of HBO treatment on *L. tropica* promastigotes

We investigated cytotoxicity and the growth inhibitory effect of HBO treatment on *L. tropica*

promastigotes. After 2 h of HBO treatment, no significant differences (P > 0.05) in the motility and viability of the promastigotes were detected. A significant difference was observed in the decreasing of cell viability after 4 and 6 h (P < 0.001), as shown by the trypan blue exclusion test (Figure 1). Moreover, the motility of HBO treated promastigotes at 4 and 6 h of treatment became more circular. In contrast, untreated promastigotes in the control group remained 95% viable, highly motile, and spindle-shaped (Figure 2). Pretreatment of the cells with the antioxidant NAC (30 mM) prior to exposure could completely abrogate the effect of HBO on motility and cell viability. The cytotoxic effect of the increasing O$_2$ tension on *L. tropica* promastigotes was examined separately on days 1, 2, and 3. The cytotoxic effect that was observed on day 3 was 73.3%, and it was significantly higher than those observed on previous days (P > 0.05). It was observed that the metabolic products of MTT were not formed in the control
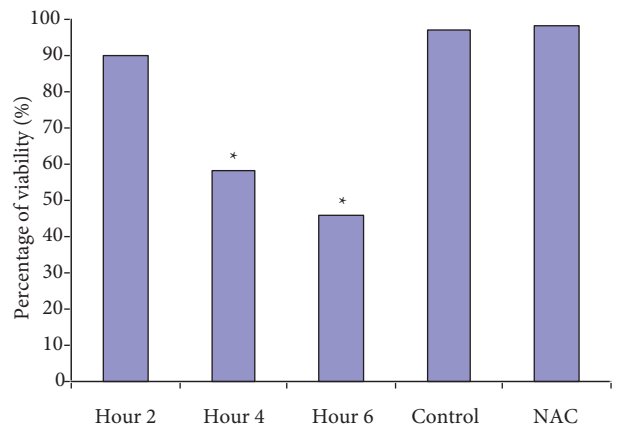
Figure 1. Cell viability was measured using the trypan blue viability test. *L. tropica* promastigotes in culture, both untreated (control) and pretreated with NAC, were exposed to HBO for 2, 4, and 6 h. Cell viability rates were expressed as a percentage of the untreated cells. There was a significant difference between the treated cells and the untreated cells (*P < 0.001). The data depict the mean ± SD of 3 separate experiments.
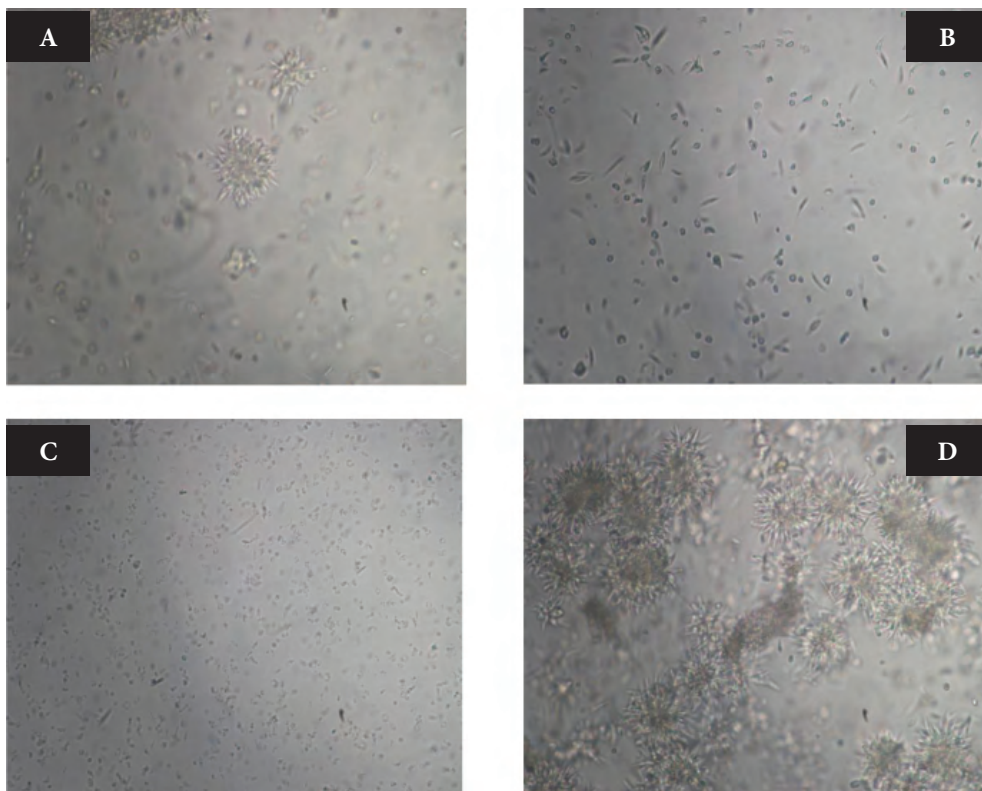
Figure 2. The microscopic views of *L. tropica* promastigotes in culture, with and without HBO (control), at 2, 4, and 6 h (40×): A) 2 h, B) 4 h, C) 6 h, and D) control. It was observed that *L. tropica* promastigotes lost their motility and became more circular after exposure to HBO for 4 and 6 h. In contrast, untreated promastigotes in the control group remained 95% viable, highly mobile, and spindle-shaped.

