VE TPGS-Loaded Silk Fibroin/Hydroxybutyl Chitosan Nanofibrous Scaffolds for Skin Care Application

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Abstract: Vitamin E (VE) is an ideal antioxidant and a stabilizing agent in biological membranes. In this study, silk fibroin (SF) / hydroxybutyl chitosan (HBC) nanofibrous scaffolds are loaded with VE tocopherol polyethylene glycol 1000 succinate (VE TPGS) via electrospinning. SEM images show that the average nanofibrous diameter has no significant difference when the content of VE TPGS increases to 4.0% (SF/HBC). However, the average nanofibrous diameter decreases largely to 200 nm when the VE TPGS content reaches 6.0%. Furthermore, VE TPGS presents a sustained release behavior from the nanofibrous scaffolds. Cell viability studies of mouse skin fibroblasts (L929) demonstrate that VE TPGS loaded SF/HBC nanofibrous scaffolds present good cellular compatibility. Moreover, the incorporation of VE TPGS could strengthen the ability of SF/HBC nanofibrous scaffolds on protecting the cells against oxidation stress using the Tertbutyl hydroperoxide (t-BHP) – induced oxidative injury model. Therefore, VE TPGS-loaded SF/HBC nanofibrous scaffolds might be potential candidates for personal skin care, wound dressing and skin tissue engineering scaffolds.

Key words: *vitamin E tocopherol polyethylene glycol* 1000 *succinate* (*VE TPGS*); *silk fibroin* (*SF*) */hydroxybutyl chitosan* (*HBC*) *nanofibrous scaffolds*; *resistance to oxidation*; *biocompatibility*

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Introduction

Skin acts as a barrier between the organism and the environment protecting the organism from injuries or damage, however, its functions are continuously interfered by environmental pollutants, such as ultraviolet (UV) radiation, volatile organic compound, and ozone. These result in skin disorders or diseases including skin aging and skin cancer [1-3]. Vitamin E (VE) has been found to be of high potential of antioxidant in biomembranes, particularly with respect to lipid peroxidation. The oxidation of human low density lipoprotein (LDL) is sponsored by free radical initiator, and its oxidation is inhibited by VE. Moreover, VE has excellent anti-tumorigenic and skin barrier stabilizing properties, which commonly is used for skin care products [4-6]. Compared fat-soluble VE tocopherol polyethylene glycol 1000 succinate (VE TPGS), a water-soluble derivative of VE has proved to be intracellularly hydrolyzed more easily releasing tocopherol after crossing cell membranes [7-8], making VE TPGS more suitable for skin care application.

Silk fibroin (SF) is an attractive natural fibrous protein for biomedical applications or cosmetic due to its unique properties, including good biocompatibility, biodegradability, desirable oxygen and water permeability, lower inflammatory and commercial availability at relatively lower cost [9-11]. Chitosan, a kind of natural polysaccharide, has also been widely applied in pharmaceutical, tissue engineering and cosmetic fields due to good biocompatibility, biodegradability, antibacterial activity, moisturized property [12-13]. Meanwhile, chitosan was reported to be capable of inhibiting the activity of melanin form enzyme-tyrosinase [14]. In previous study, we successfully fabricated silk fibroin (SF)/ hydroxybutyl chitosan (HBC) blended nanofibrous scaffolds to mimic the native extracellular matrix (ECM) structurally and chemically. And they presented good biocompatibility both in *vitro* and in *vivo* [15-16]. Thus, VE loaded SF/HBC nanofibrous scaffolds may be beneficial to skin care, regeneration and wound healing. In the present study, VE TPGS-loaded SF/HBC blened nanofibrous scaffolds are fabricated. Sustained release of VE TPGS from nanofibrous scaffolds, the cytocompatibility and the anti-oxidation of nanofibrous scaffolds are investigated.

1 Experimental

1.1 Materials

Cocoons of *Bombys mori* silkworm are kindly supplied by Jiaxing Silk Co., China. VE TPGS are purchased from Eurochem Asia Ltd. Each gram of VE TPGS contains VE 387 IU (260 mg). HBC is kindly provided by Shanghai Qisheng Biological Agents Co., China. Two kinds of solvents 1, 1, 1, 3, 3, 3-hexafluoroisopropanol (HFIP) from Fluorochem (United Kingdom) and trifluoroacetic acid (TFA) from Sinopharm Chemical Reagent Co., China. Tertbutyl hydroperoxide (t-BHP) are purchased from Sigma-Aldrich China Inc. L929 cells are provided by Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China).

