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RESEARCH ARTICLE

Effect of Silk protein (sericin) on the growth and proliferation of Leishmania donovani, the causative agent of visceral leishmaniasis

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Abstract

Silk protein (Sericin) is a protein removed from the silk cocoons which possesses many biological activities. In this study, we investigated the anti-leishmanial properties of Sericin from the VL endemic area of Bihar, India and its effect on Leishmania donovani (Ld) promastigotes in vitro at 24h, 48h and 72 h after initiation of culture. Sericin, a silk protein (SP), is one of the main constituents of silk cocoons, comprising 20-30% of total cocoon weight [1]. We further studied the minimum effective concentrations as well as the effect on promastigotes viability and the cell cytotoxicity on human peripheral blood mono nuclear cells on sericin extracts that induced significant promastigotes killing. Effect-based dose finding analysis revealed that the threshold concentration of Sericin 10 µg/ml after 48h culture. The present study provides scientific data that support the protective effect of silk protein (sericin) against visceral leishmaniasis (VL) control.

Introduction

Visceral leishmaniasis (VL), also known as Kala azar, is a major public health problem in India and many parts of the world [2], more than 20,000 cases of VL are reported annually in India alone [3]. The disease is fatal if left untreated. Sericin is a silk protein woven from silkworm cocoons (Antheraea mylitta D). The present study investigated the effects of Sericin on human peripheral blood mononuclear cells (PBMCs) compared to normal healthy control. Sericin is a family of adhesive silk protein synthesized in middle silk glands of silkworms [4] that envelops fibroin fibers in cocoon [5]. Usually it constitutes 20–30% of silk protein in cocoon [6-7] and consists of amino acids most of which have strong polar side groups such as hydroxyl, carboxyl, and amino groups with high serine content contributing to its high hydrophilicity [8]. It is non toxic, antioxidant agent, anti-aging properties like vitamin C [9-11], with antibacterial, UV resistant and anti apoptotic properties [6]. Other biological activities it comprises are anti tyrosinase, anticoagulation and anti-cancerous activities [6-12] such as colon tumorigenesis [1, 13] and supports cell growth and differentiation and has been considered to act as cell culture supplement in serum free media [14]. Sericin also helps in reduction of cholesterol [15].

In this study, we assessed the leishmanicidal activities of sericin against *Leishmania donovani.* Studies on Silk Protein (sericin) extracts were extended to establish their minimum effective concentrations (MECs), their leishmanicidal effects on promastigote and their cytotoxic effects against human peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1 Preparation of crude Silk Protein (sericin)

Silk Protein (sericin) was extracted with deionized water from raw silk yarns of the silkworm Antheraea mylitta D under high pressure and high temperature. The Silk Protein extract was later dried at 130 ºC, and then

ground and sieved through a 0.75 mm screen. The resulting Silk Protein (sericin) powder was sealed in sterile plastic bags and kept at room temperature until used. The Silk Protein (sericin) was used by reconstituting it in phosphate-buffered saline (PBS) and was sterilized by autoclaving at 121°C for 15 minutes [16].

2.2 Promastigote culture of *Leishmania donovani*

*I*n this study WHO reference strain (MHOM/IN/80/DD8) isolates of *L. donovani* from the various hospitals of Bihar, India were used. The *L. donovani* promastigote strains were maintained in RPMI-1640 medium (Sigma-Aldrich, USA) containing 20% heat inactivated fetal bovine serum (FBS; Himedia, India), pH 7.2–7.4, at 24±2^{0}C. Cultures were maintained through serial sub-culturing for further studies [17].

2.3. Treatment of *L. donovani* promastigotes with Silk Protein (sericin)

To identify Sericin's anti-leishmanial activity, the effects of SilkProtein (sericin) on the growth on the promastigotes was measured. For these tests, a total of 2x10^6/ml early stationary phase *L. donovani* promastigotes (24 h, 48, and 72h culture) in RPMI-1640 complete media with 20% FBS were dispensed into 24-well culture plates. The culture was supplemented with sericin at different concentrations of 5µg/ml, 10µg/ml and 20 µg/ml in duplicate series and cultures were incubated at 24 ± 2^{0}C and microscopic analysis was performed after 24h, 48h and 72h (Fig 1) using a 0.1-mm Naubauer Chamber [18].

