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Reversible inhibition of contractions of mammalian cardiomyocytes and of smooth muscle by the protistan parasite *Leishmania major*

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Abstract

Myotropic neuropeptides have been isolated from vertebrates and invertebrates. Recently, a myoinhibitory peptide from the protist *Leishmania major* was isolated, and its function in the sand fly vector was described. Similar lysates of cultured *L. major* were tested for their ability to inhibit contractions in mammalian cell and tissue preparations. *L. major* proteins (LMP) (34 μg/ml) completely stopped spontaneous contractions of cultured rat cardiomyocytes; cells resumed contracting after a saline wash. An application of 880 μg/ml LMP significantly decreased force of contractions (36%) in strips of guinea pig ileum precontracted with nicotine ($p < 0.01$) but not with acetylcholine ($p > 0.01$). Ileal strips rinsed with Tyrode's solution and again stimulated with nicotine contracted normally. Contractile force of ileal strips electrically stimulated with 40 V was reduced in a dose-dependent manner (30, 76, and 100%) ($p < 0.01$) by increasing concentrations of LMP (220, 440, and 880 μg/ml). This ileal preparation resumed contracting after rinsing with Tyrode's solution. Oxytocin-induced contractions of guinea pig uterine strips were reduced significantly in a dose-dependent manner (21 and 55%) ($p < 0.01$) by increasing concentrations (170 and 310 μg/ml) of LMP and resumed contracting normally after rinsing with Tyrode's solution. Modes of action for *L. major* myoinhibitory factors may include either decreasing Ca^{2+} influx or increasing Ca^{2+} efflux in susceptible muscle. Protistan-induced inotropism is discussed in light of exacerbating pathology of disease.

Keywords: Myoinhibitory; Nicotinic stimulation; Acetylcholine stimulation; Oxytocin; *Leishmania major*; Leishmaniasis

1. Introduction

Female sand flies ingest amastigotes of *Leishmania major*, the causative agent of enzootic cutaneous leishmaniasis, when imbibing blood from an infected vertebrate reservoir. Intracellular amastigotes emerge from macrophages in the fly midgut and elongate to form extracellular, motile promastigotes. Promastigotes undergo a complex cycle of growth and differentiation within the sand fly gut, migrating forward to the fly pharynx, from whence they are transmitted to a new host when the fly blood feeds

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again (Sacks and Kamhawi, 2001). After the sand fly inoculates metacyclic forms into a susceptible host, promastigotes bind to host macrophages, enter, transform into amastigotes, and reside and multiply within phagolysosomes (Chang and Fong, 1983).

A novel hydrophobic peptide isolated from cultured promastigotes reversibly inhibited sand fly hindgut contractions and resulted in significant midgut and hindgut distension. By mimicking the effect of an insect myoinhibitory peptide, parasites paralyzed and expanded the fly gut, providing more volume in which to develop and preventing expulsion after blood meal digestion (Vaidyanathan, 2005). Lysates of *L. major* also inhibited spontaneous contractions of visceral muscle preparations of seven other insect species (Vaidyanathan, 2004).

There is evidence that infection with the protistan parasites *Trypanosoma brucei* and *Eimeria tenella* results in localized vasodilation and exacerbated pathology in their vertebrate hosts (Viswambharan et al., 2003; Allen, 1997). In addition, assays with the dog heartworm *Dirofilaria immitis* revealed that application of lysates of adult nematodes resulted in modification of vertebrate muscle contractility (Kitoh et al., 2001).

The purpose of this study was to test if *L. major* also might modify vertebrate muscle contractility. Fragments of *L. major* myoinhibitory peptide predicted from de novo sequencing of mass spectrometric data are currently available. This screening of total *L. major* proteins (LMP) is the first step toward evaluating further purification fractions for increasing specific activity using a mammalian bioassay. Total LMP reversibly inhibited spontaneous contractions of rat ventricular myocytes, nicotine-induced contractions of guinea pig ileum, and oxytocin-induced contractions of guinea pig uterine muscle. However, LMP had no effect on acetylcholine-induced contractions of guinea pig ileum. Possible modes of action and other examples of parasite-induced inotropism are discussed.

