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Original Research Article

Anticancer effects of silkworm protein (sericin) in lung cancer cells

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Abstract
Background: Lung cancer is a wide-spread malignancy across the world. With limited availability of therapeutic options, 5-year survival is low in advanced stages of lung cancer. Sericin, a biowaste protein from silkworm cocoon has shown anticancer potential against various tumors in pre-clinical research with minimal side-effects. The study was designed to determine cytotoxic effects and expression modulations imposed by sericin in lung cancer cells.
Methods: Sericin from local cocoons was extracted by degumming process. Human lung cancer cells (H1299) were cultured and exposed to different concentrations of extracted and a commercially available sericin (0.03-1 mg/ml) for 24-72 hours. Effects on cell proliferation were determined by MTT dye reduction assay. Following the total RNA extraction and cDNA synthesis, expressional changes in cell cycle (CDKN1A, CDKN1B) and stress (GADD45A, GADD45B) related genes were determined via real-time PCR methodology.
Results: Sericin exposure induced concentration and time dependent inhibitory effects on cancer cell proliferation. Comparable results from commercially available sericin and local extracted sericin confirmed the reliable extraction process adopted in this study. CDKN family of genes (cell cycle inhibitors) was up regulated in response to sericin exposure. GADD genes (markers of cell stress) were also induced in cancer cells in response to sericin exposure.
Conclusion: Lung cancer cells were responsive towards sericin treatment as observed by inhibition of the proliferation. Sericin interfered with expression levels of cell cycle inhibitor and stress related gene families. Further studies are needed to understand the anticancer potential of sericin against lung cancer cells.
Key Words: Lung cancer, Sericin, Silk protein, Anticancer, Cell cycle, Cell stress

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INTRODUCTION

Lung cancer is a major cause of cancer associated deaths in the world [1]. Behavioral, environmental and genetic risk factors are all known to contribute towards the development of lung cancer. Overall, 5 years low survival rate with lung cancer has barely changed over the years [2, 3]. Surgery, radiotherapy, chemotherapy and targeted therapy are treatment options for lung carcinoma [4, 5]. Due to limited treatment options and associated side-effects, there is a dire need for new cost effective and safer anticancer agents to treat lung cancer. In this context, sericin, an integral part of silkworm cocoon, is a promising protein with significant potential as an anticancer agent.

An economically significant worm called silkworm (*Bombyx mori)*, feeds on mulberry leaves while in its larval stage and spins a cocoon of silk around. The cocoon of this worm is composed of spherical proteins called fibroin and sericin. When silk is produced, sericin occupies space around the two fibroin threads, thus providing protection. This also leads to the fusion of the two threads to make silk yarn. This is where the silkworm develops and completes its life cycle from larva to adulthood [6, 7]. Sericin is around 15-30% of the dry cocoon weight and connects the filaments of silk fibroin together [8]. Overall, *B. mori* cocoon is formed of 65-85% fibroin, 15-30% sericin, 1-5% non-sericin components including pigments, wax, carbohydrate and impurities. Sericin is a natural protein with multiple biological properties [9, 10]. It is a water soluble protein having molecular mass of 20-400kDa [11]. Sericin is being wasted in the silk industry during the degumming process to improve value of silk as textile fiber.

Anticancer properties of sericin have been studied in multiple cancer cell lines including colorectal, prostate and cervical cancers. Sericin was also tested *in vivo* to suppress skin tumors in the mice model induced via 7,12-dimethylbenz(α) anthracene (DMBA) and 12 o-tetradecanoyl phorbol 13-acetata. Sericin has also

been showed to induce apoptosis and cell cycle arrest in cancer cells [12]. Sericin and sericin based nanoparticles have been used in experiments to see molecular changes in the human breast cancer cell lines with remarkably reduced cell proliferation [13]. Antineoplastic effects of sericin against various other cancer cells including human breast carcinoma (MCF-7), squamous carcinoma (A431) and oral carcinoma (SAS) cells led to the suppressed cell growth [14]. Another study showed the anticancer effects of sericin in triple negative breast cancer cells (MDA-MB-468) by inhibiting P13K/AKT pathway. Different functional assays confirmed that sericin causes suppression of proliferation in breast cancer cells, induces cell cycle arrest in G_0/G_1 phase and promotes cellular apoptosis [15]. Sericin has also shown anti-proliferative effects by enhancing caspase 3 activity and suppression of BCL2 against colorectal cancer cells (SW480) [16].