1.2 Preparation of regenerated SF

Raw silk is degummed three times with 0.5% Na_2CO_3 solution at 100 ℃ for 30 min and then washed with distilled water. Degummed silk is dissolved in a ternary solvent system of $CaCl_2/H_2O/EtOH$ solution (1/8/2 in mol ratio) for 1 h at 70 ℃. After dialysis with cellulose tubular membrane (Sigma) in distilled water for 3 d at room temperature, the SF solution is filtered and lyophilized to obtain the regenerated SF sponges.

1.3 Preparation of VE TPGS loaded SF/HBC nanofibrous scaffolds

VE TPGS is dissolved in HFIP with different concentrations at ultrasonic treatment and then the regenerated SF sponges is dissolved into VE TPGS HFIP solution to render SF at the concentration of 12%, the content of VE TPGS is 2.0%, 4.0% and 6.0 % based on the weight of SF and HBC, respectively. HBC is dissolved in HFIP/TFA mixture (90:10) for 6% concentration, respectively. The two solutions are blended at the volume ratios of 8:2 with sufficient stirring at room temperature. The blended solution is loaded into a 2.5 mL

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plastic syringe with a blunt-ended needle with an inner diameter of 0.21 mm. The needle is located 150 mm above a grounded solid collector. A syringe pump (789100C, cole-pamer, America) is used to feed solutions to the needle tip at a feed rate of 0.5 – 1.0 mL/h. A high voltage of 20 kV is generated between the needle and ground collector using a high-voltage power supply (BGG6-358, BMEICO, China). To improve water-resistant ability, the collected nanofibrous scaffolds are treated with 75% ethanol vapor for 24 h as described before [17], and then dried under vacuum for 1 week at room temperature.

1.4 Characterization of the nanofibrous scaffolds

The morphology and diameter of the electrospun fibers are observed with a scanning electronic microscope (SEM, JSM-5600) at an acceleration voltage of 10 kV. The diameter range of the nanofibers is measured basing on the SEM images using the image visualization software ImageJ 1.34 s (National Institutes of Health) and calculated by selecting 100 fibers randomly observed on the SEM images.

1.5 Release studies *in vitro*

A certain amount nanofibrous scaffolds after treatment are dissolved in proteinase K solution (0.5 mg/mL in 12.5 mol/L Tris-HCl buffer solution, pH 8.0) at 37 ℃ for 6 h. The solution is filtrated through a 0.22 μm filter (RF-jet) and then is measured at 284 nm using a UV spectrophotometer. The actual amount of VE TPGS in the nanofibrous mats was determined from the obtained data against a predetermined calibration curve for VE TPGS. The encapsulation efficiency (E) was then calculated as follows:

$$E/\% = \frac{C_a}{C_i} \times 100, \quad (1)$$

where C_a is the calculated amount of the encapsulated VE TPGS and C_i is the initial amount of VE TPGS used for electrospinning. The release of VE TPGS from SF/HBC nanofibrous scaffolds is carried out over a period of 72 h. Samples are incubated in phosphate buffered saline (PBS) with a continuous swing of 90 r/min at 37 ℃. At a specified period between 0 and 72 h, 3 mL of the release solution is withdrawn and an equal volume of PBS is refilled. The amount of VE TPGS in the sample is determined using UV detector (284 nm).

1.6 Viability study of L929 cells on nanofibrous scaffolds

L929 fibroblasts are cultured in dulbecco's modified eagle medium (DMEM) medium with 10% fetal bovine serum and 1% antibiotic-antimycotic in an atmosphere of 5% CO_2 and 37 ℃, and the medium is replenished every 3 d. Nanofibrous scaffolds prepared on circular glass coverslips (14 mm in diameter) and cover slips are treated with 75% ethanol vapor and then fixed in 24-well plates with a stainless ring. L929 fibroblasts are seeded on the nanofibrous scaffolds and cover slips at a density of 1.0×10^4 cells/well. Initially, the volume of cells and medium is 400 μL for each well, and then 200 μL of fresh medium is added to each well every 3 d. L929 fibroblasts are up to 7 d. Cells viability on nanofibrous scaffolds, coverslips are determined by MTT method.