2. Materials and methods

2.1. Parasite lysate proteins

Techniques for culturing parasites and preparing parasite lysate were described (Vaidyanathan, 2005). Reagents – including pharmaceutical grade nicotine, acetylcholine, and oxytocin – were purchased from Sigma Chemical Co. (Rehovot, Israel) unless otherwise specified. Cultures of *L. major* MHOM/IL/86/Blum (Jordan Valley strain) were obtained from the W.H.O. *Leishmania* Reference Center, the Hebrew University of Jerusalem. Parasites were grown in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Beit Haemek, Israel), 10% heat-inactivated fetal calf serum (FCS) (Biological Industries), 4 mM L-glutamine, and 2 mM adenosine. Cultures were maintained at 28 °C and passaged every 4 days. Control cultures of *Crithidia fasciculata*, an obligate kinetoplastid parasite of mosquitoes, were grown in brain-heart infusion at 28 °C and passaged daily.

Promastigote cultures containing 10^7–10^8 parasites/ml were washed twice with ice-cold mosquito saline (Hagedorn et al., 1977) with protease inhibitors and lysed by three cycles of freeze–thawing. Crude homogenates were centrifuged 12,000 × *g* for 30 min. Concentrations of LMP in the supernatant were quantified by the Bradford method (Bradford, 1976). Based on previous studies (Vaidyanathan, 2004, 2005), only lysate supernatant was used for mammalian bioassays.

2.2. Neonatal rat cardiomyocyte assay

Rat ventricular myocytes were prepared according to Pinson et al. (1987). Neonatal rats (<6 h old) were decapitated, and ventricles washed in phosphate-buffered saline (PBS), mechanically sliced, and digested with 1% trypsin. Samples were centrifuged at 1000 × *g* and resuspended in Ham F-10 (Biological Industries) supplemented with 10% horse serum (Ham, 1963). Suspensions were diluted with Ham F-10 until turbid, and 1 ml was used to seed each well of sterile 24-well plates (Corning Co., New York, USA). Monolayers of ventricular myocytes were maintained at 35 °C in an atmosphere of 3.1% CO_2. Samples of mosquito saline, LMP (34 μg/ml), or *C. fasciculata* lysates (42 μg/ml) were overlaid on cultured myocytes. These concentrations correspond to 50% contractile inhibition in a sand fly hindgut bioassay (Vaidyanathan, 2005). Samples were evenly distributed by gently swirling after application and allowed to incubate 30 min. Contractions were

counted for 10 min on a warm plate. All wells were then washed with sterile saline, reincubated for 30 min, and observed again. Data for six observations sequentially performed on the same sample of myocytes are presented.

2.3. Guinea pig visceral muscle assays

Adult male and female guinea pigs were obtained from the Animal Care Facility of Hadassah Medical School, Jerusalem, Israel. Animals were sacrificed in accordance with the Report of the Federation of European Laboratory Animal Science Associations (Anon, 1994). Segments of ileum and uterus were dissected and gently cleared of adhering fat and mesentery. Segments of 2–3 cm of each organ were tied with silk thread to the base of a 20 ml isolated organ bath containing Tyrode's solution at 35 °C, bubbled with 95% O_2: 5% CO_2. The composition of Tyrode's solution is 11.90 mM $NaHCO_3$, 1.05 mM $MgCl_2{\cdot}6H_2O$, 5.55 mM glucose, 2.68 mM KCl, 167.00 mM NaCl, 0.42 mM NaH_2PO_4, and 1.80 mM $CaCl_2{\cdot}2H_2O$, pH adjusted to 7.2 with NaOH (Webster and Prado, 1970) and filter-sterilized (0.22 μm Minisart filters, Sartorius AG, Göttingen, Germany). Bubbling in the organ bath allowed for effective mixing of drugs and LMP. The other end of the tissue was hooked to a force-displacement transducer (Coulbourn Instruments, Allentown, PA, USA) attached to a computerized polygraph to record contractions. Both ileum and uterus preparations were allowed 30 min equilibration before addition of drugs or LMP (Webster and Prado, 1970).

Segments of ileum were precontracted with 30 μl nicotine or acetylcholine per 20 ml Tyrode's solution. After repeated contractions reached a plateau, 2.0 ml of LMP (8.8 mg/ml, final concentration 880 μg/ml) were added and contractile force measured after 2 min (to allow bath temperature to equilibrate to 35 °C). The tissue samples were washed with fresh Tyrode's solution, and nicotine or acetylcholine was added again to check if myoinhibition was reversible. Tests were repeated six times.

