In vitro efficacy of silk sericin microparticles and platelet lysate for intervertebral disk regeneration

Elia Bari[a], Sara Perteghella[a,b,*], Giorgio Marrubini[a], Milena Sorrenti[a], Laura Catenacci[a], Giuseppe Tripodo[a], Maddalena Mastrogiacomo[c], Delia Mandracchia[d], Adriana Trapani[d], Silvio Faragò[e], Paolo Gaetani[b,f], Maria Luisa Torre[a,b]

[a]Department of Drug Sciences, Medicinal Chemistry and Technology Section, University of Pavia, 27100 Pavia, Italy

[b]Pharmaexceed srl, 27100 Pavia, Italy

[c]Department of Experimental Medicine, University of Genova, 16132 Genova, Italy

[d]Department of Pharmacy-Drug Sciences, University of Bari, 70125 Bari, Italy

[e]Silk Division, Innovhub, Stazioni Sperimentali per l'Industria, 20133 Milan, Italy

[f]U.O. Chirurgia Vertebrale, Istituto Clinico Città di Pavia, Gruppo San Donato, 27100 Pavia, Italy

*Corresponding author:

Sara Perteghella, Ph.D

Department of Drug Sciences, University of Pavia,

Viale Taramelli 12, 27100 Pavia, Italy

Tel.: +39 0382 987359; Fax: +39 0382 422975

e-mail: sara.perteghella@unipv.it

Declaration of interest

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Abstract

Positive effect of combined use of silk sericin (SS), platelet lysate (PL) and platelet poor plasma (PPP) on proliferation rate of nucleus pulposus (NPs) and adipose-derived mesenchymal stem/stromal (MSCs) cells has been demonstrated. ROS-scavenging activity was investigated for SS, PL and PPP, alone and in combination, and the maximum antioxidant activity was reached using SS alone at 1 % w/v or in association with PPP at 0.75 % w/v, while PL had an inhibitory effect. The simultaneous use of PL and PPP promoted cell proliferation, and cellular doubling was significantly higher in cultures with PL than fetal bovine serum for both cell lines. Moreover, the mixture SS-PL showed cytoprotective properties against oxidative stress, and this effect was particularly appreciated on NPs. The combination of these substances showed different biological properties, paving the way to a new tool for both *in vitro* and *in vivo* intervertebral disk regeneration.

Keywords: silk sericin microparticles, platelet lysate, nucleus pulposus, mesenchymal stem/stromal cells, intervertebral disk, regeneration.

Abbreviations

SS: silk sericin

PL: platelet lysate

PPP: platelet poor plasma

PRP: platelet rich plasma

IVD: invertebral disk

FBS: fetal bovine serum

CD: cellular doubling

NP: nucleus pulposus cells

MSC: adipose-derived mesenchymal stem/stromal cells

ROS: radical oxygen species

BSA: bovine serum albumin

1. Introduction

Regenerative medicine requires high number of cells, generally obtained by *in vitro* cell expansion. Fetal Bovine Serum (FBS) has been widely used as medium supplement due to its simple productive process and high growth factor content [1]. However, the use of FBS is associated with some disadvantages: poorly defined composition, high batch-to-batch variability and it may be vector of xenogeneic antigens and zoonotic infections [2]. For these reasons, the expansion of cells should occur without the use of animal sera, according to the last European directive [3]. In the last years, several human-derived products, such as serum, platelet rich plasma (PRP) and platelet lysate (PL), have been proposed as FBS substitutes for a successful *in vitro* cell expansion [4]. In particular, PL is rich in growth factors that promotes cell proliferation and inhibits the apoptosis [5]. PL is obtained from platelet concentrates, unsuitable for transfusions, that are usually subjected to an activation procedure for the release of growth factors from their α-granules [6]. The effectiveness of PL to sustain the cell proliferation has been already demonstrated on different cell lines [4].