After 3 d of culturing, the electrospun fibrous scaffolds with cells are examined by SEM. The cell-seeded scaffolds and cover slips are rinsed with PBS twice and fixed in 4% glutaraldehyde (GTA) solution at 4 ℃ for 2 h. Fixed samples are rinsed with PBS twice and then dehydrated in graded concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%). Finally, the samples are freeze-dried overnight. The dry samples are sputter coated with gold and observed under the SEM at a voltage of 10 kV.

1.7 Antioxidation assay

L929 fibroblasts are seeded on nanofirbous scaffolds as described above and exposed to different concentrations of t-BHP (0, 50, 100, 200 and 400 μmol/L) for 24 h when reached a confluence about 90%. Then, t-BHP is removed and the wells are rinsed with PBS twice. After that, cell viability is quantified by MTT assay as described above.

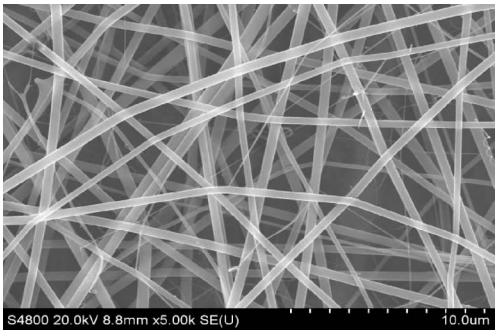
1.8 Statistical analysis

Statistical analysis is performed using Origin 7.5 (Origin Lab). Values (at least triplicate) are averaged and expressed as AD ± SD. Statistical differences are determined by one-way ANOVA and differences are considered statistically significant at $p < 0.05$.

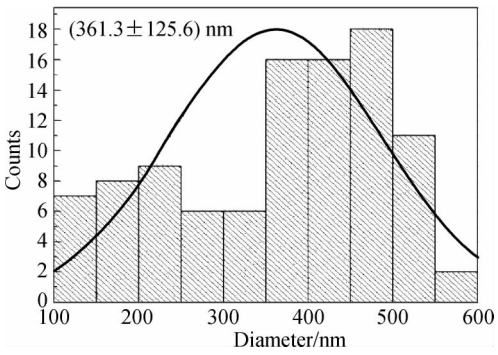
2 Results and Discussion

2.1 Morphology of VE TPGS-loaded SF/HBC nanofibers

The SEM micrographs and diameter distributions of nanofibrous scaffolds with different VE TPGS content are shown in Fig. 1. The average diameter of nanofibers with the VE TPGS content of 2.0% and 4.0% had no significant difference. However, the average diameter of nanofibers sharply decreases to 200 nm when VE TPGS content increases to 6.0%. VE TPGS consists of a big lipophilic tail (polyethylene glycol) and a hydrophobic head (tocopherol succinate) [18]. The decrease of fiber diameter might be due to the fact that big lipophilic tail (polyethylene glycol) forms strong intermolecular hydrogen bonds with SF and HBC when VE TPGS content increases to certain amount, which undermines inter reaction between SF and HBC to lead to lower solution viscosity.



(a)



(a′)

