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Released from ZrO₂/SiO₂ coating resveratrol inhibits senescence and oxidative stress of human adipose-derived stem cells (ASC)

Resveratrol-doped biomaterial rejuvenates ASC

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Abstract: The rapid aging of the population results in increased number of metabolic and degenerative disorders, especially in the elderly. Thus, a novel approach in the fields of orthopedic and reconstructive surgery for bone regeneration is strongly desirable. A new perspective in the therapy of bone fractures is tissue engineering which combines living cells with biomaterials to develop modern substitutes that can restore tissue functions. Metallic biomaterials, including stainless steel and pure titanium, have been extensively used for the fabrication of surgical implants over decades. Chemical modification of material surface for example incorporation of chemotactic factors may significantly improve the therapeutic effect. In this paper we describe titanium substrate modifications with ZrO₂/SiO₂ coating functionalized with resveratrol using a sol – gel, dip-coating technique. Moreover, we established the effects of fabricated scaffolds on adipose stem cells isolated from elderly patients. Using fluorescence imaging, polymerase chain reaction (PCR) and cytotoxicity tests, we established that 0.5 Res_ZrO₂/SiO₂ significantly reduced apoptosis and accumulation of oxidative stress factors in adipose derived stem cells (ASC). Thus exploitation of fabricated biomaterials in regenerative medicine as a strategy for rejuvenate ASC from elderly patients *in vivo*, seems fully justified.

Keywords: sol-gel; dip coating; resveratrol; biocompatibility; mesenchymal stem cells.

1 Introduction

Due to the rapid aging of the population, diseases and traumas are causing increased numbers of tissue defects and other musculoskeletal disorders [1–6]. Regenerative medicine aims to reconstruct organs and tissues by triggering a regeneration response. The special need for bone replacement exists in the fields of orthopedic and reconstructive surgery, where elderly patients comprise a major group of patients [7–9]. Tissue engineering combines living cells with biomaterials to develop modern substitutes that can restore tissue functions [10]. Scaffolds provide a mechanical stability for the transplant and surface area for proliferation, differentiation and adhesion of cells, to support regeneration of damaged tissue [11]. Moreover, by chemical modifications like incorporation of cell adhesion molecules or chemotactic factors on the biomaterial surface, tissue development may be significantly improved [12]. Extensive research has been dedicated to developing biomaterials with various composition and physical properties, like mechanical strength and topology, to control and enhance the biological response of cells [13–15].

Metallic biomaterials including stainless steel, cobalt – chromium alloys, pure titanium and titanium alloys, such as titanium – aluminum – vanadium alloy and nitinol have been extensively used for the fabrication of surgical implants over decades [16]. Due to their strength, resistance, electrical conductivity and ease of fabrication, those scaffolds are widely used in orthopedics, guaranteeing long-term implant performance. Although, due to concerns over metal corrosion and biocompatibility,

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a relatively small number of metals are currently used in medical practice [17]. 316L austenitic stainless steel (ASTM F 138/139) has been widely used in the repair of fractures to replace tissues and stabilize structures, including bone. Although, relatively non-expensive, it possess great susceptibility to corrosion when it gets into contact with biological fluid. Compared to stainless steel, titanium alloys are characterized by excellent *in vivo* corrosion resistance, due to the stable passive oxide layer forming. Titanium (Ti) alloys have strength comparable to 316L, although they are approximately 50% lighter in weight, which makes them an ideal tool in scaffold fabrication. Moreover, Ti implants are better tolerated by the immune system.

To prevent the initiation of corrosion, surface modifications such as the application of protective coatings has been performed in numerous studies. Because of non-expensive, uncomplicated and low temperature conditions required, the sol – gel technique is commonly used as a surface modification method. The coatings can be composed of organic, inorganic and polymer compounds, forming hybrid or multilayer films [18]. Sol - gel modification can provide appropriate implant – host tissue interaction. Biomaterials obtained from the same chemical compounds, but modified by a method other than sol-gel, exhibited lower bioactive properties in comparison [19]. The chemicals which create the sol layer are usually metal alkoxides. Further steps in coating manufacturing include hydrolysis and polycondensation reactions. During this process, –OH groups are formed, which allow the creation of new bonds and results in the final gel [20]. Sol-gel thin films are applied on substrates by dip – coating, spin – coating and spraying techniques [21]. Dip-coating is performed by the immersion of the substrate with constant speed and controlled time in the solution and allows a homogeneous coating of a definite thickness [22] to be obtained. The great advantage of sol – gel coating is the ability to modify the sol solution with certain materials and chemical compounds, thereby controlling the film properties. In recent years, modification of scaffold surfaces with hydroxyapatite has been proven to improve its osteoinductive properties [23,24]. Our previous studies have shown the beneficial impact of SiO₂ films functionalized with vitamin E on the proliferative potential of human adipose – derived mesenchymal stem cells (AMSCs) [25]. One of benefits of vitamin E is the reduction of oxidative stress, which may elicit cell injury and death [26]. Another group of natural antioxidants are polyphenols [27]. One of them – resveratrol, found mostly in berries, nuts and grapes was widely described as an anti-inflammatory, antioxidative and anti-aging agent [28–30].

