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Evaluating the Glucantime Concentration for the *ex vivo* Glial Cell Model of Antimony-resistant *Leishmania tropica* Amastigotes

Antimon Dirençli Leishmania tropica Amastigotlarının ex vivo Glial Hücre Modeli için Glukantim Konsantrasyonunun Değerlendirilmesi

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ABSTRACT

Objective: Because the protocols used in the treatment of leishmaniasis can be toxic and have many limitations, such as the development of resistance against such protocols, new treatment options are needed, especially against resistant patients. *Ex vivo* models may be a good source for evaluating new drug options for patients with antimony-resistant parasites. This study aimed to evaluate the Glucantime concentration for our *ex vivo* glial cell amastigote model we had defined in previous work.

Methods: We prepared the astroglial cell culture from brains of 2 to 3 day old neonatal Sprague-Dawley rats under sterile conditions by modifying McCarthy's method. Four plates of cells were infected with antimony-resistant *Leishmania tropica* promastigotes. After 24 h of incubation, we added Glucantime to 3 plates with different concentrations. After 72 h, we removed the supernatant and then dried, fixed, and stained the plates with Giemsa to count the amastigotes in the glial cells.

Results: We observed the amastigotes in glial cells in the control flask. Glial cells were ruined in flasks, which include 75 µg/mL and 37.5 µg/mL Glucantime. The number of amastigotes per 100 glial cells was 116 for the flask with 7.5 µg/mL Glucantime concentration, while 487 for the control flask.

Conclusion: We found that while high concentrations of Glucantime were toxic for glial cells, 7.5 µg/mL Glucantime concentration managed to reduce the number of *Leishmania tropica* amastigotes in glial cells.

Keywords: *Leishmania tropica*, *ex vivo*, glial cell, amastigote, antimony resistance

ÖZ

Amaç: Leishmaniasis tedavisinde kullanılan ilaçlar toksiktir ve pratikte uygulanan tedavi protokollerine karşı direnç gelişimi gibi birçok kısıtlılığı vardır. Özellikle dirençli hastalar için yeni tedavi seçeneklerine ihtiyaç vardır. *Ex vivo* modeller, antimon direnci olan hastalarda yeni ilaç seçeneklerini değerlendirmek için iyi bir kaynak olabilir. Bu çalışmada, daha önce tanımladığımız *ex vivo* glial hücre amastigot modelimizde kullanılabilecek Glukantim konsantrasyonunu değerlendirmeyi amaçladık.

Yöntemler: Astroglial hücre kültürü, McCarthy'nin yöntemi değiştirilerek steril koşullar altında 2-3 günlük yenidoğan Sprague-Dawley sıçan beyinlerinden hazırlandı. Dört hücre plağı antimon dirençli *Leishmania tropica* promastigotları ile enfekte edildi. Yirmi dört saatlik inkübasyondan sonra Glukantim farklı konsantrasyonlarda üç plakaya eklendi. Yetmiş iki saat sonra üst sıvı çıkarıldı, plaklar kurutuldu, sabitlendi ve glial hücrelerdeki amastigotları saymak için Giemsa ile boyandı.

Bulgular: Amastigotlar, kontrol plağındaki glial hücrelerde yoğun şekilde gözlendi. 75 µg/mL ve 37,5 µg/mL Glukantim içeren şişelerde glial hücreler tamamen hasarlandı. Yüz glial hücre başına amastigot sayısı, 7,5 µg/mL Glukantim konsantrasyonlu plak için 116 iken kontrol plağı için 487 idi.

Sonuç: Yüksek Glukantim konsantrasyonları glial hücreler için toksik bulundu ve 7,5 µg/mL Glukantim konsantrasyonu glial hücrelerdeki *Leishmania tropica* amastigotlarının sayısını azaltmak için yeterli bulundu.

Anahtar Kelimeler: *Leishmania tropica*, *ex vivo*, glia hücresi, amastigot, antimon direnci

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Leishmaniasis is a vector-borne zoonotic disease with different clinical features such as cutaneous, mucocutaneous, visceral and viscerotropic forms (1). Leishmaniasis is endemic in nearly 100 countries and people living in tropics, subtropics and the Mediterranean basin are under risk (2,3). The treatment regiments used against Leishmaniasis have many limitations such as toxicity and bone marrow suppression (4). One of these limitations is the resistance against the treatment regiments used in routine practice (5).

Drug resistance can be monitored with promastigotes *in vitro* and with amastigotes *in vitro* or *ex vivo*. *In vitro* models are not adequate for evaluating the resistance in human leishmaniasis while promastigotes are the forms in sand-fly vectors. Amastigote-macrophage model is used as the gold standard method for the evaluation of drug efficacy against *Leishmania* species (6-8). However, there are reports that experiments with this method yield incompatible results with clinical response (9). Previously, we defined an *ex vivo* glial cell-amastigote model (10). In this study, we aimed to define the appropriate Glucantime concentration for evaluating the resistance against *Leishmania* species in *ex vivo* glial cell-amastigote model.