Cultured cells are often subjected to oxidative stresses and this is the resultant of different aspects: cells are exposed to high oxygen levels and are not protected by physiological antioxidant systems; the culture media are poor of antioxidant molecules, while they contain metal ions required for cell proliferation that exert pro-oxidant activities [7, 8]. In this context, some natural compounds have been investigated as media supplement to protect cells from oxidative stress. Silk sericin (SS) is a globular water-soluble protein, recently proposed as useful polymer in cell cultures, tissue engineering and drug delivery fields [9, 10]. Antioxidant activity of SS is the main responsible for its biological effects: SS shows positive effects on cell viability and integrity [11], it is able to promote cell proliferation [12, 13], and it has protective effects in the cell cryopreservation [11].

The aim of this work was to assess, for the first time, whether the combined use of PL, PPP and SS could be effectively used to promote cell proliferation and to protect cells from oxidative stress, paving the way to a new tool for intervertebral disk regeneration. For these reasons, two different cell lines were considered: nucleus pulposus cells (NPs) and adipose-derived mesenchymal stem

cells (MSCs). NPs are chondrocyte-like cells isolated from herniated tissues during standard procedures of microdiscectomy [14]. NPs are characterized by low proliferative index, due to their origin from inflamed and degenerated tissues, even if they are able to remain viable under hypoxia and low glucose levels [15]. Moreover, NPs may lose their phenotype when expanded in monolayer conditions [16]. For these reasons, the regeneration of the damaged intervertebral disk is still a pending problem [17]. SS and PL+PPP mixture were tested also on MSCs which are naturally resident into intervertebral disk, where they participate to tissue regeneration by both differentiation and secretion of trophic factor which exert immunomodulatory effects [18, 19]. We chose MSCs because they are excellent candidates for allogenic cell therapies: they can be easily isolated from adipose tissue with high cell yield and proliferative rates [20]. This innovative regenerative tool, based on platelet lysate and silk sericin, should better support cell proliferation than FBS, especially for NPs, allowing to obtain an adequate number of cells in less time. Moreover, it should provide an appropriate set of substances, such as flavonoids, protecting cells from ROS. The *in vitro* effectiveness of SS, PL and PPP combination paves the way to their use as active for intervertebral disk regeneration in an *in vivo* animal model.

2. Materials and Methods

2.1 Materials

2,2-diphenyl-2-picrylhydrazyl hydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ethanol, methanol, dimethyl sulfoxide, hydrogen peroxide and type II collagenase were purchased from Sigma-Aldrich (Milan, Italy). All reagents used for cell cultures were purchased from Euroclone (Milan, Italy). A commercial platelet lysate kit (Lyset®-Kit) has been obtained by Carlo Erba Reagents (Milan, Italy): the kit is composed by two lyophilized reagents: human platelet lysate (PL) and human platelet poor plasma (PPP).

2.2 SS extraction, microparticles preparation and characterization

Bombyx mori cocoons were degummed in autoclave (Systec V-65, Wettenberg, Germany) at 120 °C for 1 h (40 mL water/g of cocoons). Sericin solution was dried to obtain microparticles using a Büchi Mini SprayDryer (Flawil, Switzerland), with below process parameters: pump, 6 ml/min; inlet temperature, 120 °C; outlet temperature, 80 °C; air pressure, 3 bar; fluid flow, 500–600 ml/h [21]. Sericin microparticles morphology was evaluated by scanning electron microscopy (JEOL JSM-6380LV, Tokyo, Japan). The samples were placed on an aluminum *stub* and working conditions were rough vacuum, 10 kV signal and back-scattered electrons. Particle size analysis was performed with a laser light scattering granulometer (Beckman Coulter LS230, Miami, Florida) equipped with a small cell volume; microparticles were suspended in ethanol (refractive index was set at 1.359) and transferred to the measurement cell.

