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Effectiveness of doxycycline for the treatment of zoonotic cutaneous leishmaniasis *in vivo*

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Research Article

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Abstract

There are available data on *in vivo* studies of monotherapy of zoonotic cutaneous leishmaniasis with some antibacterial drugs (doxycycline) and their comparison with meglumine antimoniate (glucantime). We used golden Syrian hamsters as a laboratory model. Experimental groups were formed, each of which was treated with one of the tested drugs. Infection of animals was carried out with *Leishmania major* promastigotes. We selected highly virulent strains of *L. major* culture isolated from human ulcers or rodents. Meglumine antimoniate monotherapy and doxycycline monotherapy are quite effective and do not differ by the 30th day of their use in such indicators as the average degree of local damage and the average number of *Leishmania* in the lesions. The main differences were recorded in terms of average body weight gain and average clinical recovery in favour of doxycycline. *Leishmania* in the lesion on the 60th day were completely absent in treatment with doxycycline. The experiment proved the effectiveness of doxycycline monotherapy: *Leishmania* in the lesions were absolutely absent by the end of the treatment.

Introduction

Currently, the problem of cutaneous leishmaniasis (CL) is relevant for practitioners. This phenomenon occurs due to the active migration of the population to areas endemic for leishmaniasis (tourist trips, business trips, contract work, etc.), and also due to the arrival of people from countries where this disease is recorded quite often. CL is endemic in 98 countries. More than 350 million people are at risk, more than 12 million people are already infected, and the annual number of new cases is estimated at 1.5–2 million (WHO, 2010).

Physicians have serious problems both with diagnosis (Aleksandrova *et al.*, 2008; Bodnya *et al.*, 2014; Rakhmatov *et al.*, 2014; Jalilov, 2017; Bettaieb *et al.*, 2020; De Brito *et al.*, 2020; Rostami *et al.*, 2020) and the choice of treatment method (Arteaga-Livias *et al.*, 2020; Peixoto *et al.*, 2020; Pinart *et al.*, 2020; Qurtas and Shabila, 2020). There is no single efficient therapy for CL. In this regard, each region, the Old World or the New World, has its own primary therapy regimens and its own treatment approaches, based on its own experience. In the European recommendations for the management of CL, a new species-oriented therapy approach was proposed (Blum *et al.*, 2014; Hodiamont *et al.*, 2014), which became possible thanks to more accessible methods of molecular diagnosis of species (Van der Auwera and Dujardin, 2015). The lack of an effective method or means of treatment for all types and syndromes of CL is still a serious problem (Nassif *et al.*, 2017; Kuznetsova, 2018; Mancianti *et al.*, 2020; Roatt *et al.*, 2020).

The WHO recommendations, the European recommendations for the treatment of leishmaniasis and the LeishMan guidelines for the treatment of CL suggest using meglumine antimoniate (glucantime), pentostam (stibogluconate sodium), miltefosine (impavido), amphotericin B liposomal (ambisome), paromomycin, aminochinolum and pentamidinum (Masmoudi *et al.*, 2008; Sosa *et al.*, 2013). But all of them are quite toxic, and there is no other drug effective for all *Leishmania* species.

In this regard, the publications of authors empirically treated zoonotic cutaneous leishmaniasis (ZCL) are valuable. We chose antibiotics of two various pharmacological groups: (1) doxycycline (Masmoudi *et al.*, 2008; Cortez-Maya *et al.*, 2019). A universal drug for the treatment of leishmaniasis is the preparation of 5-valent antimony. Therefore, meglumine antimoniate (glucantime) was used as a control in assessing the effectiveness of other drugs. Since it is quite toxic, our task was to find a non-less effective drug without or less pronounced side-effect.

The pentavalent antimony's mechanism of action is well studied: the effect occurs due to inhibition of a parasite adenosine triphosphate synthetase (William and Khaldoun, 2004). The effectiveness of doxycycline (71%) is quite competitive with pentavalent antimony, which has effectiveness from 60 to 90% (Masmoudi *et al.*, 2008). However, the mechanism of action of doxycycline has not been determined yet. The most popular hypotheses of doxycycline effectiveness are as the following: (1) direct effect on the body of *Leishmania* due to good intracellular penetration (Bonnetblanc, 2002); (2) tetracyclines affect protease–antiprotease imbalance,

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inhibit collagenase activity, thus exerting anti-inflammatory activity (Humbert *et al.*, 1991*a*, 1991*b*). Doxycycline may possibly represent a therapeutic alternative in the treatment of CL, especially in endemic areas, providing better tolerance and lower cost.