An additional assay entailed stimulating ileal strips to contract with regular cycles of 40 V. Cumulative samples of 0.5, 0.5, and 1.0 ml of LMP (8.8 mg/ml) were added to test for dosage response. The final protein concentration after each application was 220, 440, and 880 μg/ml. Tests were repeated three times.

Segments of guinea pig uterus were precontracted with 10 μl of oxytocin (10 units/ml) per 20 ml Tyrode's solution. Samples of LMP (3.3 mg/ml) were added 3 and 6 min after initial equilibration and allowed to act for 3 min. Final protein concentrations in the organ bath after each application were 170 and 310 μg/ml. The bath was washed afterwards with fresh Tyrode's solution and 10 μl of oxytocin was added again to test if myoinhibition was reversible. Data are presented for two repetitions.

2.4. Statistical analysis

Contractile values induced by nicotine, acetylcholine, and oxytocin with and without LMP treatment were compaired using paired *t*-tests. A probability of <0.01 was considered significant (Daniel, 1999).

3. Results

3.1. Neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes stopped contracting when incubated 30 min with 34 μg/ml LMP and resumed contracting normally when rinsed with saline (Fig. 1). Because plates were gently agitated after overlay of lysate, the entire cell monolayer surface came in equal contact with LMP, and cessation of contractions was detected regardless of where cells were observed. Inhibition of contraction was accompanied by clumping and swelling of cells. Neither mosquito saline nor *C. fasciculata* lysate proteins (42 μg/ml) had any effect on cardiomyocyte contractions.

3.2. Guinea pig ileum pre-treated with nicotine and acetylcholine

Strips of guinea pig ileum contracted spontaneously for 3 min without significant change in frequency or amplitude when suspended in oxygenated Tyrode's solution at 35 °C (Fig. 2). Ileum strips precontracted with nicotine exerted an average of 2.8 g tension. Strips treated with 880 μg/ml LMP and retreated with nicotine exerted an average of 1.8 g

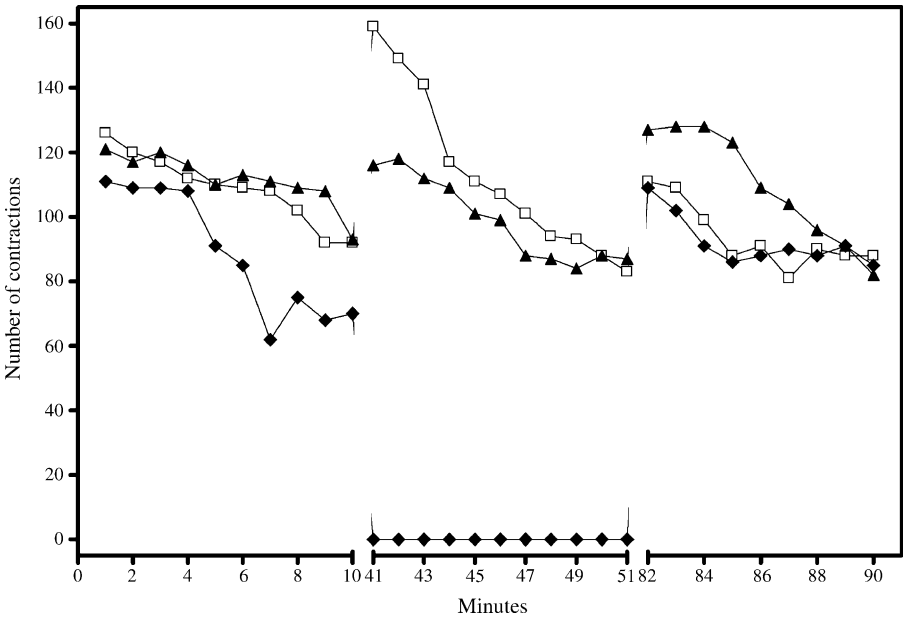

Fig. 1. Effect of saline (□), 34 μg/ml LMP (◆), and 42 μg/ml *C. fasciculata* lysate proteins (▲) on spontaneous contractions of a neonatal rat ventriculocyte monolayer. Proteins were added at point 0 and contractions counted for 10 min. Cell cultures were then incubated for 30 min (first break in *x*-axis) and contractions counted again for 10 min. Cultures were then washed with saline and incubated a further 30 min (second break in *x*-axis). Contractions were counted for 10 min after incubation.

tension, a rapid 35.7% decrease in force of contraction ($p < 0.01$) (Fig. 3). After washing in fresh Tyrode's solution and reapplication of nicotine, the strips continued contracting at 3.0 g tension or greater.