group or the promastigotes subjected to NAC. The NAC protected the promastigotes against the effect of the HBO. According to the MTT method, the percentage of the cytotoxic effect of HBO on the promastigotes compared to the control group is shown in Figure 3. The promastigotes treated with HBO were transferred to normal oxygen medium (fresh medium) after 6 h to protect the cells from the effect of the elevated partial oxygen pressure that was dissolved in the medium. The numbers of the dead and live promastigotes were counted microscopically using the trypan blue exclusion test and were calculated each day. While a slight elevation in the number of live promastigotes was observed after 2 h of incubation following the treatment, the elevation in the number of cells was significantly suppressed after 4 and 6 h of HBO treatment (P < 0.001). The growth-inhibiting effect of HBO treatment on the promastigotes is shown in Figure 4.

The effect of HBO on *L. tropica* amastigotes in the macrophages obtained from BALB/c mice

The number of parasites was lower in the cultures treated with HBO than in the untreated cultures at 2 h of treatment. It was also observed that the amastigotes died within the macrophages. Although the total number of parasites decreased after 4 and 6 h (P < 0.05), it was observed that the loss of amastigotes in the HBO-treated macrophages was higher than that of the control cells (Figure 5).

Figure 4. The effect of the growth inhibition of HBO exposure on *L. tropica* promastigotes daily in culture and those not exposed to HBO (control) at 2, 4, and 6 h. The data depict the mean ± SD of 3 separate experiments. Exposure to HBO for 4 and 6 h significantly suppressed the elevated promastigote numbers (*P < 0.001).
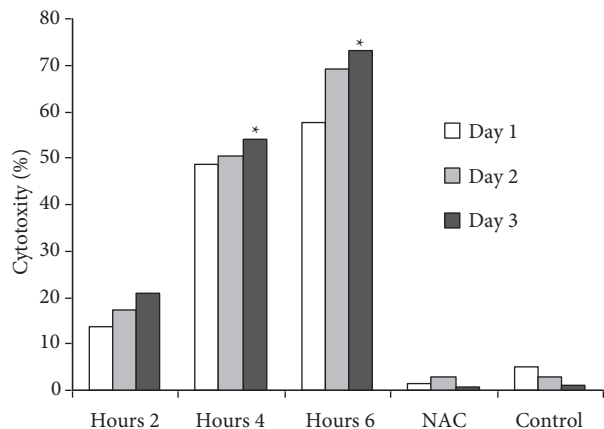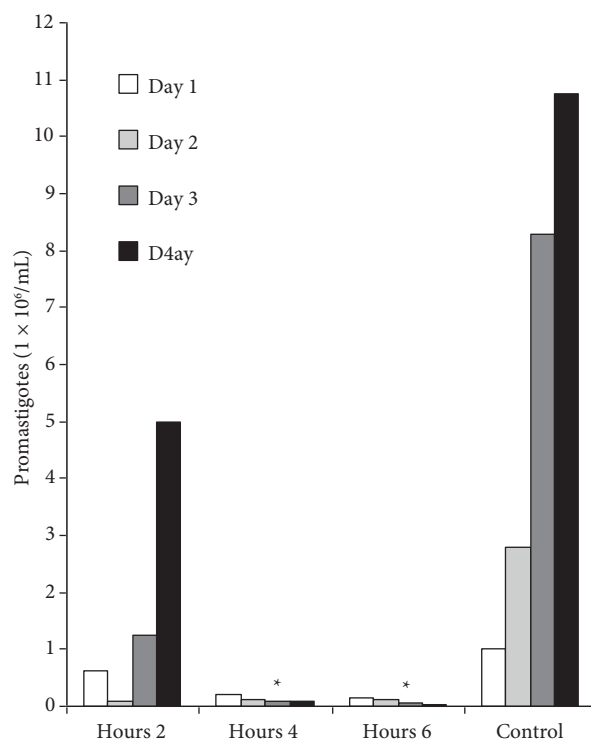
Figure 3. Cytotoxicity percentage of HBO exposure for *L. tropica* promastigotes in culture, not exposed to HBO (control) and pretreated with NAC on days 1, 2, and 3. The data shown represent the mean ± SD of 3 separate experiments. The significance of the difference between the treated and untreated cells is indicated (*P < 0.05).
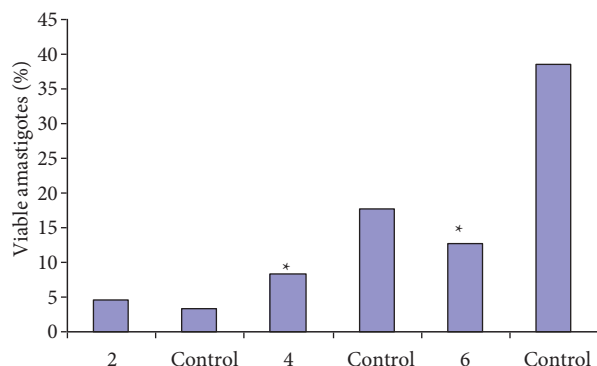
Figure 5. Comparison of HBO treatments on *L. tropica* amastigotes in mice peritoneal macrophages with the control macrophage culture cells (not exposed to HBO) at 2, 4, and 6 h. The significance of the difference between the treated and untreated cells is indicated (*P < 0.01).