2.4. Evaluation of minimum effective concentrations of Silk Protein (sericin) showing significant leishmanicidal properties

We evaluated the minimum effective concentrations (MECs) of Silk Protein (sericin) that showed significant levels of *L. donovani* killing. The leishmanicidal activity of Silk Protein (sericin) against *L. donovani* promastigotes (2x10^6/ml) were evaluated in duplicate series (Fig.2) at different concentrations of 5µg/ml, 10µg/ml and 20µg/ml. The concentration of the SilkProtein (sericin) was measured at 570nm by spectrophotometer.

2.5 Evaluation of cell toxicity of Silk Protein (sericin) at the MEC against human Peripheral Blood Mononuclear Cells.

To evaluate the cytotoxicity of SilkProtein (sericin) against human PBMCs [17-18], the mitochondrial dehydrogenase-based 3-(4,5-diamethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used. To perform this assay, MTT-based in vitro toxicology assay kit (Sigma–Aldrich, USA) was used. Briefly, peripheral blood samples taken from 5 healthy human volunteers and PBMCs were isolated by density gradient centrifugation (2000 RPM, 15 min) over Histopaque-1077 (Sigma, USA). After washing with PBS, PBMCs (1x10^{6}cells/ml) were cultured in complete RPMI-1640 with 20% FBS media (without phenol red). Further, 100 µl of MTT solution (10% of PBMCs culture) were added to PBMCs and incubated for 2 h in a CO_2 incubator at 35 °C and >95% humidity. After incubation period these cells were supplemented with 1 ml MTT solubilization solution. These cells were then vortexed to dissolve formazan crystals and analyzed with spectrophotometer at a wavelength of 570 nm, using 690 nm as reference. Values obtained using SilkProtein (sericin) treated cultures were analyzed. Mitochondrial dehydrogenases cleave the tetrazolium ring of MTT, in viable cells and yielded insoluble purple formazan crystals. The higher concentration of formazan is indicative of a high level of mitochondrial dehydrogenase activity [17].

2.6 Statistical Analysis

The results were expressed as the mean ± SD unless otherwise indicated.

3. Result

3.1 Time and dose-dependent effect on promastigote growth

The effect of Silk Protein (sericin) on the growth pattern of *L.donovani* was determined. Out of 5µg/ml, 10µg/ml and 20µg/ml, only 10µg/ml at 48h had shown maximum killing of *L.donovani* promastigote.

3.2 Cell toxicity of Sericin at the MEC against human Peripheral Blood Mononuclear cells

To analyze the safety of the SP (sericin) as having anti-leishmanial properties, we examined their cytotoxic effect. We found that Silk Protein (sericin) showed the maximum effect against promastigotes in culture at 48h and 10µg/ml. At this concentration, maximum decline in dehydrogenase activity of mitochondria and observed to be less toxic in comparison to other groups and other concentrations.

Discussion

Silk protein (Sericin) are of considerable interest of anti-leishmanial properties. Previously Silk protein (sericin) has been reported to suppress colon tumorigenesis in animal models [20-21] and many other important roles like antioxidant agent, non-toxic [9,10], antibacterial [6], reduction in cholesterol [14] and cell culture supplement in serum free media [13]. However, there is still a limited number of studies that explain SP (sericin's) role in treatment of visceral leishamaniasis. SP has anti-leishmanial effect that will help in the effective treatment of visceral leishmaniasis. This present study gives new insight whether SilkProtein (sericin) has any effects on VL in Indian kala-azar patients.

Figure 1. Treatment of *L. donovani* promastigotes with Silk Protein (sericin).