Ileum strips precontracted with acetylcholine exerted an average of 4.3 g tension. Strips treated with 880 μg/ml LMP and acetylcholine exerted an average of 3.8 g tension (Fig. 4). This reduction was not significant ($p > 0.01$). After washing in fresh Tyrode's solution and reapplication of acetylcholine, the strips continued contracting at 4.2 g or greater.

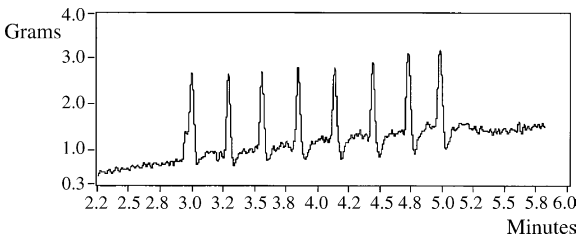

Fig. 2. Spontaneous contractions of the guinea pig ileal strip, without drugs, LMP, or electric stimulation.

3.3. Guinea pig ileum electrically stimulated

Mean peak value of 3.7 g during regular 40 V electric stimulation was immediately reduced to 2.6 g after addition of 220 μg/ml LMP, a 29.7% decrease in contractile activity ($p < 0.01$) (Fig. 5). Although the amplitude decreased after addition of LMP, frequency remained constant. Increasing concentration to 440 μg/ml resulted in a mean peak value of 0.9 g, a total decrease of 75.6% from the original ($p < 0.01$) and a decrease in frequency. A further increase in concentration to 880 μg/ml obliterated contractions despite regular 40 V electric stimulation. The mean peak value of 0.5 g was close to the baseline value of 0.2 g.

3.4. Guinea pig uterus pre-treated with oxytocin

Strips of uterus precontracted with oxytocin repeatedly exerted 3.1 g tension (results not shown). Treatment with 170 μg/ml LMP resulted in an immediate reduction to 2.6 g tension, a 21.2% decrease in contractile activity ($p < 0.01$) (Fig. 6).

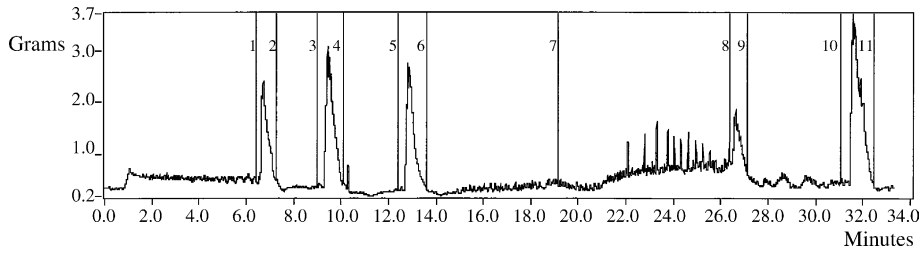

Fig. 3. LMP effect on ileum strips precontracted with nicotine. (1, 3, 5, 8, 10) Nicotine application; (2, 4, 6, 9, 11) wash in Tyrode's solution; and (7) application of 880 μg/ml LMP. Note spontaneous contractions between 22 and 26 min.

An increase to 310 μg/ml LMP resulted in a further reduction to 1.5 g tension, a significant reduction of 54.5% relative to the Tyrode's solution controls ($p < 0.01$). After washing in fresh Tyrode's solution and reapplication of 10 μl oxytocin, the strips continued contracting at 4.0 g tension or greater.

4. Discussion

The goal of this project was to assay lysates of *L. major* for inotropic effect using mammalian models. Previous studies with LMP in insect visceral muscle bioassays revealed a broad range of activity against different insect species and different muscle preparations (Vaidyanathan, 2004). A myoinhibitory peptide was isolated from *L. major*, but bioassays revealed several chromatographic fractions with myoinhibitory activity (Vaidyanathan, 2005). This study was undertaken with whole parasite lysates because peptide sequence data are still fragmented. The current paper also expands the number of potential tissues affected by *L. major* myoinhibitory factors.

