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Optimizing Electros pinning Parameters of Polymeric Scaffold Using Anfis for Amd Treatment

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Abstract
Among wide range application of stem cells in tissue engineering, using them in Age-related macular degeneration (AMD) has recently payed attention. AMD is one of the retinal degenerative diseases associated with some degree of dysfunction and loss of retinal pigmented epithelium (RPE) cells and leads to permanent sight loss. In this study, ANFIS was used for optimization the electrospinning's parameters to make scaffold. For better adhesion and proliferation of cells, the polycaprolactone scaffold's surface was modified by alkaline hydrolysis. Some analyses, such as water uptake and biodegradation rate were done. Then, differentiated human embryonic stem cells (hESCs) were cultured on several groups of scaffolds. Finally, viability, proliferation, and morphology of differentiated hESC-RPE cells on all groups of the scaffolds were investigated. The nanofibers' diameter was minimized by optimization of voltage and solution concentration with fuzzy model for the first time, which obtained 110.5 nm, 19.1 kV, and 0.065 g/mL (w/v), respectively. Immersion time of scaffold in alkaline solution and concentration of solution during surface modification were achieved 104 minutes and 4.3 M, respectively. Results of the MTT assay showed that the hydrolyzed group had a high proliferation of cells. Scanning electron microscopy observation of cell morphology after two months confirmed this result. In conclusion, our results demonstrate that the hydrolyzed scaffold is a suitable bed for cell proliferation, which can be a good option for AMD treatment.
Keywords: ANFIS, Age-related macular degeneration, Polycaprolactone, Optimization, hESCs, Fuzzy model

1. Introduction
Age-related macular degeneration (AMD) is one of the age-related diseases of the retina, which leads to blindness in elderly (over 65 years of age) population worldwide (Kashani et al., 2018; Thomas et al., 2016). In AMD, retinal pigment epithelium (RPE) cells -a pigmented cell monolayer that supports the photoreceptors and sits on a pentalaminar sheet called Bruch's membrane (BM)- are degraded irreversibly (Mitchell et al., 2018; Sadda et al., 2018; Zadeh et al., 2019). The fibers' diameter of BM range from 50 to 500 nm and the membrane's thickness is less than 5μm. BM helps RPE cells maintain their monolayer structure and transfer nutrients and metabolites to and from choriocapillaris located on the other side of BM (Hotaling et al., 2016). Unlike fish and amphibians, mammals can't regenerate the damaged retina (Di Foggia et al., 2016; Shahmoradi, Hatamian, et al., 2015). Human embryonic stem cells (hESCs) are a promising source of RPE cells for treating common and incurable forms of severe visual impairment, such as AMD (Mazzilli et al., 2020; Song et al., 2015). In tissue engineering, using scaffold as a bed to proliferate and differentiate cells has gained huge attention nowadays. The scaffold consists of nanofibers that help cells proliferate and make a layer to substitute the disrupted RPE layer (Croze &

Clegg, 2014; Forest et al., 2015). Scaffolds show the advantages of surface topology in nanoscale and induce desirable cellular responses. Nanoscale topological cues are able to influence the morphology and differentiation of cell types (Tan et al., 2019).
Pennington et al. believed that the culture of stem cell-derived RPE monolayers on scaffolds is a great approach for the treatment of AMD and other retina diseases. Pluripotent stem cells can potentially be used as an unlimited source of RPE cells (Pennington & Clegg, 2016). In another study, porous honeycomb-like films were used as scaffold materials for human embryonic stem cell-derived RPE (hESC-RPE) cells. A high permeable film with dip-coating of collagen type IV fabricated the homogeneous surface. Cells were cultured on this surface and the differentiation of hESC-RPE cells was confirmed by detection of specific RPE markers. Since porous honeycomb films enabling the free flow of ions and molecules across the material, they are suggested for application in hESC-RPE tissue engineering (Maria Teresa Calejo et al., 2016). Additionally, in a study polybutylene succinate (PBSU) films were used as a polymeric surface for supporting adhesion of hESC-RPE cells. Results showed that the films' physical properties and biocompatibility are highly dependent on the adopting casting method (M. Teresa Calejo et al., 2019).
Among different polymers that are used to fabricate scaffolds polycaprolactone (PCL) is selected because of its biocompatibility, biodegradability, and high tensile strength. Electrospinning of this polymer enables us to make a scaffold with different diameters and porosity. Previous studies showed that the relationship between the voltage of electrospinning, the solution's concentration, and nanofibers' diameter is nonlinear (Shahmoradi et al., 2017). A neuro-fuzzy model is a powerful tool that can use the information from expert's knowledge, observations, and data to model a system's behavior and the relationship between its parameters (Hafizi et al., 2014). In this study, an Adaptive Neuro Fuzzy Inference System (ANFIS) structure was applied to model the relationship between the electrospinning parameters and scaffold diameter and to find the best electrospinning condition. After surface modification of the resulted scaffold using these parameters, differentiated human embryonic stem cells were cultured on this scaffold and the behavior of this scaffold was investigated.

