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RESEARCH ARTICLE

Assessment of leishmanicidal effects of Silk protein (Sericin) as an alternative treatment option for the Visceral leishmaniasis (Kala-azar).

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Abstract

The potential of Silk protein Sericin for the treatment of visceral leishmaniasis (VL) was assessed in this study. Silk protein (SP), Sericin is a protein removed from the silk cocoons which possesses many biological activities. The present study provides scientific data that support the protective effect of SP, Sericin against VL (Kala-azar) control. In this study, we investigated the anti-leishmanial properties of SP, Sericin which reduced very less dehydrogenase actitivity as compared to PBMCs without SP but in presence of Sericin in PBMCs culture from VL patients, maximum decline in dehydrogenase activity of mitochondria was observed and found to be less toxic as compared to without Sericin. In other groups like Con-A and Amp-B with or without Sericin, there were very less significant changes were observed. It suggested SP, Sericin might play important role in alternative chemotherapeutic agent against VL

INTRODUCTION

Visceral leishmaniasis(VL) is commonly known as Kala-azar, a vector borne disease transmitted by sandflies (of the genus Phlebotomus and Lutzomyia). Leishmaniasis is a protozoan parasitic disease endemic to the tropical and subtropical regions of the world, with three major clinical forms, self-healing cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL).The disease is fatal if left untreated. Sericin, a silk protein (SP), is one of the main constituents of silk cocoons, comprising 20-30% of total cocoon weight [1]. SP, Sericin is a woven from silkworm cocoons of *Antheraea mylitta D* and family of adhesive silk protein synthesized in middle silk glands of silkworms [2] that envelops fibroin fibers in cocoon [3]. It usually constitutes 20–30% of silk protein in cocoon [4-5] 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 [6]. It is non toxic, antioxidant agent, anti-aging properties like vitamin C [7-9], with antibacterial, UV resistant and anti apoptotic properties. Many other biological activities it comprises are anti tyrosinase, anticoagulation and anti-cancerous activities [10] such as colon tumorigenesis [11] and supports cell growth and differentiation and has been considered to act as cell culture supplement in serum free media [12] and also helps in reduction of cholesterol [13].

In our previous study, we assessed the leishmanicidal activities of SP, Sericin on VL [14]. In present study we tested their leishmanicidal activities against *Leishmania donovani* promastigotes *in-vitro* in comparison to Amphotericin B (Amp-B) and concanavalin A (Con A). Studies on SP, Sericin were established their minimum effective concentrations (MECs), their leishmanicidal effects on promastigotes and their cytotoxic effects against human peripheral blood mononuclear cells (PBMCs).

Currently, the only treatment for leishmaniasis is drug treatment like sodium antimony gluconate (SAG) and Amphotericin B (Amp-B). However, either prolonged use or inefficient drug therapy has resulted in drug resistance.

Treatments for VL are expensive and often result in the development of drug resistance. At present more than 60% of the clinical cases are resistant to the first line drug, antimony [15]. There are no vaccines available at present for the treatment of VL. Attempts to develop vaccines for such parasitic agents such as heat killed, subunit, or DNA vaccines have not resulted in a successful vaccine candidate that could be applicable to humans [16–18].
In this context, we identified SP, Sericin significantly inhibited the growth of promastigotes in addition to Con-A and Amp-B. We further studied the minimum effective concentrations (MECs) as well as the effect on promastigotes viability and the cell cytotoxicity on human peripheral blood that induced significant promastigotes killing. Effect-based dose finding analysis revealed that the thresold concentration of Sericin was 10 µg/ml after 48 h culture. Sericin eliminated *L. donovani* promastigotes after 48 h at concentrations of 0.1 and 0.5 mg/ml, respectively. Sericin eliminated the promastigotes at a concentration of 0.5 mg/ml within 24 h. Sericin, led to approximate 2.5- and 1.3-fold declines in mitochondrial dehydrogenase activity compared with control. Sericin stimulation resulted in an up-regulation of dehydrogenase activity.

2. Materials and methods

2.1. Crude Silk Protein (Sericin) preparation:-

SP, 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 SP, Sericin powder was sealed in sterile plastic bags and kept at room temperature until used. SP, Sericin was used by reconstituting it in phosphate-buffered saline (PBS) and was sterilized by autoclaving at 121°C for 15 minutes [19].