Qualitative estimation of sericin purity was evaluated determining the A280-ratio. This test is based on the Lambert-Beer Law A=ebc, where A is the sample absorbance, *e* is the molar extinction coefficient, *b* is the cell path length and *c* is the sample concentration. For proteins at a concentration of 1 mg/ml, the *e* values are 0.57 and 1.00 at 260 and 280 nm, respectively. Therefore, an A-ratio above 0.57 corresponds to a sample that is free of contamination [22]. For the determination of total protein content, a micro BCA-Protein Assay Kit (Thermo Scientific, Milan, Italy) was used according to the manufacturer's specifications. The absorbance–concentration calibration curve was generated using bovine serum albumin (BSA) standards. Each sample was tested in triplicate.

2.3 Chemical-physical characterization of SS microparticles, PL+PPP and their physical mixture

Fourier transform infrared (FT-IR) spectra were obtained using a Spectrum One Perkin-Elmer spectrophotometer (Wellesley, MA, USA) equipped with a MIRacle^{TM} ATR device (Pike Technologies, Madison, WI, USA). The IR spectra in transmittance mode was obtained in the spectral region of 650–4000 cm^{-1} with a resolution of 4 cm^{-1}. Measurements were carried out in

triplicate and the characteristic absorption bands were recorded for SS microparticles, PL+PPP and their physical mixture.

2.4 ROS-scavenging activity of SS microparticles, PL and PPP

To evaluate ROS-scavenging properties, the DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) method was used, according to [21]. Briefly, 1800 µl of DPPH (0.028% w/v methanolic solution) were mixed with 200 µl of each sample; reaction mixtures were incubated in the dark for 20 minutes at room temperature, centrifuged at 3000 g for 10 min, and spectrophometrically analyzed at 515 nm (Uvikin 930, Kontron Instruments, USA). PL and PPP, alone and in combination, were analyzed considering the common concentration used in cell culture and four different dilutions: PL at concentrations 0.050, 0.070, 0.50, 0.70 and 1.0 % v/v, and PPP at concentrations 0.20, 0.27, 2.0, 2.7, 4.0 % v/v. SS microparticles were tested at five concentrations (0.075, 0.10, 0.75, 1.0 and 1.5% w/v) after dissolution and dilution with distilled water or with PL+PPP. Reaction mixture, without sample, was used as negative control, while ascorbic acid was used as positive control at the same sample concentration. ROS-scavenging activity was calculated with the following formula: % activity = $(A - B)/A \times 100$, where A is the absorbance of the negative control and B is the absorbance of the tested solution. Analyses were performed in three replicates.

2.5 NPs isolation and culture

Intervertebral disk (IVD) samples were harvested during surgery for herniated disc disease standard microdiscectomy. Nucleus pulposus and annulus fibrosus were separated; after saline washing, nucleus pulposus tissues were suspended in Phosphate-Buffered saline (PBS) with 1% penicillin/streptomycin for the transport to the laboratory. Samples were minced to 1-2 mm^3 segments and digested with trypsin-EDTA, for 1 h at 37 °C, 5% CO_2, followed by overnight incubation with 0.075% type II collagenase. In order to eliminate undigested tissue, suspension was filtered through 70 µm nylon mesh (Greiner Bio-One GmbH, Kremsmunster, Austria) and cells were centrifuged at 300 g for 5 min. NP cells were seeded onto flasks (10,000 cells/cm^2) with

Dulbecco's Modified Eagle's Medium High Glucose (DMEM-HG), 10% FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml).

2.6 MSCs isolation and culture

Subcutaneous adipose tissues were obtained, from informed donors, during abdominoplasty surgery; samples were preserved in PBS, containing penicillin 5,000 IU/ml, streptomycin 625 µg/ml and amphotericin B 51 µg/ml, and carried to laboratory. Each adipose tissue was processed as reported by Faustini et colleagues [23]. Briefly, the sample was cut and treated with 0.075% w/v type II collagenase at 37°C until complete digestion. In order to inhibit the enzymatic action, 10% v/v of FBS was added, and cell suspension was filtered, centrifuged, at 300 g for 10 min, and washed three times in PBS. The obtained cell vascular fraction was seeded (100,000 cells/cm^2) and cultured with DMEM-F12 1:1, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. For subsequent passages, after detaching with trypsin 0.05% and EDTA 0.02%, MSCs were seeded at a density of 10,000 cells/cm^2, and cultured until confluence.