2. Materials and methods

2.1 Designing experiments

To obtain a clear electrospinning solution, the polycaprolactone (PCL) (Mn= 80000 Da, Sigma Aldrich Co.) in pellet form was dissolved in dimethylformamide (DMF, Merck Co.) and chloroform (CHL, Merck Co.) with 2:8 volume ratio for 4 hours at room temperature with stirrer (Qin & Wu, 2012). This solution was electrospun using a device with a 10 mm diameter and 3 mL volume syringe and some adjustable parameters (Shahmoradi et al., 2017).
Since smaller diameters of nanofibers are ideal for cell culture, significant adjustable electrospinning device parameters on the nanofibers' diameter were selected. Finally, a set of experiments was designed using the response surface method (RSM). The Voltage with the range of [10, 25] kV and concentration of the solution with the range of [0.05,0.15] g PCL mL-1 were considered as significant variables in the experimental design process (Baker et al., 2016; Shahmoradi, Hatamina, et al., 2015). Other parameters such as tip to collector

distance (10 cm), solution temperature (27 °C), speed of drum (800 rpm), and rate of solution (0.6 mL/h) were assumed to be constant.
Designed experiments were performed to create the scaffolds according to each set of parameters. Then, Scanning electronic microscopy (SEM) images of fabricated scaffolds were analyzed, and the diameter of nanofibers was determined. After that, a set of input-output pairs were created to be used in neuro-fuzzy modeling of the electrospinning process.

2.2 ANFIS structure

ANFIS, one of the famous neuro-fuzzy systems, was initiated by Jang in 1993 (Jang, 1993). This system implements a Takagi-Sugeno fuzzy inference system. Assume that fuzzy inference system has 2 input, x1 and x2 and one output y. A typical rule-based of Takagi-Sugeno fuzzy inference system with m rule can be expressed as:

Rule#1: if x1 is A1 and x2 is B1 then: y1= α1 x1+β1x2+γ1
Rule#2: if x1 is A2 and x2 is B2 then: y2= α2 x1+β2x2+γ2
.Rule#M: if x1 is Am and x2 is BM then: ym= αm x1+βmx2+γm

In which A and B are fuzzy membership functions for x1 and x2 respectively and y is the output of the network which is linear combination of the inputs.
ANFIS structure is comprised of 5 layers (Chang & Chang, 2006). Thes layers are as follows:
Layer 1: This layer is the fuzzification layer in which each neuron has a membership function; the output of this neuron is the membership degree of the crisp value of the input to each membership function of the neurons.
Layer 2: The second layer neurons contain a t-norm operator such as the product operator, which calculates the product of membership degree of the inputs in their associated fuzzy membership functions as a scale of rule firing strength W_i:

$$W_i = \mu_{A_i}(x_1) \times \mu_{B_i}(x_2) \tag{1}$$

The firing strength rule is a measure of satisfaction of the antecedent part of a fuzzy rule with the input vector.
Layer 3: The third layer is the normalization layer, which normalizes each rule's firing strengths calculated in the previous layer. The normalized output of this Layer ($\overline{W_i}$) is computed as the ratio of each rule firing strength to the sum of all rules firing strength:

$$\overline{W_i} = \frac{W_i}{\sum_{i=1}^{m} W_i} \tag{2}$$

Layer 4: This layer computes the output of each rule based on Eq.3

$$y_i = \overline{W_i}(\alpha_i x_1 + \beta_i x_2 + \gamma_i) \tag{3}$$

Layer 5: This layer calculates the final output of the system with a summation of all previous layer outputs

$$y = \sum_{i=1}^{m} y_i = \frac{W_i(\alpha_i x_1 + \beta_i x_2 + \gamma_i)}{\sum_{i=1}^{m} W_i} \quad (4)$$

The structure of the ANFIS is shown in Fig. 1a.
The membership functions' parameters in the first layer, called antecedent parameters and the fourth layer ($\alpha_i, \beta_i, \gamma_i$) which are called consequent parameters, must be appropriately determined to achieve a good modeling performance. These parameters in ANFIS structure are tuned based on observed input-output pairs from the system and with the aid of a hybrid learning algorithm. The last layer parameters are tuned based on the least squares method and the first layer parameters are tuned using gradient descent and backpropagation error algorithm. More details about the hybrid algorithm can be found in (Jang, 1993).
In this study, the electrospinning voltage and the solution concentration were considered as the ANFIS model input and nanofibers'diameter was considered as the output of it. Also, three triangular fuzzy membership functions are used for each input in the first layer and the product is used as the t-norm operator. The 40 generated input-output pairs in the previous section were used to tune the ANFIS parameters with the aid of hybrid learning algorithm. 80% percent of the input-output pairs were randomly selected as the training set and 20% were selected as the test set.
After tuning the ANFIS model's parameter using training data, the input values of validation input-output pairs were fed to the ANFIS model and the outputs of the ANFIS were compared with the actual nanofibers' diameter resulted from electrospinning experiments. Fig1.b shows a perfect agreement (the coefficient of determination of train and test was 1) between the predicted value and actual values of nanofibers' diameter for all 40 experiments and its generalization performance.
Finally, the fuzzy model was used to estimate the nanofibers' diameter based on arbitrary values of the voltage of electrospinning and concentration of the solution. So, these parameters' optimum values to reach the minimum diameter of nanofibers can be determined based on this model. This optimum condition is shown in Fig. 1c.

