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Original Research Article

Does intrapleural sericin administration have an effect on rat plasma thiol disulphide redox homeostasis in accordance with antioxidative functions or cancer preventive features?

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Abstract: Sericin is a natural, gum-like, protein synthesized from silkworms, which has antioxidative efficiency, accelerates wound healing, and stimulates proliferation *in vitro*. Thiol disulphide redox homeostasis plays important roles in the antioxidant defense, detoxification, acceleration of tissue proliferation and regeneration, and has cancer-preventive features. The aim of the study was to investigate the effects of intrapleural sericin administration on plasma native thiol, total thiol, and disulphide levels in rats. Twelve-week-old, male, adult Wistar albino rats were used in the present study (n=12). The rats were divided into two equal groups as the sericin group and the control group. After intramuscular anesthetic agent administration, the rats were intubated, mechanically ventilated and a left thoracotomy was performed. Subsequently, 30 mg sericin powder was administered in the thoraxes of the sericin group; the remaining rats were allocated as sham thoracotomy. The rats were sacrificed eight days later; the preferred method was cardiac puncture. The plasma of rat blood was separated from cells, and a biochemical examination for the parameters of native thiol, total thiol, and disulphide was performed. The rate of native thiol level was significantly higher in the sericin group (sericin: 110.06 µmol/L; control: 75.70 µmol/L; $p < 0.05$). Similarly, total thiol level was higher in the sericin group, but this was within the limit, according to the independent t-test ($p = 0.056$). Intrapleural sericin application was found to cause a significant change in the thiol disulphide redox homeostasis in rat plasma. Sericin is a protein that has antioxidative properties and is known to facilitate wound healing. Thiol disulphide redox homeostasis is known to induce tissue proliferation and regeneration and to have antioxidative and cancer prevention properties. Since the amounts of native thiol and total thiol were increased in the subjects that received sericin, it can be predicted that this protein can be used in the future as an antioxidative and cancer preventing agent, facilitating lung tissue proliferation.
Keywords: Sericin, Thiol, Disulphide, Intrapleural, Homeostasis

INTRODUCTION

The cocoon shell of silkworms, *Bombyx mori*, is composed of two natural macromolecular proteins called fibroin and sericin [1, 2]. Fibroin is produced from the posterior silk gland of the silkworms, and accounts for 75% of all silk protein and fundamental macromolecular protein used in the textile industry [3]. Sericin is a textile by-product, which has a wide molecular weight range from 30 to 400 kDa, and has strong polar side groups and hydrophilic properties [4-6]. Good hydrophilic properties and high water solubility are essential features in the pharmaceutical and cosmetic sectors, which are the most common application fields of the protein [5]. Sericin can also be used as an antioxidant and has antimicrobial activity [4]. In addition to this features, it has a wide spectrum of feasibility as a cell culture media, cell protective activity, and increasing effect on cell movement and proliferation [7, 8]. The favorable effects of sericin on wound size reduction and the healing process have also been demonstrated in several animal studies [1, 7-9]. Sericin also increases fibroblastic activity and fibrosis in the visceral pleura without significant adverse effect

on lung parenchyma [2]. Therefore, sericin has a wide feasibility spectrum in the multiple sections of science.

In organic chemistry, thiol groups, usually also known as mercaptans, are a compound that contains the functional group composed of a sulfur atom and a hydrogen atom (-SH) [10]. In thiols (R-SH and -C-SH), the -OH group of alcohols is replaced by the -SH group; being the sulfur analogue of an alcohol is referred to as a sulfhydryl group. Various proteins including sulfhydryl groups are present in the human body. These proteins mediate the functions of thiol compounds and increase the effects of thiols. These proteins are primarily albumin and cysteine amino acid, glutathione, homocysteine, thioredoxin, N-acetlycysteine, cysteinylglycine and ɣ-glutamyl cysteine.