2.7 Cellular doubling and morphology of NPs and MSCs

NPs and MSCs were cultured at a density of 10,000 cells/cm^2 from P1 to P9 in their specific media, supplemented with 1 % v/v PL and 4 % v/v PPP. For both cell lines, at least two donors have been considered. Every 7 days, until the tenth passage, cells were detached and counted to calculate cellular doubling (CD) using the following equation: $CD = \log(N/N_0)*\log 2$, where N = number of counted cells and N_0 = number of seeded cells. The same procedure was performed on cells cultured in standard conditions (10% FBS). At each passage, adherent cells have been observed under a microscope (JuLY™, Smart Fluorescent Cell Analyzer, Ruskinn, USA) and cellular morphology was evaluated.

2.8 Cytotoxicity assay

In order to define the cytocompatible concentration of SS microparticles, they were solubilized at five different concentrations (0.075, 0.1, 0.75, 1 and 1.5 % w/v) in three different media: without

supplementation (CTR-medium), with 5 % PL+PPP (PL-medium) or with 10 % FBS (FBS-medium). Sericin solutions were sterilized by filtration (0.22 µm pore size) before use.

NPs and MSCs were seeded in 96-well plate (10,000 cells/cm^2). After 24 hours, media were discarded and 100 µL of sericin solutions were added for 72 hours of incubation. Then, cells were washed with PBS and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) were added in each well. After 3 hours, the medium was removed and the cells were mixed with 100 µL of dimethyl sulfoxide (DMSO). The optical density was measured using a microplate reader (Synergy HT, BioTek, United Kingdom) at 570 nm and 670 nm (reference wavelength). Each condition was tested in triplicate. Cell viability (%) was calculated as follows: 100 × (ODs/ODc), where ODs is the mean value of the measured optical density of the tested sample and ODc is the mean value of the measured optical density of cells incubated with FBS without SS and/or PL+PPP.

2.9 Cytoprotective effect against oxidative stress

NPs and MSCs were seeded in 96-well plate (10,000 cells/cm^2). After 24 hours, media were discarded and 100 µL of sericin solution (1.5 % w/v) were added, considering the same media used for cytotoxicity test (CTR-medium, PL-medium or FBS-medium), for 24 hours; after this time, media were eliminated and 100 µL of hydrogen peroxide (1 mM) solution was added to each well. An MTT test was performed, as previously reported, after 24 hours of incubation with H_2O_2 and the optical density was measured. Untreated Cells were considered as control. Each condition was tested in triplicate.

2.10 Statistical analysis

All calculations were performed using STATGRAPHICS XVII (Statpoint Technologies, Inc., Warrenton, Virginia, U.S.), Microsoft Excel and R version 3.1.0 (2014-04-10) Copyright (C) 2014 The R Foundation for Statistical Computing. R-based chemometric software routines were used for design of experiments calculations. The R-based software has been developed by the Group of

Chemometrics of the Italian Chemical Society [http://gruppochemiometria.it/gruppo-lavoro-r-in-chemiometria.html].

ROS-scavenging activity results were fitted by a quadratic D-optimal design computed to study the information contained in the data collected and summarized in Table S1. The concentration values of the three factors in Table S1 were coded between -1 and +1 before running the Fedorov algorithm for selecting the D-optimal set of experiments. The equation of the model postulated for the function of the median Antioxidant Activity % (AA%) and the concentrations of Sericin (S), PL and PPP components is the following: $AA\% = intercept + b1 \cdot S + b2 \cdot PL + b3 \cdot PPP + b12 \cdot S \cdot PL + b13 \cdot S \cdot PPP + b23 \cdot PL \cdot PPP + b11 \cdot S^2 + b22 \cdot PL^2 + b33 \cdot PPP^2$.

The comparison between mean volume-weighted diameter ($d_{4,3}$) and mean surface-weight diameter $d_{3,2}$ of sericin microparticulate systems, was performed by a t-test for means with different variances.