Materials and methods

Laboratory model

The drugs were tested *in vivo* using a laboratory model of ZCL. We used golden Syrian hamsters (GSH) as a laboratory model. They represent a classic model of visceral leishmaniasis due to their greater susceptibility to the disease, and in our study, we aimed to obtain the most pronounced and vivid clinical picture compared to BALB/c mice. After hamsters and mice were infected, the pathological process in hamsters was more striking. A few studies documented a transient or sporadic dissemination of *Leishmania major* amastigotes distant from the inoculation site (Stenger *et al.*, 1996; Nicolas *et al.*, 2000). It was also a benefit for us. All research studies with laboratory animals are carried out in accordance with generally accepted ethical standards for the treatment of animals, based on standard operating procedures adopted by the research manufacturing organization, which comply with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123).

The animals were purchased from the Pushchino laboratory animal nursery, had a veterinary passport, and the contents and nutrition were in accordance with regulatory documents.

In the *in vivo* experiments, infection of animals was carried out on 2-month-old animals, the mass of which was about 30 g. The incubation period and the development of the classical clinical picture of the disease had taken some time, so by the beginning of therapy with the tested drugs (doxycycline and meglumine antimoniate), the weight of the animals was 100 g or little more.

We used 122 GSH, divided into four experimental groups of 28 animals, each of which was treated with one of the tested drugs, and one untreated group of 10. To standardize the experiments, we used the preclinical drug study guide edited by Mironov *et al.* (2012). At the end of the experiment, in animals posthumously, we determined the presence or absence of *Leishmania* in the internal organs (liver, spleen and kidney). There was no pathological visceral process.

Infection of animals was carried out with *L. major* promastigotes, which is closer to natural, since it is the promastigotes that enter the macroorganism when the vector bites. These forms of *Leishmania* are easy to cultivate on artificial substratum and dose under the control of Goryaev's chamber. We selected highly virulent strains of *L. major* culture isolated from human ulcers or rodents, which were stored in the cryobank of Martsinovsky Institute of Medical Parasitology, Tropical and Vector-borne Diseases. *Leishmania* was cultured at a temperature of 24°C on NNN medium (Mironov *et al.*, 2012). Considering that during prolonged cultivation of promastigotes on artificial nutrient media, their virulence is lost, we used the biomass after 1–2 passages, i.e. 10–14-day-old culture. The minimum infectious dose should contain 10^6 parasites in a volume of 0.05 mL. A two-millionth suspension of the promastigotes of the *L. major* culture was administered intradermally once at a time to each animal. The injection sites were symmetrical: groin (the least indumentum) and ears (the most frequent localization of lesions in animals in natural conditions) with the formation of the effect of 'lemon peel'. After the incubation period, a typical 'leishmanioma' forms in animals at the injection site of the culture.

The administration of the test drugs can begin both on the day of infection (preventive study) and at different stages of infection (medical study). We started the treatment after the incubation period and the transformation of most tubercles into ulcers. It was usually a month or more after infection.

Representative experimental groups

The incubation period of experimental ZCL in hamsters ranged from 1 to 8 months, averaging 77.1 ± 20.3 days (Supplementary Table 1). In more than half of the animals (58.1%), clinical manifestations of ZCL occurred within 2 months, and in 1/3 of animals (33.8%), within 4 months. The rest of the animals (8.1%) were affected within 4–8 months.

For the purpose of representativeness, the formation of all experimental groups was carried out taking into account the body weight of the animal, the uniformity of clinical manifestations on the skin and the duration of the ulcer. The main criterion for selecting animals to start therapy was the clinical picture of leishmaniasis with a pronounced skin process, confirmed microscopically – the detection of amastigotes in a smear from the lesion. The average size of ulcers before therapy was 0.40 cm^2. Thus, we began the therapy as the pathological process developed. To verify the diagnosis, scarificates were made from the torus-shaped borders of the ulcer. *Leishmania* were also found in large numbers in serous fluid obtained by pressing on the crust. A large number of *Leishmania* located in macrophages and freely lying testified to the presence of a specific process. Animals were included in the experimental section of the research to study the antileishmanial activity of the drugs only after microscopic detection of *Leishmania* in the lesion foci.

Study drugs

We studied the effectiveness of Meglumine antimonate (MA), doxycycline, monotherapy in a ZCL model. The single therapeutic dose of drugs was determined by the maximum concentration recommended by the manufacturer's instructions of the drug for each substance for a person with an average weight of 60 kg for intramuscular administration and corresponded to that used in medical practice. For the GSH, this dose was determined per 100 g of body weight. The multiplicity of drugs administration depended on their effectiveness and tolerance. For assessment, we used the method of two samples assuming equal variances.