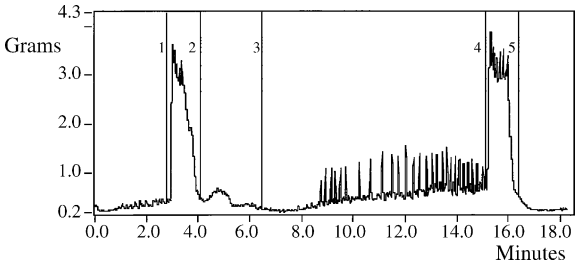

Fig. 4. LMP effect on ileum strips precontracted with acetylcholine. (1 and 4) Acetylcholine application; (2 and 5) wash in Tyrode's solution; (3) application of 880 μg/ml LMP. Note spontaneous contractions between 9 and 15 min.

Incubation with LMP completely inhibited contractions of isolated rat ventricular myocytes (Fig. 1). Heart cells contract essentially like striated muscle, except for the extraordinarily large influx of Ca^{2+} during the action potential plateau. Voltage-gated Ca^{2+} channels and the Ca^{2+}–Na^{+} exchange pump control intracellular Ca^{2+} concentration and are inhibited when antagonists enter the cell and bind intracellularly (Catterall, 1993). LMP might interfere with voltage-gated Ca^{2+} channels or the Ca^{2+}–Na^{+} exchange pump. An increase in intracellular Ca^{2+} would cause an influx of water across the plasma membrane, resulting in the swollen cells seen after 30 min incubation. Similar distension was observed in the sand fly midgut and hindgut treated with LMP (Vaidyanathan, 2005).

To distinguish between spontaneous versus induced contractions, the former are presented for comparison in a control ileum preparation (Fig. 2), between 22 and 26 min in nicotine-treated samples (Fig. 3) and between 9 and 15 min in acetylcholine-treated samples (Fig. 4).

LMP significantly ($p < 0.01$) inhibited nicotine-induced contractions in a guinea pig ileum preparation (Fig. 3). No significant myoinhibition was detected on acetylcholine-induced contractions ($p > 0.01$) (Fig. 4). This indicates that LMP acts on ganglionic receptors in the muscle and not on cholinergic synapses. Acetylcholine acts through muscarinic and nicotinic receptors. Muscarinic stimulation is mostly parasympathetic and plays a role in gastrointestinal motility and smooth muscle contraction. When muscarinic receptors are blocked, nicotinic receptors stimulate autonomic ganglia and voluntary muscle. Nicotinic receptors in the guinea pig ileum are localized in the somatodendritic region of excitatory longitudinal muscle motoneurons. They are inhibited

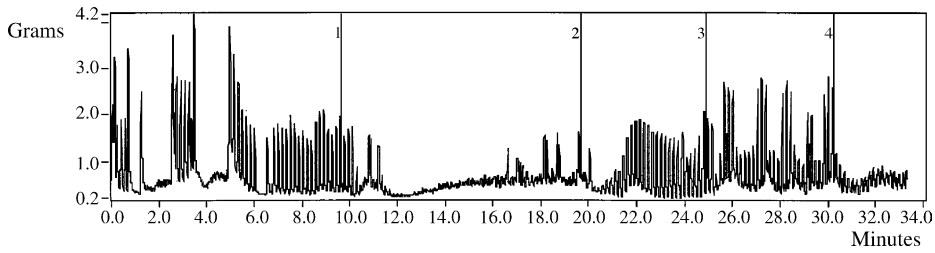

Fig. 5. Cumulative dosage effect of LMP on ileum strips repeatedly stimulated with 40 V; first 10 min constitute electric stimulation without LMP treatment. (1) 220 μg/ml LMP; (2) regular 40 V stimulation; (3) 440 μg/ml LMP; and (4) 880 μg/ml LMP.

by antagonists, such as tetrazepam, which is believed to reduce ileal contractions through a reduction of Ca^{2+} influx through Ca^{2+} channels (Perez-Guerrero et al., 1997). Treatments with LMP were as effective (35.7%) in reversibly inhibiting nicotine-induced contractions as pteleprenine, a specific antagonist of nicotinic receptors (Seya et al., 1998).

Electric stimulation of 30 V or greater on guinea pig myenteric plexus longitudinal muscle activates nerve impulses, potentiating muscarinic transmission and Ca^{2+} uptake (Kadlec et al., 1990). The inhibitory effect of LMP on guinea pig ileum stimulated with 40 V cycles (Fig. 5) indicates a perturbation in Ca^{2+} uptake.