Cellular doubling results were analyzed using a multifactor analysis of variance (ANOVA), considering CD as response variable while the type of supplement (FBS or PL+PPP), the culture passage and the donor as fixed factor. Results of cytotoxicity and oxidative stress test were processed with a multifactor analysis of variance (ANOVA) considering cell viability (%) and optical density as response variable, respectively. Culture medium was considered as fixed factor for cytotoxicity test, while, for oxidative stress test, we considered hydrogen peroxide and culture medium as fixed factor. The differences between groups were analyzed with the post hoc LSD's test for multiple comparison. The statistical significance was fixed at $p \leq 0.05$.

3. Results

Silk sericin microparticles, obtained by spray drying technique, has been characterized: SEM analysis showed that they are collapsed with a smooth surface, while some particles, with higher size, appear with a round shape (Figure 1). The mean volume-weighted diameter ($d_{4,3}$) ± confidence interval ($\alpha = 0.05$, 34 d.f.) of sericin microparticulate systems was 3.6 ± 0.1 µm, and the mean

surface-weight diameter $d_{3,2}$ ± confidence interval (α = 0.05, 34 d.f.) was 2.28 ± 0.07 µm. The $d_{4,3}$ value resulted significantly higher than the $d_{3,2}$ (p-value < 0.001), confirming the irregular shapes of microparticles. The relative span value (referred to diameter $d_{4,3}$ and calculated as $(d_{90}-d_{10})/d_{50}$, where d_{10}, d_{50} and d_{90} mean the 10^{th}, the 50^{th} and the 90^{th} percentile, respectively) was of 1.42 indicating a narrow size distribution (the values which tend to 1 indicate a narrow distribution).

The total protein content and the purity test were done on SS, previously solubilized in water: results demonstrated that the average protein content ± standard deviation was 61.8 % w/w ± 14.3 and the A-ratio ± standard deviation was 0.88 ± 0.0075, confirming that our combined process of degumming and spray-drying allows to obtain stable and pure samples.

In FTIR spectra, SS shows characteristic vibration bands at 1641.54 cm^{-1} (C-O stretching), 1526.74 cm^{-1} (N-H bending) and 1244.10 cm^{-1} (C-N and N-H functionalities) (Figure 1B). FTIR analysis of PL + PPP shows the characteristics peaks of proteins, as reported by [24]. In detail, the NH stretching vibration of Amide A and B gives rise two bands at 3277 and 3061 cm^{-1}, respectively. Absorption band at 1641 cm^{-1} is correlated to C=O stretching vibration of amide I, while peaks at 1540 and 1451 cm^{-1} are related to the amide II bending and stretching vibrations (NH and CN groups, respectively). Contributions of amide III-correlated NH bending vibration are visible in the 1400-1200 cm^{-1} region. FTIR spectrum of physical mixture of SS microparticles and PL + PPP showed a peak at 1639 cm^{-1}, related to the presence of sericin. Moreover, the physical mixture between SS microparticles and PL+PPP induced the interaction between functional groups of protein chains; in particular, it is possible to observe the presence of a well-defined peak at 1234 cm^{-1}, not visible in singular PL+PPP or sericin spectra, owing to C-N stretching vibrations in the amide III linkage [25] (Figure 1B).

The second aim of this paper was to evaluate whether the ROS-scavenging activity was enhanced by the combined use of SS and PL+PPP. The experiments, initially conducted to estimate the antioxidant activity, were not planned by any experimental design systematic approach. However, the study of the results obtained from 30 experiments with replicate measurements (N = 3) of the