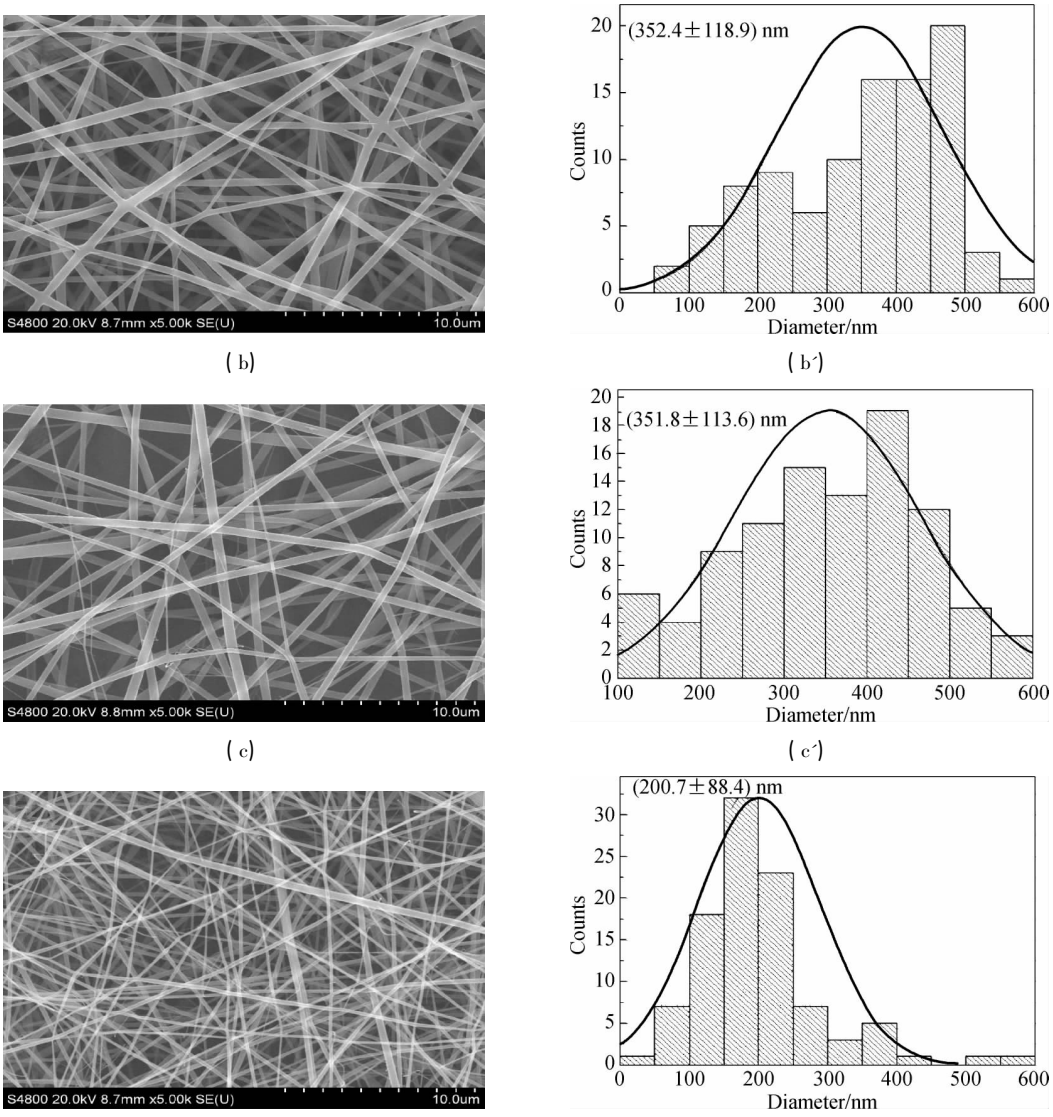


Fig. 1 SEM images and diameter distributions with different content of VE TPGS: (a) and (a′) 0.0%; (b) and (b′) 2.0%; (c) and (c′) 4.0%; (d) and (d′) 6.0%

2.2 Release of VE TPGS from the nanofibrous scaffolds in *vitro*

The actual amount of VE TPGS in nanofibrous scaffolds is determined through a certain amount of VE TPGS loaded SF/HBC nanofibrous scaffold dissolved in proteinase K solutions. The encapsulation efficiency of the scaffolds with VE TPGS contents of 2.0%, 4.0% and 6.0% are 72.53%, 74.67% and 65.02%, respectively. The cumulative release amount of VE TPGS is reported as the percentage of the actual amount of VE TPGS present in the sample. The release curves of VE TPGS are shown in Fig. 2. The release curves have no significant difference among with three VE TPGS loaded nanofibrous scaffolds. They exhibit a slightly burst release of VE TPGS during the beginning 12 h, then reach a sustain release over 72 h. However, VE TPGS presents more accumulative release when the VE TPGS content is 6.0% in nanofibrous scaffolds in comparison with 2.0% and 4.0% in nanofibrous scaffolds. This may be caused that the diameter of nanofibers containing 6.0% VE TPGS becomes thinner than 2.0% and 4.0%. Thus lead to VE TPGS quick release from nanofibers. According to previous result [15], SF/HBC nanofibrous scaffolds did not degrade in a short time. So the VE TPGS release from SF/HBC nanofibrous scaffolds mainly results from VE TPGS diffusion. The PEG lipophilic tail may form H-bond action with the amide group of SF and HBC to result in the sustained release behavior of VE TPGS from SF/HBC nanofibrous scaffolds.