The drug MA was administered once a week in a dose of 0.03 g per 100 g of the laboratory model weight. The maximum number of injections was 6–7.

The drug doxycycline was administered once a week in a dose of 0.00033 g per 100 g of the laboratory model weight. The maximum number of injections did not exceed 4–5.

The effectiveness of therapy

The effectiveness of therapy was evaluated by using the following criteria: (1) the time of clinical recovery (scarring); (2) the average increase in GSH body weight; (3) the average degree of local lesion on the skin. The degree of local lesion was determined on a special scale: '0' – no lesions; '+' – infiltration; '++' – ulceration (less than 0.2 cm); '+++' – an ulcer with a crust (0.3–0.8 cm); '++++' – extensive deep ulcer (more than 0.9 cm); '++' – dying down of the process, the beginning of epithelialization; '+' – further healing, accompanied by peeling; '0' – complete epithelialization; (4) the average number of *Leishmania* detected microscopically in smears stained with haematoxylin and eosin. For this, the number of *Leishmania* in smears was counted. We used the following scheme: '0' – no *Leishmania*; '+' – 1 *Leishmania* per 1–9 fields of view; '++' – from 2 to 10 *Leishmania* in the 1 field of view; '+++' – from 11 to 50 *Leishmania* in the 1 field of view;

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Table 1. Results of evaluating the effectiveness of MA monotherapy in 28 GSH

Observation days	The weight of the laboratory model (g)	The average size of ulcers (cm^2)	Degree of local lesion (0/+)	Microscopy/number of *Leishmania* (0/+)[a]
Before treatment	106.8 ± 1.8 ($P < 0.05$)	0.6	3.1 ± 0.7 ($P < 0.05$)	3.4 ± 0.5 ($P < 0.05$)
30th day after starting therapy	109.5 ± 1.6 ($P < 0.05$)	0.08	1.3 ± 0.7 ($P < 0.05$)	1.5 ± 0.5 ($P < 0.05$)
60th day after starting therapy	112.9 ± 1.6 ($P < 0.05$)	0	0.1 ± 0.04 ($P < 0.05$)	0.6 ± 0.07 ($P < 0.05$)
Clinical recovery period (days)	**40.6 ± 3.7 ($P < 0.05$)**			

[a]The fact that the presence of a parasite in smears does not reflect the viability of the pathogen.

Table 2. Doxycycline monotherapy efficiency assessment results in 28 GSH

Observation days	The weight of the laboratory model (g)	The average size of ulcers (cm^2)	Degree of local lesion (0/+)	Microscopy/number of *Leishmania* (0/+)[a]
Before treatment	103.5 ± 2.2 ($P < 0.05$)	0.43	3.0 ± 0.8 ($P < 0.05$)	3.6 ± 0.3 ($P < 0.05$)
30th day after starting therapy	109.0 ± 1.5 ($P < 0.05$)	0	1.05 ± 0.8 ($P < 0.05$)	1.28 ± 0.47 ($P < 0.05$)
60th day after starting therapy	114.3 ± 1.3 ($P < 0.05$)	0	0.08 ± 0.01 ($P < 0.05$)	0.03 ± 0.01 ($P < 0.05$)
Clinical recovery period (days)	**20.2 ± 2.2 ($P < 0.05$)**			

[a]The fact that the presence of a parasite in smears does not reflect the viability of the pathogen.

'++++' – more than 50 *Leishmania* in the 1 field of view; (5) the average size of a skin ulcer.

Skin ulcer in most cases had the shape of an ellipse. To measure the average area of the ulcer, we used the formula:

$$S = 1/2D \times 1/2d \times \pi,$$

where D is the length of the major axis of the ellipse, d is the length of the minor axis of the ellipse, $\pi = 3.14$.

The follow-up time for animals after drug administration was 60 days.

Negative control

In animals from the untreated group ($N = 10$), lesions were observed until the end of life and natural death of animals in laboratory conditions. Their life lasted on average 2 years.

Statistical processing of the material

The processing of the research results was carried out using the statistical software package, and parametric methods (the two-sample Student's test) to analyse normal distribution parameters. The data were rendered by the EXCEL program. Descriptive statistics of quantitative features are presented by mean and standard deviations (in the format M ± m).