Currently, adipose-derived mesenchymal stem cells (ASC), become a major source of cells in tissue engineering and cell based therapies. Those cells can be easily isolated from abundant and accessible pools of adipose tissue with non-invasive and cost-effective procedures. Numerous advantages including differentiation into multiple lineages, secretion of various cytokines and immunomodulatory effects make ASC a promising tool for regeneration of tissues and organs damaged by injuries or diseases. However, it must be considered that ASC undergoes senescence both, *in vivo* and *in vitro* [31–33]. Biological aging of the organism is a complex, not yet fully understood process, associated with functional decline, impaired homeostasis and reduced capacity of tissues to regenerate. Those unfavorable changes exert effects on tissue-resident stem cells including decline in their number and flawed functionality [34]. Numerous studies have addressed questions relating to stem cell rejuvenation, senescence and their protection from injuries [35–38]. Our own studies have shown that aging negatively affects ASC properties, leading to increased apoptosis, decreased proliferation and reduced differentiation potential [31]. The main reason of ASC deterioration is excessive accumulation of reactive oxygen species and mitochondria impairment [39–41]. Thus, when considering application of ASC from older patients with biomaterials for the tissue engineering, application of chemicals able to diminish reactive oxygen species (ROS) levels in cells seems to be fully justifiable. Our previous research indicated on protective characteristics of sol-gel derived silica coating doped with vitamin E on ASC physiological condition. Fabricated scaffold alleviated apoptosis and ROS accumulation and increased cell proliferation. Thus enrichment of biomaterials with antioxidants results in multiple beneficial effects and may become a novel strategy in implant manufacturing for tissue engineering in geriatrics.

Following our previous data, in the present study we decided to fabricate a scaffold enriched with well and widely studied antioxidant- resveratrol (RES) for aged ASC culturing. RES has been shown to exhibit anti-aging, antioxidant, antiangiogenic anticancer, cardio-protective and antioxidative effects. Moreover, recent studies have indicated its effective application in treating diabetes, neurodegenerative and metabolic disorders [42,43].

In this paper we describe titanium substrate modification with ZrO₂/SiO₂ coating functionalized with RES using sol – gel, dip-coating technique. We have investigated the relation between different surface properties with viability and proliferation of human ASCs of elderly patients. In addition, we have shown fabricated scaffold reduced accumulation of ROS in investigated cells.

2 Materials and methods

All reagents used were purchased from Sigma Aldrich, unless indicated otherwise.

2.1 Substrates

Titanium discs ($\varnothing = 13\text{mm}$) were used as a coating substrate. Before sol-gel synthesis, metallic substrates were cleaned with detergent, alcohol and distilled water by ultrasonic cleaning.

2.2 Synthesis of sol-gel

For the $\text{ZrO}_2/\text{SiO}_2$ sol preparation, the following compounds were used: zirconium (IV) isopropoxide (ZrOP, Sigma Aldrich, USA) as the zirconia precursor, tetraethyl orthosilicate (TEOS, Sigma Aldrich, USA) as the silica precursor, n-propanol (PrOH, Sigma Aldrich, USA) as a solvent and hydrochloric acid (HCl, POCh S.A., Poland) as an acid catalyst. Two different concentrations of resveratrol ($\text{C}_{14}\text{H}_{12}\text{O}_3$, Alfa Aesar, Germany) were used as a volume modification of sol. The resulting sol was obtained by magnetic stirring at a speed of 500 r/min for 2,5 hours at room temperature. Before the deposition onto the titanium discs, the sol was allowed to age for 4 hours.

2.3 Dip – coating method

The sol-gel thin layers were deposited on the titanium substrates by dip-coater (WPTL6 – 0.01, MTI Corporation, USA) with controlled dipping speed ($v = 34.26\text{ mm/min}$). The immersion time adopted were as follows; first layer: 60 s, second layer: 30 s and third layer: 15 s. The first and second deposited layers were clear $\text{ZrO}_2/\text{SiO}_2$ sol-gels, while the third layers were different for each group of samples: clear $\text{ZrO}_2/\text{SiO}_2$ film, $\text{ZrO}_2/\text{SiO}_2$ functionalized by $0.1\text{ }\mu\text{mol/mL}$ resveratrol film and $\text{ZrO}_2/\text{SiO}_2$ functionalized by $0.5\text{ }\mu\text{mol/mL}$ resveratrol film. Between each layer deposition, samples were dried in room temperature for 24 hours. The resulting sol-gel films were obtained by stabilization for 12 hours at 250°C in air atmosphere. The samples for the in vitro test were closed in sterilization pouches (Safe-seal® Duet, Medicom, Canada) and sterilized by thermal sterilization for 23 minutes at 121°C .

2.4 Surface morphology and topography

The surface morphology of sol-gel layers obtained was carried out by scanning electron microscopy (SEM, EVO LS15, Zeiss, Germany). Before the examination each sample was coated with a thin layer of gold by vacuum sputter (ScanCoat Six, Edward, Poland). Topographical analysis was performed by atomic force microscope (Dimension FastScan™ AFM, Bruker, USA) with PeakForce QNM mode. All measurements were performed by SCM PIT probe (f: 75 KHz, k: 2.8 N/m, length: $225\text{ }\mu\text{m}$) and SCANASYT AIR probe (f: 750 KHz, k: 0.4 N/m, length: $115\text{ }\mu\text{m}$).

2.5 Surface wettability properties

The wettability of the surfaces was evaluated by measuring the contact angle and surface free energy (SFE). The contact angle measurement was carried out by goniometer (OCA 35, DataPhysics Instruments GmbH, Germany) using distilled water, glycerin and diiodomethane. Each liquid was placed on the surface three times in three different places of each sample. The volume of each drop was $5\text{ }\mu\text{L}$. The van Oss-Good method was used to calculate the surface free energy.