antioxidant activity suggested that a regression model could be computed to understand the information contained in the data collected (Table S1). Therefore, a quadratic D-optimal model was postulated for studying the relationship between antioxidant activity % (AA %), the concentrations of the three factors considered (sericin, PL and PPP) and their interactions. By applying the exchange algorithm of Fedorov [26] on the data in Table S1 it was possible to extract a group of 13 experiments which constituted the D-optimal set of experiments. In the studied domain of concentrations (sericin between 0 and 1.5, PL 0 and 1, and PPP between 0 and 4 [units]), the model showed a very good fitting (Adjusted R^2 0.9988) and residuals randomly distributed around the x axis (data not shown). The model was validated, as shown by the data reported in Table S1 (compare the values in the columns Median AA, Computed AA, and Relative Error). These results evidenced that the following model equation could be used to describe quantitatively the antioxidant activity variation as a consequence of the changes made in the sample composition, in the experimental domain studied: $AA\ \% = 51.98 + 51.16^{(***)} \cdot S - 13.10^{(***)} \cdot PL + 8.45^{(***)} \cdot PPP - 14.00^{(***)} \cdot S \cdot PL + 9.30^{(***)} \cdot S \cdot PPP - 0.83 \cdot PL \cdot PPP + 6.71^{(**)} \cdot S^2 - 1.32 \cdot PL^2 + 0.33 \cdot PPP^2$.

The effect of sericin is very important and, with the additional effect provided by PPP, these two factors determined the overall AA% measured in the samples. Therefore, the maximum AA% was obtained at the highest level of concentration of sericin and by controlling the concentration of PPP, while excluding the PL factor from the sample composition. The effect of the PL resulted detrimental to the overall AA% observed in the samples. The AA% was affected even by a small concentration of the PL. Figure 2 shows the plot of the model function: when PL is excluded from the preparation (Figure 2A), the maximum AA% can be obtained using sericin alone at about 1.1 % w/v or in the region from 1.1 to 1.5 % w/v of sericin + amounts of PPP ranging from 0 to about 0.75 % w/v. When PL is at the intermediate concentration level of 0.50 %w/v (Figure 2B), the maximum AA % is observed only if both sericin and PPP are present at the highest concentration levels in the samples. If PL is introduced in the samples at the maximum concentration (1.0 %w/v), the AA% is

generally low and never exceeds the 85% level even if the highest concentrations of sericin and PPP are present in the sample (Figure 2C).

The presented data provide strong evidence that the most important factor, which influences the antioxidant activity, with a positive correlation, is the sericin concentration (coefficient 51.16). The PL component has an inhibitory effect: in fact, increasing its concentration, the antioxidant activity decreased, due both to the PL effect and the PL-SS interaction. Higher antioxidant activity was instead observed by increasing the PPP concentration, due both to the PPP effect and to the PPP-SS interaction.

The *in vitro* tests were performed on NPs and MSCs and the effect of PL and PPP has been evaluated for cell expansion: cells were cultured from P1 to P9 in their specific media, supplemented with PL+PPP or FBS (as control). For both cell lines, cellular doubling (CD) was independent from the donor, suggesting that our culture method could overcome the individual cell variability, thus allowing the reproducibility of the experimental conditions. Moreover, the CD was significantly higher in cultures with PL+PPP than FBS for both NPs and MSCs (ANOVA, $p<0.0001$). Particularly, a significant effect was observed from third passage in NP cultures (ANOVA, $p = 0.0111$) (Figure 3A), while the higher CD was appreciated since first passage for MSCs cultured with PL+PPP than serum (ANOVA, $p < 0.0001$) (Figure 3B). Comparing the cellular doubling of two cell lines cultured with PL+PPP, the overall mean values were 1.83 and 2.80, for NPs and MSCs respectively, confirming higher ability of stem cells to proliferate. These results were also confirmed by observing, at each passage, cells under optical microscope: cell morphology was not influenced by the type of supplement in culture media; NPs showed a typical round-shaped chondrocyte-like morphology, while MSCs displayed a characteristic spindle shape morphology (data not shown). The cell density was higher in the presence of PL+PPP than FBS cultures. These results confirmed that PL+PPP was able to better guarantee the proliferation of both NPs and MSCs, when compared to standard cultures supplemented with FBS.