Treatment with LMP resulted in significant ($p < 0.01$), reversible, dose-dependent inhibition of oxytocin-induced contractions of uterine muscle (Fig. 6). Oxytocin is the sole physiological inducer of uterine contractions and acts by inhibiting Ca^{2+}–Mg^{2+} ATPase, the pump system that extrudes Ca^{2+} from uterine smooth muscle (Soloff and Sweet, 1982; Sakamoto and Huszar, 1984). By inhibiting Ca^{2+} efflux, oxytocin allows a temporary, sustained rise in intracellular Ca^{2+} concentration, thereby prolonging a contractile state (Soloff, 1989).

Uterine muscle contractions originate in the muscle itself and are not abolished by interference with the nerve supply. Tocolytic agents inhibit oxytocin-induced contractions; they act as selective β_2-adrenoreceptor agonists, promoting cAMP-mediated inhibition of contractility. Tocolytic agents inhibit Ca^{2+}-calmodulin function or Ca^{2+} influx by selectively blocking Ca^{2+} channels (Huszar and Walsh, 1989; Rang et al., 1998). Given how tocolytic agents function, it is reasonable to postulate a mode of action for LMP which depends on decreasing Ca^{2+} influx or increasing Ca^{2+} efflux to inhibit uterine muscle contraction. Results from guinea pig ileum precontracted with nicotine and uterus precontracted with oxytocin suggest that LMP interferes with Ca^{2+} flux.

Using mammalian muscle bioassays, inotropic effects from other parasites or parasite-derived factors have been reported. Immunoreactivity against an FMRFamide peptide, an insect myotropin, was detected in the nematodes *Dirofilaria immitis* and *Brugia pahangi* (Warbrick et al., 1992), and extracts of adult *D. immitis* contracted strips of dog aorta at low concentrations and relaxed aortic strips at higher concentrations (Kitoh et al., 2001). The pathogenic

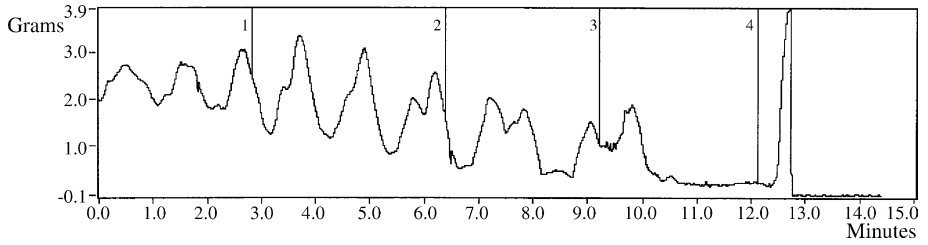

Fig. 6. Dosage effect of LMP on uterine muscle precontracted with oxytocin. (1) 170 μg/ml LMP; (2) 310 μg/ml LMP (cumulative); (3) wash; and (4) oxytocin re-application.

fungus *Candida albicans* inhibited endothelium-dependent relaxation of thoracic aorta in rabbits, with yeast infiltrating into vascular smooth muscle (Ataoglu et al., 1999).

Few studies have examined vertebrate muscle dilation by protist or protistan-derived factors. Infection by the kinetoplastid parasite *Trypanosoma brucei* enhanced endothelial NO-synthase expression in mouse aorta, resulting in endothelium-dependent, NO-mediated relaxation in the presence of acetylcholine (Viswambharan et al., 2003). Vascular permeability induced by *T. brucei* infection apparently facilitated parasite penetration into adjacent tissues. In a similar manner, infection of chickens with *Eimeria tenella* resulted in increased plasma levels of NO metabolites, leading to vasodilation of the endothelium of cecal blood vessels and hemorrhage (Allen, 1997). Chronic infection with *L. donovani* in hamsters reduces peak tension in response to tetanic stimulation of fast-twitch muscle (Drew et al., 1988), and canine infections with *L. infantum* result in myofibril necrosis and atrophy (Vamvakidis et al., 2000).

The intracellular amastigote form is the *Leishmania* stage in vertebrates. The current study was performed with lysates of promastigotes, which have different peptide profiles from amastigotes. *Leishmania* promastigotes induced inotropic effects, but further work with amastigote-derived factors is necessary to examine their role in pathology of leishmaniasis. Hopefully, these results will encourage prospecting for inotropic effects in other protistan parasites and protistan-derived factors.

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