Discussion

In the present study, the toxic effect of HBO on *L. tropica* promastigote and amastigote forms was demonstrated. The cytotoxic effect of HBO began 2 h after treatment and reached a maximum level after 6 h (54.2%) in the treated groups. The rate of HBO effect on *L. tropica* amastigotes increased as time passed, from 3.3% at 2 h to 38.5% at 6 h (Figure 5). The other significant observation was that HBO-mediated toxicity is irreversible even after 2 h of treatment.

Muhvich et al. examined the effect of HBO on *L. braziliensis panamensis* and found that 90% of the promastigotes died after 24 h of HBO treatment (25). In our study, we detected that 54.2% of the promastigotes died after 6 h and growth inhibition was observed in most of the promastigotes. As is widely known, a period of 24 h is quite a long time for HBO treatment. Detection of inhibition in the growth of promastigotes even after 2 h suggests that a shorter duration of treatment may be clinically effective. Arrais-Silva et al. (23) examined the effect of HBO on *L. amazonensis* and obtained results consistent with ours, yet our results were somewhat higher than those obtained by Arrais-Silva et al. (23). The reason for this is that we subjected the cell culture plaques to a constant HBO treatment for a 3-day period without replacing the fresh culture medium. We conducted an MTT assay every day at 2, 4, and 6 h and recorded the results. Of course, partially dissolved O_2 may exist within the cell culture plaques, so the effect may be larger. As a result, we determined that the majority of the promastigotes may die from a short duration of HBO treatment, such as 2 h/day for 3 days. We also observed that HBO treatment became influential for the macrophage cells obtained from mice infected by *L. tropica* amastigotes after 2 h of treatment, but when this time was extended, the control macrophage cells were also affected. We think that the effect of HBO treatment on the amastigote forms in a short time,

2 h, was a remarkable advantage. In other studies, it was also shown that the promastigote forms were more sensitive to the treatment when compared to the amastigote forms. The reason for this was that the amastigote forms develop self-defense mechanisms with antioxidant defense molecules like glutathione peroxidase and intracellular thiol against macrophage toxic oxidant products like hydrogen peroxide (H_2O_2) and superoxide (O_2-) (17,28-30). Das et al. reported that H_2O_2 caused an apoptosis-like death in *L. donovani* promastigotes (31). Obviously, superoxide and hydrogen peroxide are the main products formed by the HBO treatment. In our study, it was also proven that the effect of the HBO treatment was inhibited when used along with NAC, which has an antioxidant property. However, as NAC cannot overcome the reactive oxygen products under an adequate pressure, it cannot prevent the inhibiting effect of HBO on the growth of the promastigotes (23). HBO treatment plays a great role in clinical treatment, especially in the healing of wounds (32).

In conclusion, the cytotoxic effect of HBO treatment on the *L. tropica* promastigote and amastigote forms within the macrophages was demonstrated in this study. Since the inhibitory effect of the treatment on the growth of the promastigotes occurred in a short time, 2 h, the dose and duration of the application could be tolerated by humans, and this method could be used for the treatment of cutaneous leishmaniasis with chemotherapy. We believe that further studies are needed for its more reliable use in the treatment of human patients.

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