Results and discussions

Results of evaluating the effectiveness of treatment with meglumine antimoniate (control group) of experimental ZCL in GSH

To study the effectiveness of MA monotherapy in the ZCL model, 28 GSH were used. In the treatment with MA animal, mortality was not registered. During the first 2 weeks, a decrease in animal activity, loss of appetite and a slight decrease in body weight were noted. However, the condition of the animals subsequently stabilized.

The results of evaluating the effectiveness of MA monotherapy in GSH ($N = 28$) are presented in Table 1.

On the 30th day after the start of MA administration, no decrease in body weight of the animals was recorded; the average increase in body weight was 2.7 ± 0.6 g ($P > 0.05$). Ulcer scarring occurred in 5 (17.9%) animals; in the remaining 23 (82.1%), the average size of the ulcer area decreased by 7.5 times (0.08 *vs* 0.6 cm^2) ($P < 0.05$). The degree of local lesion decreased by 2.4 times (1.3 ± 0.7 *vs* 3.1 ± 0.7) ($P < 0.05$), and the number of *Leishmania* in the lesions decreased by 2.3 times (1.5 ± 0.5 *vs* 3.4 ± 0.4) ($P < 0.05$).

On the 60th day, compared with the beginning of the experiment, the average increase in body weight was 6.1 ± 1.3 g. Ulcer scarring occurred in all hamsters, which corresponded to a local lesion degree of 0. The degree of local lesion decreased by 31 times (0.1 ± 0.04 *vs* 3.1 ± 0.7) ($P < 0.05$). Ulcer scarring occurred in all GSH, only a slight infiltration was maintained.

The presence of a parasite in smears does not reflect the viability of the pathogen.

Considering that animals treated with MA are a control group, when comparing with the experimental groups we used indicators: the degree of local damage after 30 days (1.3 ± 0.7) and after 60 days (0.1 ± 0.04).

Results of evaluating the effectiveness of treatment with doxycycline experimental ZCL in GSH

In the treatment with doxycycline, animal mortality was not registered. Scarring the lesions took place during the month, on average, therefore the clinical recovery period was for 20.2 ± 2.2 days, which was significantly two times faster than in the control group (MA 40.6 ± 3.7 days) ($P < 0.05$). From the very beginning, animals tolerated treatment without any adverse reactions. The results of evaluating the effectiveness of doxycycline monotherapy in GSH ($N = 28$) are presented in Table 2.

On the 30th day, compared to the beginning of the experiment, the average weight gain of hamsters was 5.5 ± 2.8 g. All ulcers healed within 3 weeks; therefore, the average size of the ulcer was 0 cm. Skin manifestations were represented only by a slight infiltration at the site of the ulcer. Comparison of the degree of local lesion in this experimental group and the control group differed by 0.25 in favour of doxycycline, and there were no

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animals with ulcers. Active scarring of ulcers took place. The results of microscopic examination corresponded to 1.28 ± 0.47 *Leishmania* in a smear from the lesion. This is 2.8 times less than before therapy (3.6 ± 0.3) ($P < 0.05$) and 0.22 less than with meglumine antimoniate (1.5 ± 0.5) ($P > 0.05$).

On the 60th day of the experiment, compared with its beginning, the animals gained an average weight of 10.8 ± 3.1 g. The degree of local damage was minimal at 0.08 ± 0.01, while for control at 0.1 ± 0.04. The results of microscopic examination were 0.03 ± 0.01 *Leishmania* in a smear from the lesion, which is 20 times less than in the control (0.6 ± 0.7) ($P < 0.05$).

The results and success of doxycycline correlate with those in humans (Lial, 1999; Masmoudi *et al.*, 2005, 2008). However, these data are very scarce, and there is no information on preclinical studies. We need further research studies to determine the doses and optimal regimen of treatment both in animals and humans. The clinical effect of doxycycline could be due to: (1) direct effect on *Leishmania* because of good intracellular penetration (Bonnetblanc, 2002); (2) tetracyclines affect protease–antiprotease imbalance, inhibit collagenase activity, thus exerting anti-inflammatory activity (Humbert *et al.*, 1991*a*, 1991*b*).

Conclusion

Based on the results of the study, doxycycline is more effective than MA, which is the 'gold standard' in the treatment of leishmaniasis. The resolution of the ulcerative process occurred at a rate twice faster when compared to the control (40.6 ± 3.7 *vs* 20.2 ± 2.2, $P < 0.05$). No side-effects were detected.

Doxycycline may be employed as a therapeutic alternative in the treatment of CL, especially in endemic areas. It has better tolerability, no side-effects, and more rapid scarring.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020002152

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Conflict of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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