Once demonstrated that PL+PPP increased cell proliferation, the final goal of this study was to confirm that SS should exert antioxidant activities on cells in order to protect them from oxidative stresses. For this reason, NPs and MSCs were cultured in media without supplementation (CTR-medium) or with PL+PPP (PL-medium) or with FBS (FBS-medium), in which SS was added. Cells were then treated with 1 mM hydrogen peroxide (H_2O_2) and the cell viability has been calculated. SS resulted cytocompatible at all tested concentrations (0.075-1.5 % w/v): in fact, the cell viability, for both NPs and MSCs, was higher than 80%. Therefore, we selected the higher SS concentration (1.5% w/v) for the evaluation of cytoprotective effect against oxidative stress. Statistical analysis showed a significant effect of both culture medium and the treatment with hydrogen peroxide (ANOVA, $p<0.0001$); these effects have been appreciated for both NPs and MSCs. H_2O_2 significantly reduced the metabolic activity of both cell lines cultured in CTR-medium or FBS-medium (Figure 4A-B), confirming the suitability of our experimental conditions. Higher optical density was observed for NPs and MSCs cultured in PL-medium than CTR-medium and FBS-medium. In particular, NPs cultured in PL-medium and treated with hydrogen peroxide have a significant reduction of metabolic activity than untreated cells, but this effect was not observed using SS: in fact, no differences were appreciated between NPs cultured in PL-medium and SS, before and after treatment with H_2O_2, confirming the cytoprotective role of SS against oxidative stress (Figure 4A). Different results were obtained for MSCs cultures, in which we did not observe an effect of hydrogen peroxide in cells cultured in PL-medium, but a significant reduction of metabolic activity was shown after H_2O_2 treatment in PL-medium and SS, even if higher optical density was highlighted than FBS-medium (Figure 4B).

4. Discussion

For this study, SS has been obtained by a water-based degumming process and, then, the solution was spray-dried to obtain a fine, stable and well-storable microparticle-based powder. The collapsed aspect of particles is the consequence of the rapid solvent evaporation, which could be related to an

elevate sericin solution dilution and to the absence of bulking. The microparticle aggregation could be related to the remaining moisture and Van der Waals forces that have been established between their surfaces [27]. Granulometric analysis showed that the spray drying technique is a reproducible method to obtain particles with a unimodal distribution. Moreover, our combined processes of degumming and spray-drying allows to obtain a highly pure protein and its content was over 60% w/w. The non-protein fraction could be based on carbohydrates, wax, mineral salts and flavonoids, which contribute to its peculiar antioxidant activity. In this work, the antioxidant properties of SS have been confirmed: results showed that SS has excellent *in vitro* antioxidant properties, as it is able to interact with reactive oxygen species (ROS) transforming them in their reduced form. This property is probably related to its aminoacidic sequence, which allows to eliminate free radicals and ROS and which increases the antioxidant activity of enzymes, such as the superoxide dismutase and the glutathione peroxidase [28]. Particularly, the large amount of hydroxyl groups of SS contributes to this activity, chelating trace elements such as iron, zinc and copper [13]. In a previous work, our group already demonstrated that SS shows ROS-scavenging and immunomodulatory activities, and these biological property is related to the *B. mori* strain [21, 29]. With the same assay, we also evaluated the antioxidant effect of a PL and PPP, but they showed a low reducing activity. Our results are not contradictory with literature as, to the best of our knowledge, no work reported that PL or PPP exert antioxidant activity. Instead, for the first time, we highlighted a strong antioxidant effect in the mixture SS-PL+PPP.