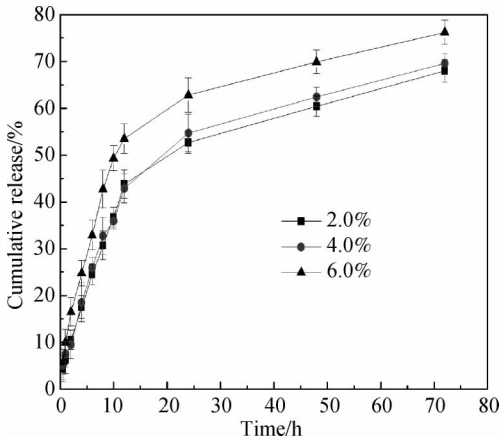


Fig. 2 Release curves of VE TPGS from SF/HBC nanofibrous scaffolds containing different amount of VE TPGS *in vitro*

2.3 Viablity of L929 cells on VE TPGS loaded nanofibrous scaffolds

VE TPGS loaded SF/HBC nanofbrous scaffolds could provide a preferable matrix for cell adhesion and proliferation by mimicking the native ECM. L929 fibroblasts are seeded to evaluate cell proliferation on the electrospun VE TPGS loaded SF/HBC nanofibrous scaffolds. The viability of L929 cells on days 1, 3, 5 and 7 after seeding on various nanofibrous scaffolds is shown in Fig. 3. Data are expressed as AD ± SD ($n=4$). Statistical difference between groups is indicated ($^{*}p<0.05$, $^{**}p<0.01$). It is revealed that all nanofibrous scaffolds have improved biocompatibility in comparison with coverslip. On day 3, proliferation on nanofibreous scaffolds increases significantly ($p<0.05$) compared to coverslips. On day 5 and 7, proliferation on nanofibrous scaffolds is also significantly higher ($p<0.01$) than on coverslips. The results show that SF/HBC and VE TPGS loaded SF/HBC nanofibrous scaffolds could promote greater cell growth and proliferation. Cell morphology and the interaction between cells and nanofibrous scaffolds are observed for 3 d, as shown in Fig. 4. Cells present fusiform spreading largely morphology on the nanofibrous scaffold and bridge each other. These data clarify that VE TPGS addition does not influence on the good biocompatibility of SF/HBC nanofibrous scaffolds, which could be benefical to the cell-cell and which could be beneficial to the cell-cell and cel-matrix signal transduction [19].

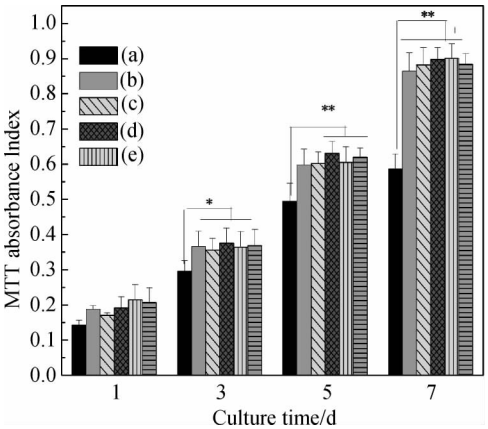
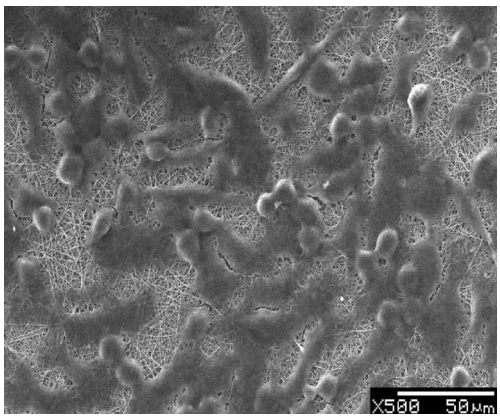
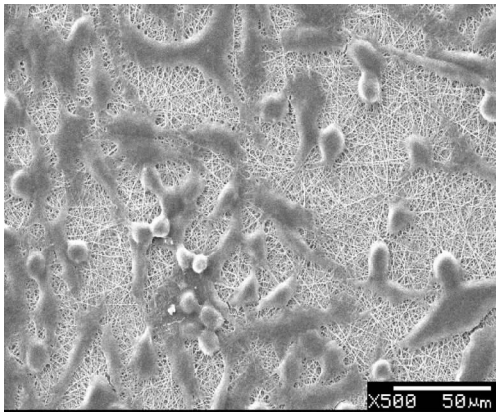


Fig. 3 Proliferation of L929 cells cultured on SF/HBC nanofibrous scaffolds with different VE TPGS content and coverslips for 1, 3, 5, and 7 d: (a) cover slips; (b) 0.0%; (c) 2.0%; (d) 4.0%; (e) 6.0%