The regulation of thiol disulphide redox homeostasis (TDRH) is critically important for multiple metabolic, signaling, and transcriptional processes in mammalian cells [11, 12]. Thiol groups are highly reactive and susceptible to oxidation; therefore, thiol chemistry is a rapidly growing field in basic and applied bioscience [10, 11, 13]. Dynamic TDRH plays a critical role in numerous intracellular enzymatic pathways including antioxidant defense, detoxification, and signal transduction, regulation of enzymatic activity, apoptosis, and cellular signaling mechanisms [12, 14]. Moreover, native thiols also have proliferative effects and elevated thiol levels in the tissue accelerate proliferation and regeneration [15, 16]. Additionally, biologically active thiol groups prevent free radical-induced damage in the body and have cancer-preventive features [17-20]. Therefore, dynamic TDRH and thiol groups play major roles in the human organism in accordance with tissue regeneration, antioxidative functions, and anti-carcinogenetic features.

Consequently, sericin has antioxidative effect, enhances wound healing, and increases fibroblastic activity and proliferation; thiol compounds have antioxidative activity, accelerate tissue proliferation and regeneration, and have cancer-preventive features. Therefore, does intrapleural sericin application have an effect on plasma thiol disulphide redox homeostasis? If it has an effect, can these two properties be joined? However, there are no studies in the English literature describing the effects of sericin application on plasma TDRH. The aim of the present study is to evaluate the effects of sericin application on rat plasma native thiol, total thiol, and disulphide levels. Considering the fact that sericin has antioxidative value and accelerates wound healing, a possible increasing effect of rat plasma thiol levels might have an effect on the acceleration of proliferation, have antioxidative value, may prevent free radical induced damage and regeneration in the lung tissue, and finally, may cease carcinogenesis. As sericin is a by-product of the textile industry and has a low cost value, the demonstration of intrapleural sericin application on plasma thiol levels may increase the feasibility of sericin protein and may decrease the possibility of carcinogenesis.

MATERIALS AND METHODS

Sericin powder is a commercially available product. As sericin is a by-product of the textile industry, sericin powder has a fair price. The sericin powder used in the present study was purchased from Zhejiang Yong Yi Chemical Co. Ltd., China. The animal study was designed in order to demonstrate the effectiveness of intrapleural sericin application on plasma thiol levels and the rat model was selected. The presence of a valid laboratory license for this animal, technical feasibility, and similar physiological characteristics when compared with humans are the reasons for choosing the rat model.

Animals

The animal study was approved by the Ethical Committee of Gazi University (GU-ET-14.003). Twelve-week-old, male, adult Wistar albino rats were used in the study (n = 12). The weight of rats was ranged between 257 g and 395 g. All animals were obtained from the Gazi University Animal Research Laboratory in Ankara, Turkey. The rats were randomly divided into two equal groups; the sericin group and control group. All animals had human care in accordance with Turkish Government's Animal Protection and Management Law.

Power analyses

In this study, a power analyses can be the most suitable measurement for deciding how large a sample size we need for performing the planned experiment? An experiment that is too small may fail to observe biologically important aspects; however, an experiment which is too large may lead to wasting the animals. In order to balance those two sensitive concepts, scientists often asked to justify the number of the animals they plan to use. This is also important as a part of the ethical review process. In a study performed by Demirel *et al.;* sample sizes were given as six for one-tailed and seven for two-tailed in the paired t-test for $1\text{-}\beta = 0{,}80$ power level in $d = 0{,}5$ values for 1% significance level [21]. According to the animal ethics committee directive of Gazi University, the most suitable animal model must be selected with the minimum number of animals [22]. The maximum number of animals has been selected according to the framework of the ethics council principles and power analyses.

Technique

Sericin powder was divided into 30 mg packs and placed in Eppendorf tubes for intrapleural administration. The rats were anesthetized using intramuscular injections of 5-mg/kg xylazine