Subsequently, SS and PL+PPP have been used as medium additive for NPs and MSCs cultures. NPs are characterized by low proliferative index as they are isolated from damaged and inflamed tissues. Consequently, their employment in cell therapy for intervertebral disc (IVD) regeneration is limited, because of the excessively long time required to obtain an adequate number of cells [15]. The effectiveness of platelet-derived products has been already demonstrated on NPs: for example, Akeda K. et al. (2006) have assessed the *in vitro* effect of PRP on the production of extracellular matrix by porcine IVD cells encapsulated in alginate beads. The results of this study have shown

that the PRP is effective in the stimulation of the proliferation of IVD cells and in increasing the synthesis of collagen and proteoglycans [30]. Obata et al. (2012) have instead highlighted the *in vivo* PRP ability to induce a regeneration of degenerated IVD in a rabbit model [31]. However, to the best of our knowledge, the ability of PL and/or PPP to support the *in vitro* proliferation of NP cells has not been investigated yet. Our experiments indicate that PL+PPP is a feasible alternative to FBS because it is able to promote cell proliferation, highlighting a less time required to reach the cell confluence. This is an important goal in the regenerative medicine field, as it allows to obtain an adequate cell number with a reduction of time and production costs. In addition to its ability to protect cells from the damaging action of oxidative stress, SS has often been added to the culture media also for its mitogen effects, which occur according to yet unidentified mechanisms and which differ from the cell line [13]. Terada et al. demonstrated that sericin-S (MW 5- 100 kDa) acts as a mitogen factor in serum-free media [32]. Also Cao et al. observed that sericin-S is suitable for cell culture [33]. In this work, SS was employed in three different media in order to assess the effect of the only sericin protein or the synergies with FBS or with PL+PPP. Results showed that SS is cytocompatible, and higher cell viability was reached in combination with PL+PPP. Moreover, the mixture SS-PL+PPP showed excellent cytoprotective properties against oxidative stress, and surprisingly this effect was more appreciated on NPs. Similar results were obtained on fibroblasts exposed to hydrogen peroxide, confirming the ability of SS to inhibit oxidative stress [34, 35].

5. Conclusion

We demonstrated that the combined use of silk sericin microparticles, platelet lysate and platelet poor plasma appears the most promising formulation for cell cultures *in vitro*, showing the highest proliferation index and the capability to inhibit oxidative damages, both for nucleus pulposus and MSCs. Thanks to our results and the already demonstrated effect of platelet rich plasma on an *in vivo* animal model of intervertebral disk degeneration, we retain that there are the premises to test the synergic effect of silk sericin and PL+PPP *in vivo* on an animal model. In particular, the

matched use of PL+PPP and sericin microparticles could enhances the cell proliferation (thanks to both bioactive components) and, at the same time, protects cells from oxidative stress and exerts antiapoptotic effects (thanks to sericin). This effect would not be exercised only on NP cells, but also on the resident population of MSCs, thus increasing the amount of secreted trophic factors that, as widely demonstrated, can modulate the inflammation process.

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Declaration of interest

SP, PG and MLT are memberships of the advisory board of the company Pharmaexceed.

Author contributions

EB and SP performed all cellular experiments and wrote the paper, GM carried out statistical analysis on experimental data, MS and LC performed the chemical-physical characterizations, GT, MM, DM, AT critically read and revised the manuscript, SF, PG and MLT conceived and designed the experiments.

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Captions

Figure 1. A) Characterization of SS powder: morphology evaluation obtained by scanning electron microscopy (SEM), scale bar 10 µm; B) Fourier transform infrared (FT-IR) spectrum in the region 650-4000 cm^{-1} of SS microparticles, PL+PPP and their physical mixture.

Figure 2. Effect of the three factors considered on the antioxidant activity (AA%): (A) PL is excluded from the preparation; (B) PL is at the intermediate concentration level of 0.50 % w/v; (C) PL is at the maximum concentration (1.0 %w/v).

Figure 3. Mean values and standard deviation of cellular doubling for NPs (A) and MSCs (B), from passage 1 to 9, cultured in their specific media, supplemented with PL+PPP or FBS.

Figure 4. Mean values and standard deviation of optical density obtained by MTT assay: NPs (A) and MSCs (B) were cultured in their specific media, without supplementation (CTR), with 5 % PL+PPP (PL) or with 10 % FBS (FBS), in addition of SS (1.5 % w/v). Cells were treated with hydrogen peroxide (1 mM) and untreated cells were considered as control. Each condition was tested in triplicate.