2.2 Culture maintenance of Leishmania donovani's promastigote:-

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 for further studies [20].

2.3 *L. donovani promastigotes's treatment with SP, Sericin:-*

In previous study we identified Sericin's anti-leishmanial activity and the effects of SP, Sericin on the growth on the promastigotes was measured. For these tests, a total of 2x10^6/ml early stationary phase *L. donovani* promastigotes (24h, 48h, and 72h culture) in RPMI-1640 complete media with 20% FBS were cultured into 24-well culture plates. The culture was supplemented with SP, 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 0.1mm Naubauer Chamber [21].

2.4. Minimum effective concentrations of Silk Protein, Sericin:-

The minimum effective concentrations (MECs) of SP, Sericin was observed in previous study that showed significant levels of *L. donovani* killing. The leishmanicidal activity of SP, 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 SP, Sericin was measured at 570 nm by spectrophotometer.

2.5 Cell toxicity of Silk Protein, Sericin at the Minimum effective concentrations against healthy human PBMCs and PBMCs from VL patients

The cytotoxicity evaluation of SP, Sericin against human PBMCs [21], 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 SP, 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.

2.7. Statistical analysis

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

3. Results

3.1 Time and dose-dependent effect on promastigote growth

The effect of SP, 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 (Fig 1).

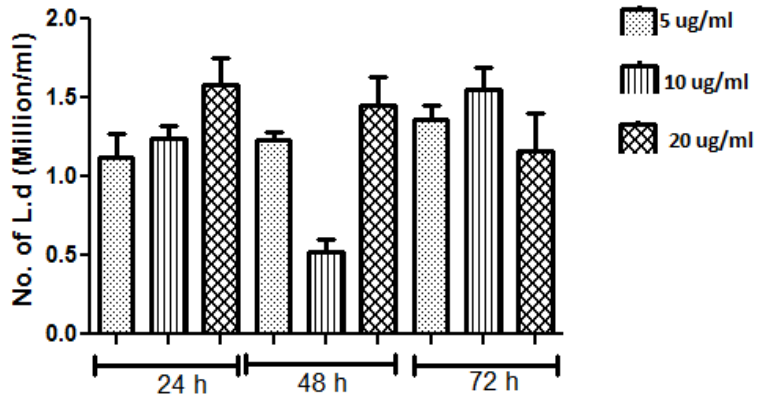

Figure 1. Treatment of *L. donovani* promastigotes with SP, Sericin

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

Previously it was found that SP, 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 (Fig 2).

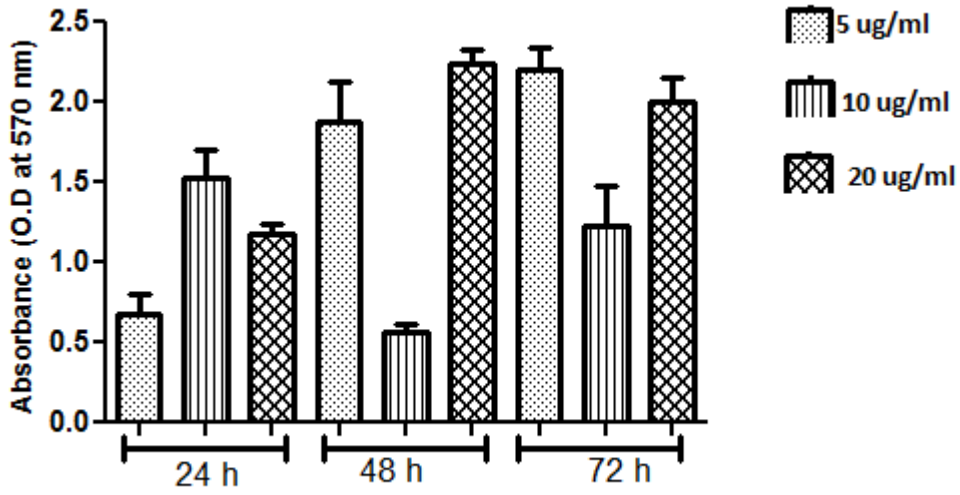

Figure 2 Evaluation of minimum effective concentrations of SP, Sericin showing significant leishmanicidal properties.