This study was proposed to determine the cytotoxic effects of sericin in lung cancer cells (H1299). Additionally, expression modulations in two important gene families (CDKN and GADD) were determined to provide mechanistic reasoning. The findings led to a better understanding about antineoplastic effects of sericin in cancer cells and support its potential clinical application in the future.

METHODS

Extraction and Purification of Sericin

Sericin was extracted by using degumming process from the locally collected cocoon. Briefly, silkworm cocoons were cut into pieces, washed with deionized water along with continuous stirring followed by autoclaving at 121°C for 45-60 minutes. Afterward, the solution having soluble sericin was filtered to remove fibroin. The filtrate was processed for lyophilization to obtained sericin powder (S-Extract) and stored at 4°C until further use. Purified sericin (S-Pure) was purchased from Sigma company (S5201-5G) to compare the effects in parallel.

Cell Culture

Lung cancer cell line (H1299) was cultured in the media (RPMI-1640) along with recommended essential supplements including FBS (10%), L-glutamine (2mM), streptomycin (100ug/ml) and penicillin (100 IU/ml). The cells were maintained at standard humidified condition (5% CO2, 37^0C) for growth purposes.

MTT Dye Reduction Assay

MTT dye reduction assay was used to measure the cytotoxic effects of extracted and purified sericin in lung cancer cells. For this purpose, the cells were cultured in 96-well culture plates (3000cells/well/ 100ul medium) for overnight period. Next day, the cells were exposed to different concentrations (0.03-1.0mg/ml) of S-Extract and S-Pure dissolved in culture medium for three different time intervals (24, 48, 72 hours). Following exposure periods, cytotoxic effects were determined by adding MTT solution (10mg/ml in PBS) in each well (10ul/well). MTT solution entered cells and converted into formazan crystal in the viable cell. The crystals were dissolved by adding DMSO (50ul/well). Intensity of the color of solution reflected the number of viable cells. Untreated cells were grown in parallel as control during the experimental procedures.

Expression Analysis

Lung cancer cell line was cultured in 6-well culture plates (150,000 cells/well/2ml medium) and exposed to a relatively low, medium and high concentrations (0.25, 0.5 and 1.0mg/ml) of S-Extract and S-Pure for 48 hours. A commercially available kit (Thermo Fisher Scientific, K0731) was used for extraction of RNA from collected cell pellets according to recommended protocol. Quality and quantity of extracted RNA was estimated by Nanodrop spectrophotometer. cDNA was synthesized by using 500ng RNA per sample along with commercially available kit (Thermo Fisher Scientific, K1622). cDNA samples along with specific optimized primers (Table 1) were used for analyzing the expression profile of selected genes by using SybrGreen master mix (Thermo Fisher Scientific, K0221) and QuantStudio-3 real-time PCR machine. Samples were amplified in triplicate and expression levels of reference gene (GAPDH) were used for normalization of the data.

Presentation of Data

Cytotoxicity data generated from MTT assay was presented as percentages. For real time PCR analysis, fold changes were calculated by Livak method by comparing Ct values of treated and untreated control samples.

Table 1: Primers sequences of the selected human genes

Genes	Forward Primer	Reverse Primer
GADD45A	CGCTAGGCTTAATCGCGTT	TTCGAAGCTGGTTCGAATGC
GADD45B	TGAATGTGGACCCAGACAGC	GTCCGTGTGAGGGTTCGT
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
CDKN2A	CCCTCAGAAATGATCGGAAA	CAGCTTGCGATAACCAAAGG
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT

RESULTS

Cytotoxic Effects of Sericin

Lung cancer cells were grown in 96-well plates at pre-optimized cell densities in RPMI-1640 medium and were treated with the fraction of S-Pure and S-Extract (0.03-1.0mg/ml) 24-72 hours. Afterwards, MTT assay was performed to assess the growth inhibitory effects. As shown in Figure 1, inhibitory effects were calculated numerically as percentages of untreated controls. Clearly, sericin inhibited the proliferation of lung cancer cells. Cytotoxic effects were concentration dependent since the suppression of cellular proliferation became more intense as concentration was increased especially for late treatment intervals (48 and 72 hours). Furthermore, the effects of S-Extract and S-Pure were comparable in the cells. Overall, sericin inhibited the proliferation of lung cancer cells (H1299).