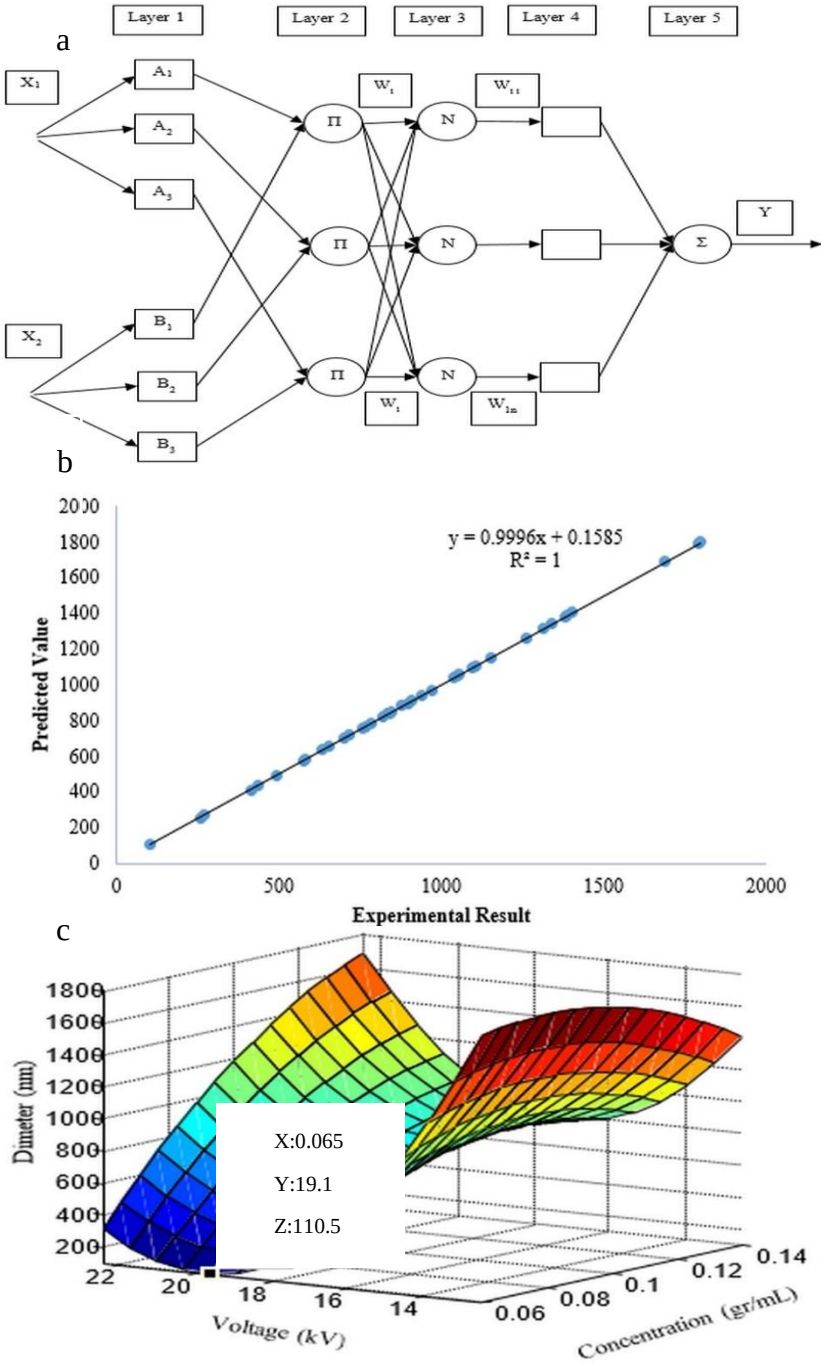

Fig. 1: a) The structure of the ANFIS, b) Predicted values versus experimental results of electrospinning process, c) 3D surface related to nanofiber diameter, solution concentration and voltage. The optimum condition was obtained which was concentration of solution=0.065 g PCL mL-1 solvent, voltage=19.1 kV and diameter of fibers=110.5 nm.

2.3. Surface modification and optimization

PCL is a hydrophobic polymer which needs to be hydrophilic to improve cell adhesion and proliferation. Alkaline hydrolysis is a method for increasing hydrophilicity of PCL which hydrolyses ester bonds and creates hydrophilic carboxylic groups. Hence, sodium hydroxide (NaOH, Merck Co.) was dissolved in distilled water and alkaline solution made. A set of experiments was designed to reach the optimized conditions of concentration of alkaline solution (with the range of 0.5-6.5 M) and exposure time of scaffolds in alkaline solution (with the range of 30-150 min). Ultimately, treated scaffolds were rinsed three times with distilled water to return pH 7 (Shahmoradi, Hatamina, et al., 2015).

Contact angle measurement was the evaluation method for finding the optimum condition of alkaline hydrolysis which was used. Four µl volume of deionized water was dropped on the surface of treated scaffolds and the contact angle of water was measured with an optical bench-type contact angle goniometer (Dataphysics, CA 15 plus) (Kosorn et al., 2013). To investigate the effect of using two types of treatment on cell culture, plasma treatment was carried out in low pressure radio frequency (RF) oxygen discharge. So, after hydrolyzing scaffolds some samples were put in chamber and treated at 150 W for 15 min (Uppanan et al., 2015).

2.4 Water adsorption

The amount of water trapped by the scaffolds was determined as $\frac{w_2 - w_1}{w_1}$ where w_1 and w_2 are weight of dried scaffold and weight of soaked one in phosphate-buffered saline (PBS) overnight, respectively (Qi et al., 2016; Zhu et al., 2002). In this analysis, scaffolds were cut in to 1×1 cm2.