hydrochloride (Alfasan International B.V., Woerden, and Holland) and 45-mg/kg ketamine hydrochloride (Richter Pharma A.G., Wels, Austria). After the administration of intramuscular anesthetic agents, the left hemi-thorax and neck was shaved for surgical incision. Shaving was followed by skin preparation with a povidone-iodine solution. Subsequently, the tracheotomy was performed with an incision starting from the neck. The muscular layers of the neck were bluntly dissected to expose the cervical trachea. After dissection, a 20-gauge plastic catheter (OpSet cannula, Esdoornlaan, Holland) was deployed into the tracheal lumen. All rats were intubated with the same technique and mechanically ventilated from this tracheotomy catheter (Inspira ASV, at 70 – 100 inspiration per minute, tidal volume 0.6-2.0 ml/kg, and FiO_2 60%). Subsequently, the rats were placed in the right-lateral decubitus position and a left thoracotomy incision (4-cm skin incision) was performed at the mid-section between the spine and sternum. The muscle layers were bluntly dissected in order to allow the exposure of the parietal pleura. After visualization of the parietal pleura, under direct inspection, it was bluntly opened and 30 mg sericin powder was administered into the pleural space of each rat in the sericin group (n=6). The remaining rats were allocated as the sham thoracotomy group (n=6). After sericin administration, the muscle layers were sutured using the running suturing technique (Vicryl 3/0; coated, braided, polyglycolic acid; Dogsan, Trabzon, Turkey). The skin incision was sutured by using interrupted suturing technique (Vicryl 4/0; coated, braided, polyglycolic acid; Dogsan, Trabzon, Turkey). The pneumothorax was drained using a 20-gauge plastic catheter connected to a syringe applying negative pressure. This catheter was removed immediately after the closure of the thoracotomy. Finally, the tracheostomy catheter was removed and the tracheostomy canal was closed with 3/0 polypropylene (Orhan Boz AS, Ankara, Turkey) using a single suture. The skin incision of the neck was also closed by using an interrupted suturing technique (Vicryl 4/0; coated, braided, polyglycolic acid; Dogsan, Trabzon, Turkey).

After the operation, the rats were closely monitored for any clinical evidence of pain or other abnormalities, such as vocalization, tachypnea, or restlessness. An antero-posterior chest x-ray was obtained immediately after the operation and at postoperative day three, in order to check for any signs of pneumothorax and/or hemothorax. The animals were housed in individual stainless steel cages; the room temperature was kept at 20^0C (± 2^0C), and relative humidity was approximately 60% (± 10%) and the rats remained under simulated daylight condition. The rats were given ad-libitum access to food and water. The rats that died within the first 24 hours following the operation were replaced with new rats. Only one rat in the control group died on day two in the sham thoracotomy group and it was replaced immediately. The rats were sacrificed at postoperative day eight; the preferred scarification method was cardiac puncture. After intramuscular injection of anesthetic agents, 5-mg/kg xylazine hydrochloride (Alfasan International B.V., Woerden, Holland) and 45-mg/kg ketamine hydrochloride (Richter Pharma A.G., Wels, Austria) a 22-gauge syringe was used for cardiac puncture. In general, cardiac puncture is recommended for the terminal stage of the study to collect a single, good quality and a large volume of blood from the experimental animal [23]. The blood sample was taken slowly from the ventricle of the heart to avoid collapsing the heart. An approximately 8 – 10 ml blood sample was taken from each rat; this terminal method was the scarification of the rats.

Biochemical examination

Blood samples were collected into tubes containing EDTA. Plasma samples were separated from cells by centrifugation at 1800 g for 10 min (Hethich Universal, 320 R, and Zentrifugen). After centrifugation, plasma samples were separated from the cells and poured into Eppendorf tubes. Those Eppendorf tubes were stored at – 80^oC for analyses. The biochemistry doctors (investigators ME, CK, and OE) were kept blind to the study groups.

Plasma thiol disulphide redox homeostasis was studied with a new automatic measurement method with an automated analyzer (Roche, Cobas501, Mannheim, Germany) and results were presented as μmol/L. The principle of the novel assay is sodium borohydride ($NaBH_4$), which is used to reduce the disulphide bonds to the thiol groups. The sum of existing thiol groups and reduced thiol groups gives the total thiol. The $NaBH_4$ residuals that are not used are completely removed by formaldehyde. Thus, the extra reduction of 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB) is prevented. The native thiol is measured using a modified Ellman's reagent that was obtained by adding a formaldehyde solution into the classical Ellman's reagent. The difference between the total thiol with native thiol is divided by two to obtain the amount of the disulphide bond. The disulphide/native thiol (-S-S-) / (-SH), disulphide/ total thiol (-S-S-) / (-SH + -S-S-), and native thiol/total thiol (-SH) / (-SH + -S-S-) ratios were calculated.