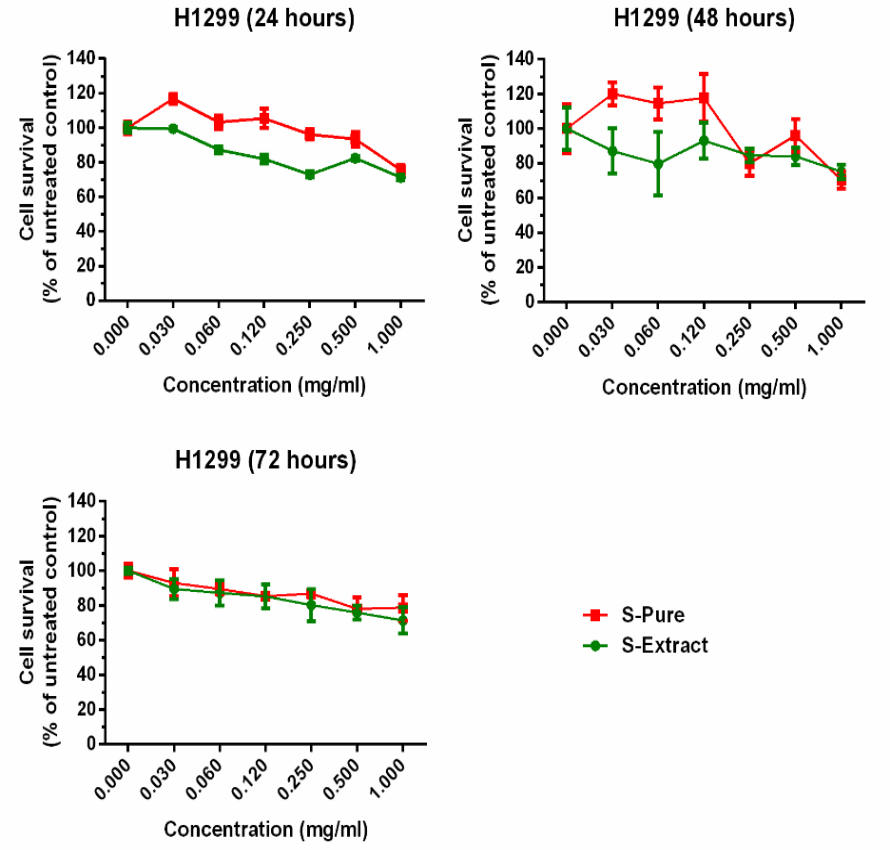

Figure 1: MTT result of H1299 cell lines after 24-, 48- and 72-hours treatment with pure and extracted sericin. The cells were exposed to different concentrations (0.03-1.0 mg/ml) of the sericin fractions and resulting effects on cell proliferation were identified by MTT dye reduction assay. Sericin exposure inhibited lung cancer cell proliferation consistently.

Expression Modulations in Cell Cycle Genes

The cDNA samples from cells treated with different concentrations of sericin fractions were used to determine expressional profile of cell cycle genes (CDKN1A and CDKN2A) by qRT-PCR methodology. For this purpose, the cells were treated with three different concentrations of S-Extract and S-Pure (0.25, 0.5 and 1.0mg/ml). Selected genes, CDKN1A and CDKN2A, were upregulated in the cells. More specifically, CDKN1A was clearly upregulated (up to 2.7fold) in lung cancer cells (H1299) as shown in Figure 2. Both sericin fractions (pure and extracted) induced CDKN1A gene in H1299 cells. Interestingly, this induction was more pronounced at lower concentrations of sericin. CDKN2A was also consistently up regulated up to 3fold as shown in Figure 2 in the lung cancer cells. Overall, sericin induced the cell cycle inhibitor genes (CDKN1A, CDKN2A) in the lung cancer cells.