2.6 Isolation of human-adipose derived mesenchymal stem cells (huASC) and cell culture

Human adipose-derived mesenchymal stem cells were isolated under sterile conditions following the previously described protocol by Grzesiak et al. [21]. The fragment of human adipose tissue was placed in Hank's balanced salt solution (HBSS, Sigma – Aldrich, Poland), supplemented with 1% antibiotics (P/S/A; penicillin/streptomycin/amphotericin B) and washed. Tissue samples were cut into small pieces and digested with 1 mg/mL collagenase type I for 40 minutes at 37°C . A cell pellet was suspended in the culture medium and plated onto culture flasks. The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma – Aldrich, Poland) with nutrient F-12 Ham, 10% of Fetal Bovine Serum (FBS) and 1% solution of P/S/A. The medium was changed every two days. Passage was performed after the cells reached 80% confluence using trypsin solution (TrypLE, Life Technologies, Denmark) in accordance with the manufacturer's protocol.

2.7 Immunophenotyping and multipotency assay

To characterize huASC, analysis of the cell surface antigens were performed. The following antigens were investigated: CD29, CD34, CD44, CD45, CD73, CD90 and CD105. To perform flow cytometry analysis, cells were incubated with the appropriate antibodies (at 4°C for 15 minutes) conjugated with phycoerythrin (PE), fluorescein isothiocyanate (FITC), allophycocyanin (AP) and peridinin-chlorophyll-protein (PerPC). At least ten thousand cells were scored and analyzed using Becton Dickinson FACs Calibur flow cytometer.

For chondrogenic, osteogenic, and adipogenic differentiation, huASCs were cultured in commercially available media (StemPro®, Life Technologies, Denmark) onto 24-well plates at concentration of 30×10^3 cells per well. The media was changed every three days. Chondrogenic and osteogenic stimulation was conducted for 16 days, whereas adipogenic differentiation lasted 14 days. Cells in control medium (10% FBS, 1% P/S/A, Dulbecco's Modified Eagle's Medium (DMEM) with nutrient F-12 Ham served as control to establish the effectiveness of differentiation. Prior to staining, cells were fixed with 4% paraformaldehyde (PFA). To visualize chondrogenic differentiation, specimens were stained with 0.1% solution of Safranin O (specific for proteoglycans). Osteogenic differentiation was proved by staining for extracellular mineralized matrix with Alizarin Red. Lipid droplets formed during adipogenesis were stained with Oil Red O. Photographs were acquired using a PowerShot Camera (Cannon, Germany) and an inverted fluorescence microscope (AxioObserverA1, Carl Zeiss, Jena, Germany).

2.8 Proliferation rate

The proliferation rate of cells was established using resazurin-based assay (Alamar blue) in accordance to manufacturer's instructions. Prior the experiment, cells were seeded in 24-well plate onto the synthesized materials at a concentration of 2×10^4 per well. Tests were performed after 24, 48 and 120 hours of culture. To perform the assay, the culture medium was replaced with a medium containing 10% resazurin dye, following 2 hours incubation at 37°C. Next, absorbance levels of the supernatants were determined using spectrometer (BMG Labtech, Germany) at wavelengths 600 nm for resazurin and 690 nm as a background absorbance. The number of cells was established based on the growth curve generated during the experiment. In order to prepare the

standard curve, cells were seeded at the density of 1×10^4 , 3×10^4 and 5×10^4 and absorbance levels were assigned to certain cell number. A linear trend line equation allowed for the estimation of cell numbers at various time intervals throughout the experiment. PDT calculation online software was used to calculate the population doubling time (PDT) [22]. The proliferation factor (PF) of cells reflects the metabolic activity of cells cultured on resveratrol-doped biomaterials in relation to pure $\text{ZrO}_2/\text{SiO}_2$ scaffold with an arbitrary value equaled 1.

Staining for Ki67 was performed using anti-Ki67 antibodies (dilution 1:500, anti-Ki67 antibody produced in rabbit, Abcam). Additionally, ratio of Ki67 positive cells to all nuclei stained with DAPI was estimated based on representative images. All the procedures were performed following manufacturers' protocols.

2.9 Visualization of cells morphology

Prior fluorescence stainings samples were washed with HBSS and fixed with 4% PFA. Then cells were permeabilized for 15 minutes with 0.1% Triton X-100 at room temperature and washed again. Atto-520-labeled phalloidin (1:1000 in HBSS) was used to visualize actin filaments. The cells nuclei were counterstained for 5 minutes at room temperature in the dark with diamidino-2-phenylindole (DAPI, 1:1000 in HBSS). Using the ImageJ software, the area covered by cells was calculated. Mitochondria were stained using Mito Red dye, while visualization of the endoplasmic reticulum was carried out using ER Tracker (Life Technologies) (1:500 in culture medium, 30 min, 37°C). All the procedures were performed in accordance with the manufacturer's protocol.