STATISTICAL ANALYSES

The mean weight score of rats was calculated for each group. The biochemical data was expressed using statistical analyses, and conducted using the SPSS statistical software package (version 17.0, SPSS, Chicago, IL, USA). In this study, the results were controlled using an independent t-test, and because of the importance of the subject, a p value of less than 0.05 was considered statistically significant.

RESULTS

All of the rats returned to their normal feeding and activities immediately after the operation. Eleven rats tolerated the operation well; whereas, one rat in the sham thoracotomy group died on the second day. According to guidelines for the design and statistical analyses of experiments using laboratory animals published by Festing and Altman, the animals should be replaced with less sentient alternatives after mortality, such as intervertebrates or in vitro methods whenever possible [24]. Therefore, it was replaced immediately. As mortality was in the sham thoracotomy group, and there were no signs of any allergic reaction, there was no requirement to perform a necropsy.

The control chest x-rays of the animals were obtained on day one and three after the operation. None of those x-rays revealed any signs of pneumothorax, hemothorax or any other pathological findings, in both groups. The mean weight was 300.3 g (257 – 331) in the sericin group and 327.1 (270 – 395) in the control group; there was no statistically significant difference between the two groups.

The biochemical examination revealed that the mean score of rat plasma native thiol (-SH) levels was calculated as 110.06 µmol/L in the sericin group, and 75.70 µmol/L in the control group. The difference between the two groups was significantly higher in the sericin group ($p<0.05$; $p=0.037$). The mean score of rat plasma total thiol (-SH + -S-S-) levels was calculated as 147.81 µmol/L in the sericin group, and 118.51 µmol/L in the control group. Similarly, total thiol levels were higher in the sericin group, but this was within the limit according to the independent t-test ($p=0.056$). Finally, disulphide (-S-S-) levels of both groups were also calculated. The mean score of rat plasma disulphide levels were calculated as 21.36 µmol/L in the sericin group, and 18.87 µmol/L in the control group. According to the independent t-test, disulphide levels were not statistically significant between groups.

The ratios between native thiol, total thiol, and disulphide were also calculated. Disulphide/Native thiol (-S-S-) / (-SH), Disulphide/Total thiol (-S-S-) / (-SH + -S-S-), and Native thiol/Total thiol (-SH) / (-SH + -S-S-) were also evaluated; none of the ratios were statistically significant according to the independent t-test. The analyses results of thiol and disulphide parameters in the rat plasma with ratios are presented in Table-1.

Table 1: The analyses of thiol parameters in the rat plasma after intrapleural sericin application

Parameter	Sericin (n=6)	Control (n=6)	p
Native thiol (-SH) (µmol/L)	110.06	75.70	$p<0.05$ ($p=0.037$)
Total thiol (-SH + -S-S-) (µmol/L)	147.81	118.51	$p=0.056$
Disulphide (-S-S-) (µmol/L)	21.36	18.87	Not significant
Disulphide/Native thiol (-S-S-) / (-SH)	0.204	0.282	Not significant
Disulphide/Total thiol (-S-S-) / (-SH + -S-S-)	0.133	0.177	Not significant
Native thiol/Total thiol (-SH) / (-SH + -S-S-)	0.732	0.645	Not significant

DISCUSSION

Sericin is a natural, gum-like protein that coats the surfaces of fibers of fibroin polymers, which holds them together in order to perform a robust cocoon shell. The removal of sericin protein from fibroin polymers has been performed by a specialized process called “degumming”. The predominant amino acid in the structure of sericin protein is serine amino acid (almost 40% of the structure), followed by glycine (16-20%), glutamic acid, aspartic acid, threonine, and tyrosine [6]. The biochemical molecular structure of sericin has strongly polar side groups, such as hydroxyl, carboxyl, and amino groups [4, 6]. Those biochemically active groups lead to easy cross-linking and copolymerization, which improves the biocompatibility and biodegradation of sericin protein [2, 4]. According to this biocompatibility, and biodegradation sericin has many application fields in science and industry. Sericin is a hydrophilic molecule; thus, sericin’s highly water soluble property gives it an application field in the pharmaceuticals and cosmetic sectors due to its moisturizing effect. Sericin is useable as an anti-wrinkle and anti-aging agent in various kinds of lotions, creams, and ointments [1, 5]. Additionally, sericin has antioxidant effects, antibacterial activity in air-conditioning systems, and is a food additive in the food industry [4, 5]. In an experiment on silk sericin and applications, Sarovart *et al.;* concluded that sericin-coated nylon and polyester fibers had a strong antimicrobial potential when used for indoor air filters; a reduced amount of toxic free radicals and several kinds of microorganisms have been reported [4]. In addition to this feasibility in several industrial sectors, sericin has been a widely feasible and useful additive in laboratory studies due to its cell protective activity, and stimulating effect in the cell movement and proliferation [25, 26, 27]. Terada *et al.;* revealed the accelerating effect of the proliferation of several mammalian cell lines including a hybridoma when sericin was added into the culture, *in vitro* [26].