Expression Modulations in Cell Stress Genes

The modulation pattern of GADD45A and GADD45B gene in response to S-Pure and S-Extract exposure showed similar pattern as compared to CDKNIA and CDKN2A genes. There was a consistent upregulation of GADD45A gene after the sericin exposure as shown in Figure 3. Similarly, there was a uniform consistency as far as expression alterations are concerned for GADD45B gene in the cells (Figure 3). This induction was considerable in the cells (maximum of 2.9fold) in response to exposure to sericin. All in all, sericin induced the expression of GADD family of genes in the lung cancer cells.

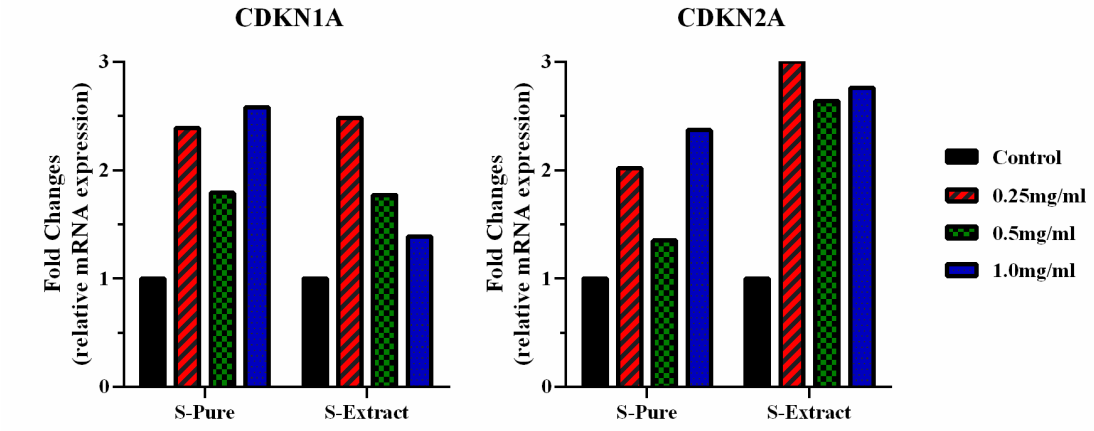

Figure 2: Expression modulation in CDKN1A and CDKN2A genes in lung cancer cells identified via real-time PCR. The cells were exposed to three different concentrations (0.25, 0.5, 1.0 mg/ml) for 48 hours and expression modulations were determined. Sericin exposure induced the levels of CDKN1A and CDKN2A transcript in lung cancer cells.

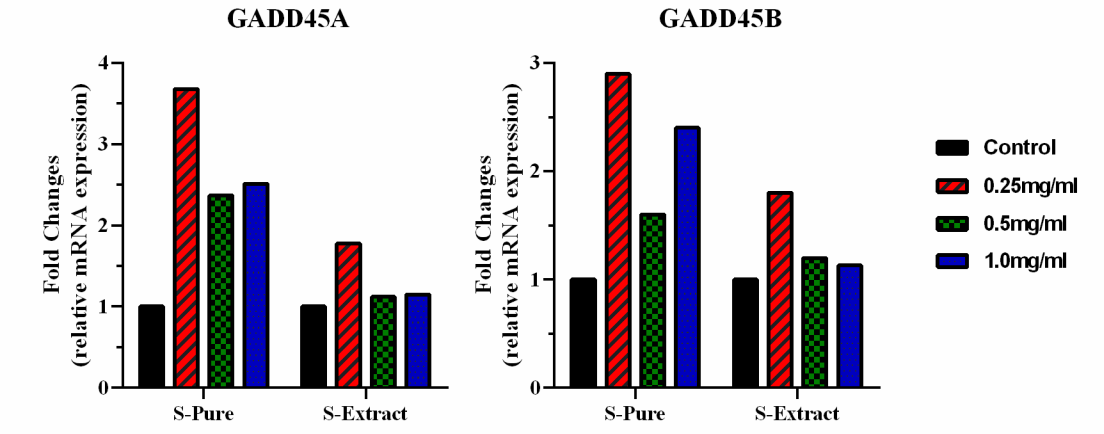

Figure 3: Expression modulation in GADD45A and GADD45B genes in lung cancer cells identified via real-time PCR. The cells were exposed to different concentrations (0.25, 0.5, 1.0 mg/ml) for 48 hours and expression modulations were determined. Sericin exposure induced the levels of GADD45A and GADD45B transcript in the lung cancer cells.