2.10 Oxidative stress factors and senescence analysis

Oxidative stress factors levels were assessed after 24, 48 and 120 hours of culture. Nitric oxide concentration was established using Griess Reagent Kit (BioVision, USA). Superoxide dismutase (SOD) was assessed using a SOD Assay Kit (Life Technologies). Reactive forms of oxygen (ROS) was measured with H₂DCF-DA assay (Life Technologies). The number of alive and dead cells was evaluated using the Cell stain Double Staining Kit. Viable cells were stained green with Calcein A.M. while the dead cells were stained orange with propidium iodide. Senescence-associated β -galactosidase accumulation was visualized using Senescence Cells Histochemical Staining

Kit. All the procedures were performed in accordance with the manufacturer’s protocol.

2.11 Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR): gene expression analysis

After the fifth day of the experiment cells were collected with trypsin and homogenized using TriReagent. Total RNA was isolated using the phenol-chloroform method as previously described by Chomczynski and Sacchi [23]. Isolated RNAs were suspended in DEPC-treated water and analysed using a nanospectrometer (WPA Biowave II). The reverse transcriptase Tetro cDNA Synthesis Kit (Bioline, United Kingdom) was used for the synthesis of complementary DNA (cDNA). For each reaction 150 ng of total RNA was used. Enzymatic digestion of Total RNA and cDNA synthesis were performed in accordance with the manufacturers’ protocols using a T100 Thermal Cycler (Bio-Rad). The qRT-PCR reactions were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) as described elsewhere [44]. The reaction mixture contained 2 µL of cDNA in a total volume of 20 µL and was conducted using SensiFast SYBR & Fluorescein Kit (Bioline, United Kingdom). Primer concentration in each reaction equaled 500 nM. All primer sequences used in individual reactions are shown in Table 1. Relative gene expression analysis (Qn) was calculated in relation to the GAPDH housekeeping gene. Following genes were analyzed: p21, p53, Bax, Bcl-2, c-Myc, cyclin D1, MNF1, Fis1, Sirt1, FOXO1, TERT, IL1-β, TNFα, IL6, IL13 and IL4. Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 Surface morphology, topography and wettability

SEM – EDX analysis revealed that ZrO₂/SiO₂ coatings on titanium substrates prepared by dip – coating method are transparent and homogeneous (Figure 1a). Element mapping confirmed presence of both, zirconium and silicon (Figure 1b - 1d). Moreover, dispersion of Zr and Si was homogenous, which indicates that this technique of producing sol – gel thin films is suitable for biomaterials manufacturing. AFM analysis showed that surface topography differs between the investigated materials (Figure 2). The results indicated

Table 1: Sequence of primers used in qRT-PCR.

Gene	Primer	Sequence 5'-3'	Accession no.
Cyclin-D1	F:	GATGCCAACCTCCTCAACGA	NM_053056.2
	R:	GGAAGCGGTCCAGGTAGTTC	
c-Myc	F:	CTTCTCTCCGTCCTCGGATTCT	NM_002467.4
	R:	GAAGGTGATCCAGACTCTGACCTT	
p21	F:	GGCAGACCAGCATGACAGATTTC	NM_001291549.1
	R:	CGGATTAGGGCTTCCTCTTGG	
p53	F:	AGTCACAGCACATGACGGAGG	NM_001126118.1
	R:	GGAGTCTTCCAGTGTGATGATGG	
BAX	F:	ACCAAGAAGCTGAGCGAGTGTCT	XM_011527191.1
	R:	ACAAAGATGGTCACGGTCTGCC	
Bcl-2	F:	ATCGCCCTGTGGATGACTGAG	NM_000633.2
	R:	CAGCCAGGAGAAATCAACAGAGG	
Mnf1	F:	GTTGCCGGGTGATAGTTGGA	XM_005247596.2
	R:	TGCCACCTTCATGTGTCTCC	
Fis1	F:	TGGTGCGGAGCAAGTACAAT	NM_016068.2
	R:	TGCCCACGAGTCCATCTTTC	
SIRT1	F:	ACAGTTGCGGGAATCCAAA	NM_001314049.1
	R:	GTTTCATCAGCTGGGCACCTA	
FOXO1	F:	CTCTGGCCCTTTCACCATA	XM_011535010.1
	R:	GCTTCTCTTGGCAGCTCG	
TERT	F:	CGGAAGAGTGTCTGGAGCAA	XM_011514106.1
	R:	GGATGAAGCGAGCTGGA	
IL1-β	F:	AAACAGATGAAGTCTCTCCAGG	XM_006712496.1
	R:	TGGAGAACCACTTGTGTCTCCA	
TNFα	F:	AGTGACAAGCCTGTAGCCCA	NM_000594.3
	R:	GTCTGGTAGGAGACGGCGAT	
IL6	F:	GAACCTCTCTCCACAAGCGCCTT	NM_000600.4
	R:	CAAAAGACCAGTGATGATTTTCACCAGG	
IL13	F:	GCAATGGCAGCATGGTATGG	NM_002188.2
	R:	AAGGAATTTTACCCTCCCTAACCC	
IL4	F:	ACATTGCTACTGCAATCGACACC	NM_000589.3
	R:	TGCTGTACGGTCAACTCGGTGC

Figure 1: SEM images of the zirconium/silica coating (a) and EDX maps of the zirconium and silicon atoms distributions (b), silicon atoms distribution (c), zirconium atoms distribution (d).

Figure 2: The images of surface topography in Atomic Force Microscope of titanium substrate (a), zirconia/silica coating (b), zirconia/silica coating modified by 0.1 µl/mL resveratrol (c) and by 0.5 µl/mL resveratrol.

that with increasing concentration of resveratrol roughness of the surface reduces, differences were observed in nanometers. Not without significance is initial roughness of titanium substrate, which directly affects the roughness of final biomaterials.