The stimulating effect of sericin on wound healing has been demonstrated by several studies [1, 7, 9]. Sericin enhances the attachment and proliferation of

skin fibroblasts; considered to play important role in the healing process. In one study, Nagai *et al.;* revealed the application of sericin solution had a potent effect in promoting wound healing and wound-size reduction in the rat debrided corneal epithelium [7]. In another study about healing effects of sericin cream, Aramwit *et al.;* demonstrated that sericin-treated wounds had much smaller inflammatory reactions, and wound-size reduction was much greater than the control group [1]. A fifteen day treatment with 8% sericin cream revealed complete healing without ulceration and an increase in collagen activity [1]. Aramwit *et al.;* performed another study, in which silk sericin promoted collagen production in the mouse cultured fibroblast cells at concentrations up to 40 ug/mL, after 24 hour incubation [27]. As sericin has an effect on fibroblast cells and improves collagen activity, it may be useable as a pleurodesis agent. For this purpose, Yazicioglu *et al.;* performed a rat study and demonstrated the increased fibroblastic activity and fibrosis in the visceral pleura after intrapleural sericin administration without adverse effects on lung parenchyma [2]. This study pioneers the feasibility of intrapleural sericin application in the fields of pulmonology and thoracic surgery.

Dynamic TDRH status plays critical roles in the antioxidant protection, detoxification, intracellular signal transduction, apoptosis, regulation of several enzymatic activities, as well as being increasingly implicated in many disorders [10, 12, 13, 16, 28-31]. Abnormal TDRH is involved in the pathogenesis of a variety of diseases, including diabetes mellitus, cardiovascular diseases, cancer pathogenesis, rheumatoid arthritis, chronic kidney disease, interstisial lung diseases, Parkinson's disease and Alzheimer's disease [11, 12, 16, 29-33]. In a review about usefulness of thiol groups, McBean *et al.;* concluded that the restoration of thiol redox balance may offer a useful approach to minimize neuronal loss during neuro-degeneration [11]. An abnormal TDRH ratio is involved in the pathogenesis of a variety of disorders including cardiovascular diseases [29]. Go *et al.;* mentioned a focus on therapies to regulate thiol dependent redox signaling may provide a useful alternative to free radical scavengers as a means to prevent and manage cardiovascular diseases [29]. Oxidant stress and thiol redox imbalance levels play an important role in the pathogenesis of rheumatoid arthritis and primary osteoarthritis patients. Tetik *et al.;* revealed the relationship between oxidant stress and inflammation by measuring thiol levels; plasma thiol levels in rheumatoid arthritis and primary osteoarthritis groups were significantly lower than in the control groups ($p < 0.0001$) [32]. In another study, the relationship between chronic kidney disease and TDRH were demonstrated. Rodrigues et al. researched plasma free cystein and free cystine levels and compared with reduction potential [33]. According to the researchers, free cysteine levels decreased ($p < 0.001$) and the reduction potential increased ($p < 0.0001$) with chronic kidney disease progression [33]. Also they found a high correlation between plasma reduction potential and creatinine concentrations; oxidative stress is primarily correlated with uremia [33]. The researchers suggested a therapy targeted to decrease the plasma cystine/cysteine ratio may present a possible mechanism to slow the rate of chronic kidney disease complications [33].