METHODS

Astroglia Cell Culture

Primary cell culture was conducted in the cell culture laboratory of Ege University Faculty of Medicine, Department of Physiology, in accordance with the principles of experimental animal use of the Helsinki Declaration.

Primary astroglia cell culture was prepared from 2-3-day old new-born Sprague Dawley rat brains under sterile conditions. The McCarthy method was used with some modifications (11,12). Briefly, new-born rat skulls were removed, and cerebral cortex was transferred to sterile petri dishes containing Dulbecco's Modified Eagle's Medium (DMEM) and placed on ice. The brain membranes and veins were removed under the stereo-zoom microscope. The resulting tissue was first cut into small pieces of 1-2 mm^3 size. Then, 5% of 0.25% trypsin-EDTA solution was subjected to enzymatic reaction to break down connective tissue. The trypsin was neutralized by adding DMEM +10% foetal calf serum + antibiotic-containing (Penicillin-Streptomycin) glia culture medium and then centrifuged at 200 g for 6 minutes at room temperature. The pellet was reconstituted, and the cells were counted on the haemocytometer, and re-diluted to a concentration of $2x10^5$. The ratio of live cells was determined by staining with Trypan blue and 6-well sterile cell culture plate was sown. Cells were grown in the above-mentioned glia culture medium, containing 5% CO_2 and 96% humid air. Cell lines were monitored daily in terms of viability, proliferation and infection in inverted microscope and their medium was changed every 2 days. It became suitable for *ex vivo* glial cell-amastigote model by multiplying enough to cover the entire floor in 7 days.

Isolation of *Leishmania tropica* Promastigotes from Antimony Resistant Cutaneous Leishmaniasis Case

Promastigotes were obtained from a lesion of an 11-year-old girl who was admitted to the Dicle University Faculty of Medicine Dermatology Outpatient Clinic in the year 2013, with a crusted papular lesion in the lower periphery of the atrophic scar, which started as a blister on the nasal dorsum two months ago. *Leishmania* spp. amastigotes were seen in the examination of the Giemsa-stained smears prepared from the lesion. Samples from the lesion were inoculated in enriched Novy-Nicolle-McNeal (NNN) medium and promastigotes were detected. The obtained promastigotes were inoculated in medium containing 10% foetal bovine serum and RPMI 1640. When it reached 10^8 promastigotes per millilitre, it was cryopreserved and stored in liquid nitrogen.

In the genotyping studies performed with primers and probes we designed specific to the ITS-1 region of the parasite, the causative species was determined to be *Leishmania tropica* (*L. tropica*) with DNA obtained both from clinical specimen and promastigotes produced in enriched NNN medium.

Intralesional meglumine antimoniate (Glucantime® injectable, Sanofi-Aventis, France) 5 millilitres per day was administered to the patient for 11 days. Since no significant improvement in the lesion was observed in the control examination performed three months later, 7 millilitres per day of intralesional Glucantime was applied again for 11 days. 4 millilitres per day intravenous sodium stibogluconate (Pentostam® injectable, GlaxoSmithKline, United Kingdom) was administered for 17 days to the patient who was admitted again in 2014 due to the non-healing of the lesion. No significant improvement was observed in the lesion 6 months after the end of treatment. Since there was no response to pentavalent antimony treatment, the patient was accepted as an "antimony resistant case".

Ex vivo Infection and Treatment Model

L. tropica promastigotes previously isolated from the above-mentioned case and cryopreserved in the Parasitic Bank of Celal Bayar University were used in our *ex vivo* infection model. Promastigotes were counted and $2x10^6$ promastigotes were prepared to achieve 1:10 glial cell promastigotes ratio. Four plates of astroglia cells, which reached sufficient density, were infected with prepared promastigotes. After 24 hours of incubation, Glucantime was added to three different plates with 75 µg/mL, 37.5 µg/mL and 7.5 µg/mL concentrations, respectively. After 72 hours of incubation, supernatant was removed, plate was dried at 25 °C, then fixed with methyl alcohol and stained with Giemsa to count *L. tropica* amastigotes in glial cells (Figure 1). The number of amastigotes per 100 glial cells were noted.

Statistical Analysis

A study that created a model and statistical analysis was not applied for such a study.

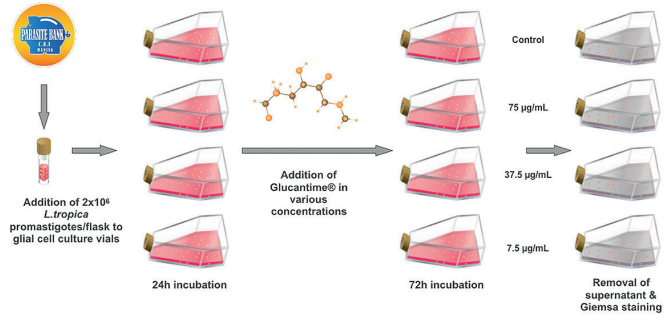

Figure 1. Trial steps of various glucantime concentrations in *ex vivo* glial cell-amastigote model

RESULTS

Amastigotes of *L. tropica* were intensely observed in glial cells in control flask (Figure 2). Glial cells were completely ruined in flasks which include 75 µg/mL and 37.5 µg/mL Glucantime, therefore no amastigotes were seen in that flasks. The number of amastigotes per 100 glial cells was 116 for flask with 7.5 µg/mL Glucantime concentration (Figure 2). The number of amastigotes per 100 glial cells was 487 for control flask (Figure 2).