3.2 Surface wettability properties

The results of water contact angle (Table 2) showed that titanium exhibits hydrophobic properties ($98 \pm 3^\circ$). Based on the analysis of contact angle it can also be stated that the properties of each sol – gel layers are hydrophilic ($\text{ZrO}_2/\text{SiO}_2$: $37 \pm 1^\circ$, $0.1\text{res_ZrO}_2/\text{SiO}_2$: $30 \pm 1^\circ$, $0.5\text{res_ZrO}_2/\text{SiO}_2$: $31 \pm 2^\circ$), which is confirmed by SFE results ($\text{ZrO}_2/\text{SiO}_2$: $94 \pm 5 \text{ mJ/m}^2$, $0.1\text{res_ZrO}_2/\text{SiO}_2$: $98 \pm 3 \text{ mJ/m}^2$, $0.5\text{res_ZrO}_2/\text{SiO}_2$: $98 \pm 4 \text{ mJ/m}^2$). The acid – base part of the SFE is higher than the dispersive part, which demonstrates the possibility of forming hydrogen bonds and shows less influence of long – range interactions on the hydrophilic properties of the tested coatings.

Table 2: Wettability properties of substrate and synthesized coatings.

	$\theta [^\circ]$	$\text{SFE} [\text{mJ/m}^2]$	$\gamma_{\text{AB}} [\text{mJ/m}^2]$	$\gamma_{\text{d}} [\text{mJ/m}^2]$
Titanium	98 ± 3	35 ± 4	33 ± 3	2 ± 1
$\text{ZrO}_2/\text{SiO}_2$	37 ± 1	94 ± 5	37 ± 1	57 ± 4
0.1res_	30 ± 1	98 ± 3	36 ± 1	62 ± 2
$\text{ZrO}_2/\text{SiO}_2$				
0.5res_	31 ± 2	98 ± 4	39 ± 1	59 ± 3
$\text{ZrO}_2/\text{SiO}_2$				

3.3 Immunophenotyping, differentiation and morphology of the investigated human AMSCs cultured on different biomaterials

Using the flow cytometry the expression of the following markers was confirmed: CD34, CD73, CD90 and CD105. Simultaneously, cells lacked the expression of CD45 surface antigen (Figure 3a). In addition multi-lineage differentiation of huASC was confirmed (Figure 3b). Using Alizarin Red, Safranin and Oil Red O, differentiation into osteogenic, chondrogenic and adipogenic lineage respectively, was confirmed.

Figure 3: Multipotency assay of ASC. Adipogenesis was confirmed by the presence of intracellular lipid droplets using Oil Red O staining. Chondrogenic differentiation were visualized by staining proteoglycans with safranin O and Alizarin Red staining was performed to confirm osteogenesis by the presence extracellular mineralized matrix (a). Flow cytometry dot plots showing surface antigens expression by ASC (b). ASCs were characterized by the expression of CD 44, CD 73, CD 90, CD 105 while lacked the expression of CD 34 and CD 45 surface marker. Magnification: x100. Scale bar 200 μm .

3.4 HuASC growth kinetics on tested biomaterials

During the 5-day experiment, the cell proliferation rate on tested biomaterials with or without resveratrol was assessed. On the initial day of the experiment the 3×10^4 cells/well were seeded onto scaffolds. According to the growth curve (Figure 4a) after 24 hours, the highest number of cells was observed on 0.5 Res_ZrO₂/SiO₂ material. Scaffold without resveratrol (ZrO₂/SiO₂) significantly reduced cell growth, which indicates the positive effects of resveratrol on cell proliferation. After 48 hours a decrease in cell number was noted in each group. Both groups with resveratrol showed a similar cell number, with a slight advantage of the 0.5 Res_ZrO₂/SiO₂ compared to 0.1 Res_ZrO₂/SiO₂. After 120 hours, increase in cell proliferation was observed, except 0.1 Res_ZrO₂/SiO₂. The greatest number of cells was observed in the group with 0.5 $\mu\text{mol/mL}$ resveratrol. Moreover, population doubling time analysis was performed (PDT, Figure 4b). The shortest PDT was observed in cells cultured on 0.5 Res_ZrO₂/SiO₂ ($p < 0.001$) in comparison to control group (ZrO₂/SiO₂). These results stand in good agreement with proliferation factor (Figure 4c). In each time point, PF in 0.5 Res_ZrO₂/SiO₂ group was

higher than 1, which indicates an increased proliferation rate. 0.1 Res_ZrO₂/SiO₂ also demonstrated increased proliferation during the whole experiment, although the level was lower than in 0.5 Res_ZrO₂/SiO₂. Based on the selected images the area covered by cells was calculated (Figure 4d). Using qRT-PCR we also evaluated the mRNA levels of cell cycle genes cyclin D1 (Figure 4e) and c-Myc (Figure 4f). Both proteins showed higher expression in cells cultured on biomaterials doped with resveratrol. Cyclin D1 for 0.5 Res_ZrO₂/SiO₂ ($p < 0.005$) and 0.1 Res_ZrO₂/SiO₂ ($p < 0.01$). c-Myc for 0.5 Res_ZrO₂/SiO₂ and 0.1 Res_ZrO₂/SiO₂ ($p < 0.05$). Immunofluorescent staining with anti-Ki67 antibodies confirmed up-regulation of proliferation in 0.1 Res_ZrO₂/SiO₂ and 0.5 Res_ZrO₂/SiO₂ (Figure 4g-h).