3.3. Cell toxicity of the selected SP, Sericin *at the MEC against human PBMCs*

To analyze the safety of the SP, Sericin identified as having anti-leishmanial properties, we examined their

cytotoxic effects against healthy human PBMCs and VL patients PBMCs (Fig. 3). We observed that PBMCs with SP, Sericin showed very less dehydrogenase actitivity as compared to PBMCs without SP. Here we found in presence of SP, Sericin in PBMCs culture of VL maximum decline in dehydrogenase activity of mitochondria and found to be less toxic as compared to without sericin. In other groups like Con-A and Amp-B with or without Sericin, there were very less significant changes were observed.

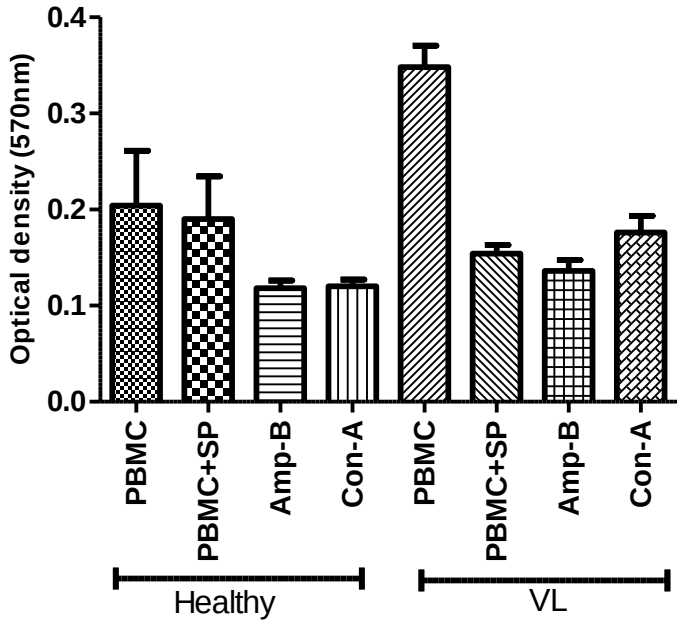

Fig 3. SP, Sericin treated or without treated PBMC of healthy and VL.

4. Discussion

SP, Sericin is of considerable interest of anti-leishmanial properties. Previously SP, Sericin has been reported to suppress colon tumorigenesis in animal models [22-23] 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 are still a limited number of studies that explain SP (Sericin's) role in treatment of VL. Anti-leishmanial effect of SP, Sericin might be help in the effective treatment of visceral leishmaniasis.

Unlike most other pathogens, *Leishmania* is never fully cleared by the immune system, depriving researchers of any natural correlate of immunity to mimic in designing a preventive vaccine that seeks to achieve sterile immunity. With vaccine candidates being tested, despite having been made empirically, detailed understanding is needed about the mechanisms by which the vaccines stimulate protective immunity. A methodical understanding of protective immune responses and generation and maintenance of the immunological memory during *Leishmania* infection is needed for a sustained long-term protective response. While developing attenuated strains of *Leishmania*, focus is required towards obtaining attenuation selectively at the intracellular stage (amastigotes), while remaining non-attenuated as promastigotes to permit large scale cultivation for use as vaccines.
The investigation of such other alternatives has become a necessity because most of the available drugs in use are costly and are typically associated with mild to severe side effects in human patients. Therefore, the goal of this study was to evaluate the leishmanicidal activities of SP, Sericin. This study shows that the SP, Sericin are capable of inhibiting the growth *L. donovani* promastigotes. It not only killed *L. donovani* promastigotes but was also found to be safe according to tests of toxicity against PBMCs of VL patients and healthy controls. The increased dehydrogenase activity induced by SP, Sericin also revealed that if it is applied in due course as a treatment option for VL, it may lead to an increase in the enzymatic activities of the mitochondria of the cells, with little or virtually negligible observed cytotoxicity against human cells.
The cytotoxicity induced by SP, Sericin is very close to Amp-B, which is currently the preferred treatment for VL. Considering the toxic effect of Amp-B, the evaluation of SP, Sericin for use as pharmacological interventions may be valid. However, a distinct time-dependent retardation in *L. donovani* inhibitory activity was observed with SP,

Sericin after 48 h of culture and its threshold concentration was found to be 10µg/ml. Hence, present study recommends further exploration of SP, Sericin as suitable chemotherapeutic alternatives for the treatment of VL.

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