DISCUSSION

There are three options for conventional treatment of leishmaniasis: pentavalent antimonial compounds, pentamidine and amphotericin B. Pentavalent antimonial compounds are still preferred due to their low cost and high cure rates despite their severe hepatic, pancreatic, cardiac, bone marrow and renal toxicity. The need for daily administration of at least three weeks and the presence of side effects such as local pain, nausea, vomiting, diarrhoea, weakness, muscle pain, rashes in intramuscular injection site led to problems in the administration of pentavalent antimony compounds (13). Increased resistance is reported due to widespread usage of pentavalent antimonial compounds in the areas such as Peru, India, and Iran where leishmaniasis is endemic (14,15).

There are three main model for both evaluating the drug resistance and testing novel drug compounds against leishmaniasis: *In vitro*, *in vivo* and *ex vivo*. The most common *in vitro* model uses promastigotes which is the form of *Leishmania* species in sandfly vectors (16-18). Therefore *in vitro* model is not adequate for evaluating the resistance in human Leishmaniasis. In the second method, *in vivo* model, although the amastigotes of the laboratory infected mice are used, incompatible results may be obtained due to host related factors such as genetical differences (19). *Ex vivo* amastigote-macrophage model is used as the gold standard method for the evaluation of drug efficacy against *Leishmania* species (6-8). However, there are reports that experiments with this method yield incompatible results with clinical response. Soleimanifard et al. (9) reported response to treatment in all cases in their *ex vivo* model with amastigotes obtained from 10 unresponsive patients and attributed this situation to the deficiencies that may occur during the application of the treatment. We developed the glial cell amastigote model, considering that the central nervous system may be a suitable area for hiding *Leishmania* species from drugs and host immune response in clinically resistant cases (10).

In this study, we assessed the effect of the various concentrations of Glucantime in our *ex vivo* glial cell-amastigote model obtained from a clinically resistant Leishmaniasis case. As there was no reference Glucantime dose present for this *ex vivo* glial cell model, we chose three Glucantime concentration in a broad range that used in various amastigote-macrophage models (20). Glial cells were completely ruined in moderate and high concentrations of Glucantime. The 7.5 µg/mL Glucantime concentration managed to reduce the number of *Leishmania tropica* amastigotes compared to control group in our *ex vivo* glial cell model but was not at a level to eradicate the infection completely. Considering that the case tested in the model was clinically resistant to Glucantime, the result we obtained was consistent with the clinical response.

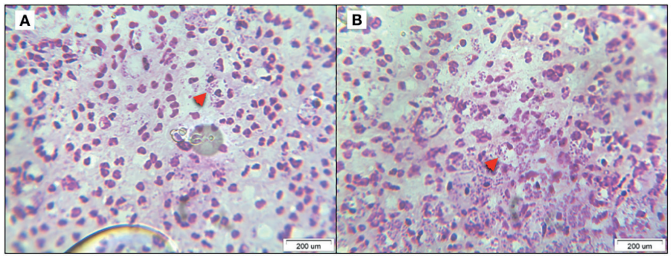

Figure 2. Amastigotes (red arrows) in glial cells in flask with 7.5 µg/mL Glucantime concentration (A) and in control flask without Glucantime (B) (400x magnification)

CONCLUSION

According to our results, *ex vivo* glial cell-amastigote model can be used for drug resistance monitoring. Comparative experiments of *ex vivo* glial cell-amastigote and amastigote-macrophage models should be done with lower concentrations of Glucantime in various incubation periods and various *Leishmanias* strains to make an approved drug resistance monitoring model of glial cells and amastigotes.

INFORMATION

This study was presented at the 38th International Congress of Turkish Society of Microbiology (November 4-8, 2018, Antalya/Turkey).

* Ethics

Ethics Committee Approval: The study was conducted in accordance with the principles of experimental animal use of the Helsinki Declaration. The authors asset that all procedures contributing to this work comply with the ethical standards of the national and institutional guides on the care and use of animals.

Informed Consent: Patient consent not applicable for this study.

Peer-review: Internally peer-reviewed.

* Authorship Contributions

Concept: O.Z., Ö.A.Y., N.T., Design: O.Z., Ö.A.Y., N.T., Data Collection or Processing: O.Z., M.H., V.E., A.Ö., Analysis or Interpretation: O.Z., V.E., M.H., A.Ö., Writing: O.Z., Ö.A.Y., N.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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