2.5 Degradation rate

Biodegradability of scaffolds was investigated with immersing them in 1 mg lysozyme/mL solution in PBS (pH = 7.4) for 1, 4, 7, 14, 21 and 28 days in an incubator at 37℃ (Zhou et al., 2013). Scaffolds were removed, washed 3 times with distilled water, dried and weighed (m_f). So, percentage of degradation (PD) was obtained from difference of weight ($m_i - m_f$) dividing to initial weight (m_i) (Cummins et al., 2015).

2.6 MTT analysis

In our previous work (Zahabi et al., 2012), we differentiated hESCs to RPE cells and they were used in this study. To investigate cell viability and proliferation on different groups of scaffolds, cells were seeded on sterilized scaffolds at a density 5×104 cell mL-1 and 200 µL fresh medium was added to the plate. The groups were control (plate without scaffold, TCP), un-treated PCL scaffold (UPCL), hydrolyzed scaffold (HPCL) and plasma on hydrolyzed scaffold (PHPCL). After 1, 4 and 7 days of incubation, media was removed from each well and 200 µL MTT was added and incubated in 37℃ and 5% CO2 for 3-4 hours till purple formazan crystals were formed due to reduction of MTT by viable cells. Then, 200 µL DMSO was added in to each well to dissolve the formazan crystals and culture plates were

shake in incubator for 20 min. After a while, absorbance of each well was read with a microplate reader at 570 nm (Gautam et al., 2013; Tezcaner et al., 2003)

2.7 Scanning electronic microscopy (SEM)

In order to evaluate cell morphology on the scaffolds after 60 days, medium was removed and scaffold were fixed in 2.5% glutaraldehyde for 2 h. Next, samples were dehydrated through a series of graded ethanol solution and dried in ambient air. Ultimately, the scaffolds were coated with gold and used for SEM (Thumann et al., 2009).

2.8 Statistical analysis

In the present study, the data were presented as mean ± standard error of the mean (SEM). Each experiment was performed three times to be calculated by one-way analysis of variance (ANOVA) followed by Tukey's multi-comparison test to analyze the statistical difference ($p < 0.05$) between groups. In all tests, the significance level was set as $p < 0.05$.

3 Results and discussion

3.1 Scaffold fabrication and optimization

The optimized dimeter of scaffold which was obtained by fuzzy system, was 110.5 nm. In the optimized situation, solution concentration and voltage were 0.065 g PCL mL-1 solvent and 19.1 kV, respectively. Xiang et al. used three different scaffolds (PCL, PCL/silk fibroin and PCL/silk fibroin/gelatin) to cultivate hESC-RPE cells that they had 157, 154 and 253 nm diameters, respectively (Xiang et al., 2014). In Park et al. study, the average diameter of nanofibers was calculated 2 µm which is too high for AMD treatment (Park et al., 2007).
The thickness of nanofibers was obtained 15±2 µm and the porosity was calculated 87%. McHugh et al. used porous polyester transwells and porous PCL as scaffolds. Their scaffolds had 64% and 90% porosity, respectively. Their results showed that the porosity of scaffold had high effect on cell proliferation after 8 weeks (McHugh et al., 2014). Also in Xiang et al. study, porosity was calculated 85%, 87% and 90% for PCL, PCL/silk fibroin and PCL/silk fibroin/gelatin scaffolds, respectively (Xiang et al., 2014).

3.2 Surface treatment and optimization

Contact angle for PCL scaffold without surface modification was measured 125.08±1.76 degree that shows high hydrophobicity of this scaffold and need of surface modification (Xiang et al., 2014).
Time and concentration of alkaline hydrolysis analysis were optimized based on minimum contact angel (4.3 M for concentration of alkaline solution and 104 mins for time of immersing scaffold in alkaline solution). Alkaline hydrolysis was led to reduction in contact angle in comparison to the contact angle of un-treated PCL scaffolds and surface allowed a drop of water to spread out that means hydrophilicity of treated surface. Additionally, plasma radiation helps scaffold to have a more water-friendly surface. Based on the measurements, the contact angle for HPCL and PHPCL groups were 14.65±0.4 and 3.98±0.02, respectively.
Guo et al. showed that alkalin hydrolysis treatment of porous poly (L-lactic acid) (PLLA) scaffolds decreases the water contact angle. So that, PLLA scaffold without surface treatment

had almost 80-degree water contact angle and after treatment it was reduced till 75 degree (Guo et al., 2013).