3.5 Oxidative stress factor and senescence analysis

The morphology of the cells in the culture was visualized using phalloidin and DAPI stainings (Figure 5a). In the ZrO₂/SiO₂ group, robust number of enlarged nuclei was observed. Moreover, the “fried egg” shape, polygonal cells were noted. The common characteristic of cells cultured

Figure 4: Growth kinetics of ASCs cultured on different sol/gel coatings. Growth curve of ASCs calculated during 5 days (a). Population Doubling Time (b) and Proliferation factor for each coating (c). Area covered by cells calculated from photographs obtained during the experiment (d). The expression levels of Cyclin D1 (e) and c-Myc (f) involved in cell cycle regulation. Ki67-DAPI staining of cultures (g) staining quantification (h). White arrows indicate the signal from the Ki67. Magnification: x100. Results expressed as mean $\pm$ SD *pvalue<0.05, **pvalue<0.01, ***pvalue<0.001.

Figure 5: Morphology of ASCs evaluated with DAPI and Phalloidin (a) - two columns from the left. Additionally Calcein-AM staining indicates live cells while propidium iodide staining signals dead cells (a) - two columns from the right. Arrows indicate dead cells upon penetration of propidium iodide. Quantification of Calcein-AM- Propidium iodide staining established with ImageJ software (b). The mRNA ratio of BAX/Bcl-2 (c) and p21/p53 (d) were evaluated in relation to mRNA level of housekeeping gen GAPDH. Magnification: x100. Results expressed as mean $\pm$ SD. **pvalue<0.01. Scale bar 250 μ m.

Figure 6: Morphology of mitochondria and endoplasmic reticulum of ASCs estimated with DAPI/MitoRed and DAPI/ER staining (a). Arrows indicate mitochondria of cells cultured on different sol/gel coatings. Expression levels of the Mnf1 (c) and Fis1 (d) genes in order to assess the balance between fission and fusion which regulates the mitochondrial dynamics. Moreover, CHOP (d) and PERK (e) mRNA levels were estimated in order to examine the condition of endoplasmic reticulum. Magnification: x100. Scale bar 250 μm. Results expressed as mean ± SD *pvalue<0.05, **pvalue<0.01, ***pvalue<0.001.

onto each of the investigated materials was centrally placed nuclei. Analysis of the Calcein-AM and propidium iodine stainings (Figure 5a,b) revealed that resveratrol doped biomaterials inhibited apoptosis in huASC. An increased number of dead cells were observed in ZrO₂/SiO₂ group. The highest number of viable cells was observed in 0.5 μmol /mL resveratrol scaffold. BAX/Bcl-2 (Figure 5c) and p21/p53 (Figure 5d) ratio was downregulated in 0.5 Res_ZrO₂/SiO₂, although with no statistical significance. Staining for mitochondria and ER revealed the highest fluorescent intensity in 0.5 Res_ZrO₂/SiO₂ which may indicate an increased metabolic activity of those organelles (Figure 6a). In addition, 0.5 Res_ZrO₂/SiO₂ exhibited significantly decreased expression of genes involved in mitochondrial stress like Mnf1 and Fis1 (Figure 6b-c). There were no differences in the expression of CHOP (Figure 6d) and PERK (Figure 6e) between resveratrol doped scaffolds although mRNA of those genes were upregulated in pure ZrO₂/SiO₂ material.

The amount of reactive oxygen species (Figure 7a), was significantly decreased in scaffolds doped with resveratrol in comparison to pure titanium disc. Cells propagated onto materials with resveratrol displayed similar ROS levels after 24 hours (p <0.05) and 120 hours (p <0.001). The lowest amount of ROS (p <0.001) was observed in 0.5 Res_ZrO₂/SiO₂ after 48 hours of culture. The amount of nitric oxide was decreased in huASC cultured on resveratrol

doped scaffolds only after 120 hours of culture (Figure 7b). Additionally the level of superoxide dismutase was examined (Figure 7c). During the experiment, the highest expression of SOD was observed in 0.5 Res_ZrO₂/SiO₂.

The accumulation of senescence associated beta-galactosidase was diminished in huASC cultured on resveratrol doped scaffolds (Figure 8a,b). Obtained data indicates that cells cultured on 0.5 Res_ZrO₂/SiO₂ accumulated the lowest amount of beta-galactosidase in comparison to other groups (Figure 8a,b). Analysis of SIRT 1 (Figure 8c) and the FOXO 1 (Figure 8d) gene expression was performed using real time PCR. The highest expression of both, SIRT 1 and FOXO 1 was observed in 0.1 Res_ZrO₂/SiO₂. On the other hand, TERT (Figure 8e) mRNA was up regulated in 0.5 Res_ZrO₂/SiO₂.

Furthermore, we analyzed the expression of pro and anti-inflammatory cytokines. No differences were observed in the expression of IL-1β (Figure 9a), while IL-6 was significantly downregulated in both resveratrol groups (Figure 9b). Additionally, no statistical significance was observed in the expression of TGF-β (Figure 9c). Moreover, 0.5 res_ZrO₂/SiO₂ showed increase transcript level of anti-inflammatory cytokines IL-13 (Figure 9d). On the other hand, increased expression of IL-4 (Figure 9d) was noted in huASC cultured on 0.1 res_ZrO₂/SiO₂ in comparison to other groups.