Thiol groups exert their effects through various proteins and primarily albumin. N-acetylcysteine (NAC), which is one of the proteins it exerts its efficacy on, is an analogue of cysteine. NAC, which is a sulfhydryl-containing compound and known as a thiol antioxidant, has many application fields in medicine. In a rat model, Chen *et al.;* researched both collagen levels in the lung tissue and the efficiency of NAC on mechanical ventilation-induced collagen accumulation [30]. As mechanical ventilation can induce collagen accumulation in the lung tissue, scientists demonstrated that NAC can be considered as a good candidate in the prevention of ventilation-induced lung fibrosis, and may helpful in the prevention of lung fibrosis for adult respiratory distress patients who received mechanical ventilation [30]. Additionally, NAC inhibits growth, proliferation, and survival of human gastrointestinal cancer cells of different phenotypes and characteristics [31]. According to Amini *et al.;* the combination therapy of NAC could have a potential value in novel therapeutic approaches to cancer [31]. NAC also has an important role in the apoptosis process. Yang *et al.;* demonstrated that NAC conjugates to phenethyl isothiocyanate, which enhances apoptosis in the growth-stimulated human lung cells [34].

The TDRH also regulates cellular redox state to constitute the major cellular protection against oxidants. An imbalance situation between cellular thiol disulphide redox state and the pulmonary defense system plays a role in the pathogenesis and progression of malignant lung and pleural diseases [19, 20]. Free radicals are linked both to carcinogenesis and tumor behavior; the most important reactive metabolites in human lung include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH-), and a number of reactive nitrogen and sulphur species (RNS, RSS) [19]. According to Toyokuni, biomolecules with redox-active thiol compounds are necessary to maintain mildly reductive cellular environments to counteract oxidative stress, and for the execution of redox reactions for metabolism and detoxification [20]. In the present study, also, the level of native thiol was significantly high. Since sericin increases plasma thiol groups when applied intrapleurally, it is possible to use it in the prevention of damage secondary to free radicals and as a cancer prevention agent in the future.

Thiol groups have tissue proliferation features, increase fibroblastic activity and fibrosis, and enhance wound healing by using the pathway including cysteine amino acids. In a rat experimental model, Saltman *et al.;* mentioned that increased intracellular glutathione levels may improve and enhance wound healing [15]. D-ribose-L-cysteine protein is a precursor of the antioxidant glutathione; thereof, the cystein compound has an effect on wound healing. A redox imbalance implicated in fibrotic lung disease pathogenesis and cystein oxidation has a role in the regulation of fibrotic lung disease patients. Janssen-Heininger *et al.;* mentioned that oxidation of cysteines in proteins is known to act as a regulatory event that affects protein functions [16]. Fibroblastic activity and fibrosis were significantly high in the visceral pleura of the rats that received sericin in a study by Yazicioglu *et al.;* [2]. Similarly, in the present study, rat plasma thiol levels were significantly increased following sericin application in the same way. In this case, thiol groups can be considered to have a significant role on the fibrosis causing effect of sericin and this effect may occur through cycteine amino acid.

The limitations of the study were that we did not perform study on a rat lung cancer model, as we were unsure whether sericin has an effect on plasma TDRH. Similarly, we did not evaluate parameters about antioxidative distress and free oxygen radicals. The demonstration of intrapleural sericin application and its effects on rat plasma native thiol, total thiol and disulphide levels were the initial goal. Researchers must answer more questions such as, if sericin increases the rate of thiols, can it be used as an agent preventing cancer or free radical damage? Therefore, more research projects are required to demonstrate the anti-cancer efficiency of sericin and thiol groups in a rat lung cancer model; antioxidative and prevention of free radical damage in another rat model. We hope that the demonstration of the feasibility of sericin protein and improving effect of plasma TDRH parameters pioneers the researches on this field.

CONCLUSION

Sericin is a new, easily available, researchable, feasible, useful, and cost-effective protein in the practice of pulmonology and thoracic surgery. Significantly higher native thiol levels in sericin-treated animals and the higher total thiol levels (at almost borderline significance) support the hypothesis that sericin application and improved thiol disulphide redox homeostasis may increase antioxidative features, and may decrease the possibility of carcinogenesis.

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