DISCUSSION

Silk is a naturally occurring macromolecular protein fiber. The bio-based substance is derived from several arthropods. Due to its ease of cultivation and high extraction yield, silk produced from *B. mori* (silkworms) cocoons is the most common form of silk. Silk sericin is an additional component of the cocoons, representing ~20–30 wt% of the total weight [17]. Sericin is a white or yellow, odorless, water-soluble protein with a pleasant flavor. Structurally, sericin is a globular protein and consist of β-sheets and random coil [18-21].The properties of the sericin may vary based on the extraction method. Thus, the application of appropriate protocols to extract and purify sericin is important and affects its molecular weight and properties. Almost ~76% of sericin consists of hydrophilic amino acids and ~24% hydrophobic amino acids. The unique amino acid combination of sericin confers the properties of oxidizing the substrates and high-water holding capacity [21, 22]. Sericin can function as a pro -oxidant B because it contain polyhydroxy amino acids like serine as well as polyphenol and flavonoids as secondary metabolites that have various biomedical properties including anticancer activity [23]. Sericin has shown anticancer effects against cancer cell lines. Sericin suppresses the proliferation related PI3K/Akt pathway, arresting the cell cycle in G_0/G_1 phase and promotes apoptosis [18]. Human oral carcinoma (SAS), breast carcinoma (MCF-7), and squamous carcinoma (A431) cells were used to study the anticancer activity of sericin. The cells were examined for anticancer activity in terms of cytotoxicity, ROS levels, cell cycle arrest, and mitochondrial membrane potential. Researchers have looked at how sericin affects the expression of p53, cytochrome C, BAX, and BCL-2 genes as well as apoptotic/anti-apoptotic proteins in cancer cells [14].

In this study, we took the human lung cancer cell line (H1299) and studied the cytotoxic effects of sericin via MTT dye reduction assay. H1299 cells were cultured under the suitable conditions and were exposed to different concentrations of the extracted sericin with various concentrations (0.03–1mg/ml for 24, 48 and 72 hours). For comparison purposes, the cancer cell line was also exposed to the commercially available purified sericin obtained from Sigma company. It was clear from the obtained result that sericin showed effective anti-proliferating effects in this cell line. Sericin does not influence the proliferation of normal cells, while cancer cells show a similar inhibition in proliferation in response to sericin exposure as reported by other researchers [13, 15, 16]. The comparable results from commercially available sericin and the local extracts confirmed the reliable extraction process adopted in this study. Overall, the cytotoxic effects were mild and concentration dependent on the cancer cells.

Identifying the molecular basis of any functional outcome is an interesting and much needed area of investigation. To figure out potential reason behind the anti-proliferative effects of sericin, expression analysis of the important cell cycle regulator genes (CDKN family) and cell stress related genes (GADD45 family) were selected in this study. CDKN family of genes was induced in the cells in response to sericin exposure, which may have contributed to slowing down the cell cycle machinery and cellular proliferation rates. As far as the GADD family of genes are concerned, the genes were unanimously induced in lung cancer cell line. GADD genes are well-known marker of cell stress and death mechanisms, while CDKN family is a well-known category of cytostatic effectors. Induction of these genes may have led to the imposed cytotoxicity and cytostatic effects via sericin exposure, respectively. If so, provided with further confirmation, sericin turned out to be a cytostatic and cytotoxic agent for lung cancer cells and may have substantial clinical applications. Findings from the current study warrant further detailed investigations to explore antineoplastic effects of sericin against lung cancer cells, while including additional representative cell lines.

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Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.