Figure 7: Analysis of the oxidative stress factors in ASCs: the levels of reactive oxygen species (a), amount of nitric oxide (b) and activity of superoxide dismutase (c). Results expressed as mean $\pm$ SD *pvalue<0.05, **pvalue<0.01, ***pvalue<0.001

Figure 8: Quantification of beta-galactosidase staining performed at wavelength of 450 nm (a) and pictures took under brightfield microscope showing the accumulation of blue dye (b). Expression levels of the SIRT 1 (c), FOXO 1 (d) and TERT genes (e) in order to assess aging of cells. Magnification: x100. Results expressed as mean $\pm$ SD *pvalue<0.05, **pvalue<0.01, ***pvalue<0.001. Scale bar 200 μ m

Figure 9: Expression level of the IL1-β (a), IL6 (b) and TNF-α (c) genes in order to assess the level of proinflammatory cytokines. Moreover, expression level of the IL13 (a) and IL4 (c) genes was performed to assess the level of anti-inflammatory cytokines. Results expressed as mean ± SD *pvalue<0.05, ***pvalue<0.001.

4 Discussion

Metallic materials are still the most common scaffolds used in orthopaedics and dental medicine. Improvement of material bioactive properties may be achieved *via* processing such as surface modification to enhance cell response and simultaneously increase materials biocompatibility. Thus, the creation of novel, smart scaffolds becomes a major challenge in the fields of cell biology, chemistry and materials science. The modern biomaterials for regenerative medicine must take advantage of different chemical substances, which are able to influence cytophysiological properties and direct the fate of cells. Bioactive substances may be deposited onto biomaterial surfaces and released in the controlled manner for better tissue rebuilding and regeneration. Recent studies, including our own, have shown that the sol-gel method is a relatively easy and non-expensive way for deposition of substances onto the surface of metallic scaffolds. In the present study, we have shown that a ZrO₂/SiO₂ coating might be used as a carrier for resveratrol (RES). SEM photographs indicated that sol-gel layers are smooth without any cracks with uniform zirconium and silica distribution. The wettability of the material surface is

mainly affected by the surface chemical functionality and topology. Wettability exerts significant effects on stem cells behaviour including proliferation rate. Study performed by Shin et al. revealed the highest number of adhered bone-marrow stem cells on a surface with intermediate wettability (57°-65°), while for the highest contact angle (97°) the cell number was decreased [45]. In Ruardy et al. [46], it was shown that the spread area of human fibroblasts increased with wettability. Similarly, Altankov et al. [47] demonstrated increased cell proliferation with increasing material surface wettability. In our study, ZrO₂/SiO₂ coating significantly increased the wettability of pure titanium alloy and further functionalization of ZrO₂/SiO₂ coating with resveratrol increased wettability even more. That fact may partially explain increased proliferation of cells cultured onto RES-doped scaffolds.

Moreover, it is now a well established fact, that rougher biomaterial surfaces, increases cell attachment and proliferation, likely due to availability of the medium and serum ingredients through the grooves underneath adhered cells [48]. Our data from AFM, indicates an increased roughness of RES doped scaffolds as the surface of these biomaterials is formed of grooves and ridges. It is even more important as collagen synthesis, extracellular

matrix, cytokines, growth factors secretion are enhanced by rough surface [49,50]. Moreover, *in vitro* studies indicated a positive correlation between surface roughness and cell proliferation [51]. Those findings stand in good agreement with our data as we observed increased cellular growth onto RES-doped scaffold, thus corroborating the data of Anselme et al. [52] and Keller et al. [53] who suggested that a high degree of microroughness increases initial cell response of adhesion and proliferation.

Due to their unique characteristics, including self-renewal and multipotency, mesenchymal stem cells have become a major tool in regenerative medicine and biomaterial science [7,8,54]. However, during aging MSC lose their stemness and ability to differentiate [31,32,40], which in consequence limits their therapeutic application. Aging is a complex, yet not fully understood process, although it is believed to be driven by genetic, epigenetic and environmental factors. During aging, the amount of reactive oxygen species (ROS) in cells increases, altering their cytophysiological characteristics and deteriorating mitochondrial functions. Simultaneously with ROS accumulation, the antioxidative defence of the organism diminishes, resulting in prevalent cellular damage, apoptosis and inflammation. Since in regenerative medicine, autologous transplant are still the most common, we decided to fabricate a novel scaffold for geriatrics, which aims to decrease oxidative stress of aged stem cells and in consequence increased tissue regeneration *in vivo*. For this purpose we isolated ASC from aged patients and cultured them onto scaffolds doped with RES, a substance proved for its antioxidative, anti-inflammatory, anti-aging and osteogenic properties [55–58].

The study performed by Lei et al. [59], revealed that RES protected bone marrow MSC derived chondrocytes cultured on chitosan-gelatin scaffolds from the inhibitory effect of interleukin-Mi1beta. Thus application of RES as a bioactive substance onto scaffolds for bone regeneration seems to be fully justified, because of pro-inflammatory microenvironment of damage tissue site. Further, more RES was proven to enhance osteogenesis of human MSC by upregulating RUNX2 gene expression via the SIRT1/FOXO3A axis [60]. Those findings correlate with our data, as we observed increased expression of both SIRT1 and FOXO genes in cells cultured on scaffolds doped with RES.