(a)



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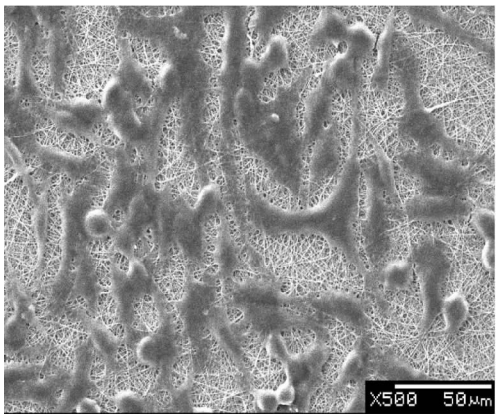

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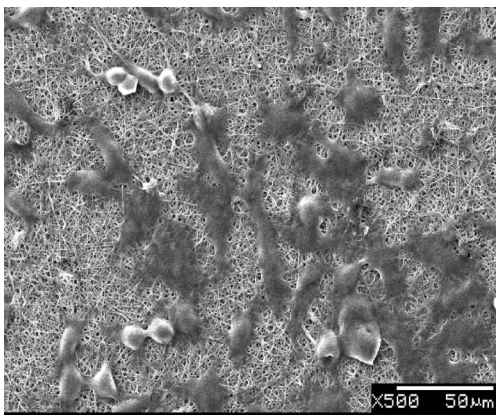

(d)

Fig. 4 SEM micrographs of L929 cells grown on SF/HBC nanofibrous scaffolds with different VE TPGS content for 3 d: (a) 0.0%; (b) 2.0%; (c) 4.0%; (d) 6.0%

2.4 Assessment of L929 cells viability on nanofibrous scaffolds under exposure to oxidative stress (OS)

The t-BHP is a potent oxidant and promote the generation of reactive oxygen species (ROS) which greatly damage cells [20]. So, the antioxidant capability of VE TPGS loaded SF/HBC nanofibrous scaffolds are evaluated using the t-BHP-induced oxidative injury model and shown in Fig. 5. The results show that t-BHP significantly affects the viability of the cells cultured on cover slips, even at lower concentration. While the viability of cells cultured on the SF/HBC nanofibrous scaffolds significant decreases when concentration of t-BHP is equal to or

greater than 200 μmol/L. This could be attributed to the tyrosine in SF possesses antioxidant function inhibiting ROS formation [21]. Moreover, chitosan could also restrain ROS formation [22]. The cells viability on VE TPGS-loaded SF/HBC nanofibrous scaffolds do not significantly decrease until the t-BHP concentration up to 400 μmol/L. The incorporation of VE TPGS is greatly beneficial to SF/HBC nanofibrous scaffolds against OS. So, VE TPGS loaded SF/HBC nanofibrous scaffolds may be a promising potential application in skin care, tissue regeneration as well as anticancer because it could significantly protect cells against damage from ROS.

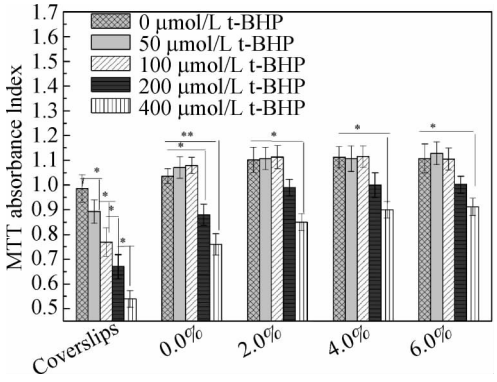

Fig. 5 The viability of L929 cells cultured on cover slips and SF/HBC nanofibrous scaffolds with different VE TPGS content exposure to t-BHP of different concentrations ($^{*}p<0.05$, $^{**}p<0.01$)

3 Conclusions

In the present study, VE TPGS-loaded SF/HBC nanofibrous scaffolds are successfully fabricated. VE TPGS exhibit a sustained release from nanofibrous scaffolds. The addition of VE TPGS into the scaffolds strengthens the survival of the cells on SF/HBC nanofibrous scaffolds against oxidative stress without compromise of biocompatibilty. These results strongly support our hypothesis that VE TPGS-loaded SF/HBC nanofibrous scaffolds have similar components and the nanometer-scale architecture of ECM and be conducive to in personal skin care, tissue regeneration.

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