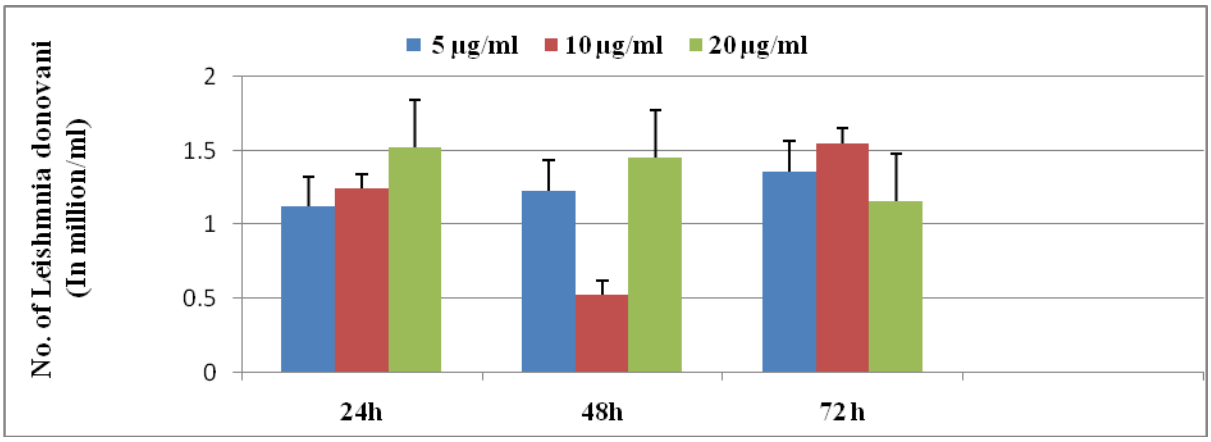
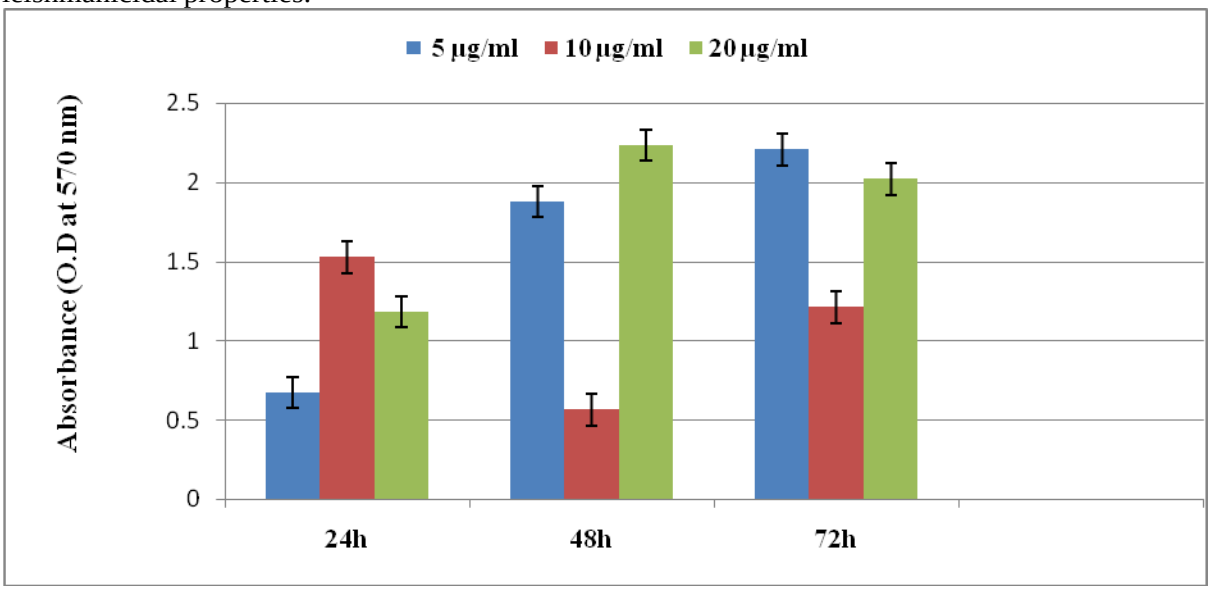


Figure 2 Evaluation of minimum effective concentrations of Silk Protein (sericin) showing significant leishmanicidal properties.



Conflict of interests

The authors declare that they have no conflict of interests.

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