In the present study, we have found that cells cultured onto RES-doped materials displayed increased proliferation rate, enhanced expression of cell cycle progression related genes (cyclin D1, c-Myc), increased accumulation of Ki67 and expression of TERT. Our data correlates with Yoon et al. [61] who established that

sustained treatment with resveratrol at early passages maintained the self-renewal potential and multipotency of MSCs up to passage 10. Moreover, cells cultured on RES scaffolds presented diminished apoptosis and senescence, these phenomenon was also observed by Peltz et al. [62], who observed that, resveratrol promotes cell self-renewal by inhibiting cellular senescence and suppresses adipogenic differentiation during short-term exposure.

Extracellular vesicles (EVs) are circular fragments of membrane which play a pivotal role in cell to cell communication. These vesicles are enriched with growth factors, anti-apoptotic and anti-inflammatory factors, all crucial for the tissue regeneration [63]. Interestingly, the paracrine effects of mesenchymal stem cells weakened during aging. Although the study conducted by Lei et al. [64] reported that resveratrol has a protective effect on senescence of ASCs and can preserve the paracrine effect of the ASCs on promoting insulin secretion of INS-1 cells via Pim-1. Moreover, cells formed dense, multi-layered aggregates and cytoskeletal projections connecting neighbouring cells.

The endoplasmic reticulum (ER) is an elaborate membrane network, synthesizing and folding secreted and membrane bound proteins. Properly folded proteins are then directed to Golgi Apparatus while misfolded ones are targeted to degradation. Unfolded protein response (UPR) pathway prevents the accumulation of dysfunctional proteins and if necessary triggers apoptosis. Although, the mechanism underlying ER-induced apoptosis are not fully understood, the body of evidence suggests that both, ER and mitochondria cooperates together to induce cell death. Activation of UPR is mediated through PERK an ER-associated transmembrane serine/threonine protein kinase and its downstream transcriptional target CHOP. In our study we have observed that RES significantly decreased the expression of both, CHOP and PERK in ASC thus protected the ASCs against ER stress-related apoptosis.

It is well known fact that during aging ROS and NO accumulates in tissues and cells including ASC, causing mitochondrial damage. For instance, Smallwood et al [65] have found an age-related increase in NO synthesis in mouse MSC-derived macrophages after lipopolysaccharide treatment. Our own studies have also shown increased accumulation of oxidative stress by ASC isolated from elderly donors [31]. Moreover, our yet unpublished data indicates that age strongly influences ASC mitochondrial dynamic and metabolic status. Thus we decided to investigate whether RES-doped scaffold affect mitochondrial fission and fusion. Both of these processes play a crucial role in maintaining healthy mitochondria during metabolic or environmental stress. Fusion helps

overcome stress by exchanging mitochondrial contents as a form of compensation while fission is needed to create new organelles and also act as a quality control mechanism allowing for damaged mitochondria removal [66]. Interestingly, RES-doped scaffolds increased the expression of both, MNF-1 and FIS-1 in ASC but only in 0.1 RES $\text{ZrO}_2/\text{SiO}_2$ coating. In scaffold doped with 0.5 μM RES both of these genes were downregulated in comparison to pure $\text{ZrO}_2/\text{SiO}_2$ surface. This indicates that RES acts in a very sensitive, dose and time dependent way on mitochondrial dynamics.

In the current research, we also investigated the amount of ROS, NO and activity of SOD in ASC cultured on $\text{ZrO}_2/\text{SiO}_2$ coated biomaterials. The data obtained clearly indicates that RES treated scaffolds significantly ameliorate the accumulation of ROS and improve the antioxidative properties of ASC. Eliminating oxidative stress may contribute to ASC rejuvenation and in consequence to their better therapeutic utility.

In addition to their differentiation potential, MSC exerts immunomodulatory properties to regulate immune response in the state of disease. For instance, MSC suppress T-cell proliferation, cytokine secretion and cytotoxicity, regulate Tregs functions and increase B-cell viability. The inflammatory environment was proven to affect immunomodulatory gene expression in MSCs or promote the cell–cell contact, resulting in increased immunosuppressive response. Thus MSC are able to modulate their effects to protect the body from disease in different situations [67]. On the other hand, RES inhibits immune cell proliferation, cell-mediated cytotoxicity, and cytokine production, at least in part through the inhibition of NF- κ B activation [67]. Since cytokines play a pivotal role in the development of immune responses, we also investigated the effect of RES doped scaffolds on the production of IL-1 β , IL-6, TNF α , IL-13 and IL-4 by ASC. Cells cultured on RES-doped scaffold were characterized by increased expression of IL-6 and increased expression of anti-inflammatory IL-13 and IL-4. Thus combining ASC and RES results in a robust expression of anti-inflammatory cytokines which is especially important if application of scaffold for in vivo tissue regeneration is considered.

5 Conclusion

In the present study, we have investigated the influence of novel, RES doped scaffold on proliferation, apoptosis and oxidative stress of ASC isolated from elderly patients. Cells cultured on RES-biomaterials were characterized

by increased proliferation, decreased apoptosis and ROS accumulation. Moreover, they secreted robust amount of anti-inflammatory cytokines. Thus exploitation of fabricated scaffold in regenerative medicine as a strategy for rejuvenate ASC from elderly patients in vivo, seems to fully justified.

Conflict of interest: Authors declare there is no conflict of interest.

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