3.3 water adsorption

Calculation of water adsorption showed that with surface modification of scaffold, amount of hydrophilicity and water uptake will be increased. Hydrophilicity improve water adsorption of scaffold, so the weight of scaffold will be increased. Therefore, using two methods simultaneously for surface modification because of having more hydrophilic groups, have more effect. Surface treatment had positive effect on the amount of water adsorption. So that, PHPCL group which was modified by two types of method had higher percentage of water adsorption, such that they were 43, 64, 87 for UPCL, HPCL and PHPCL, respectively.
This was proved by the study of Abedalwafa et al. which showed amount of water adsorption will be increased by using surface modification (Abedalwafa et al., 2013). In Guo et al. study, surface alkalin hydrolysis treatment of porous PLLA scaffold increases the water absorption rate which indicates that the hydrophilicity of PLLA has been enhanced significantly (Guo et al., 2013).

3.4 Biodegradability

Fig. 2 shows that treating the surface of scaffold, leads to high degradation rate during 28 days. So, using two types of modification seems to effect on weigh reduction. This happens due to increase in hydrophilicity and higher water uptake (Fig 2).
According to the Fig. 2, weight of scaffold will be decreased during 28 days. At first, since surface treatment is associated with weight reduction, PHPCL group had the lowest weight comparing to HPCL and UPCL group.

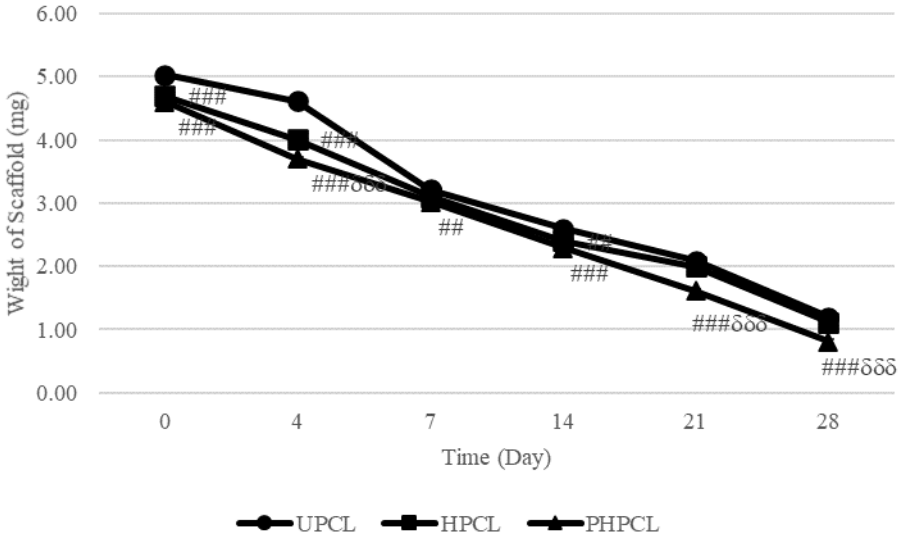

Fig. 2: Biodegradation rate of different groups of scaffolds. Weight of scaffolds were reduced during a month. Accordingly, surface modification has positive effect on degradation rate which is vital in in vivo. So that, PHPCL has higher biodegradability in comparison with HPCL Difference between UPCL and other groups is significant at P<0.01 (##), P<0.001 (###), and difference between HPCL and PHPCL is significant at P<0.001 (δδδ).

Sant et al. used surface treatment for scaffolds which were made by PCL and PGS (2:1 ratio). It had suitable effect on degradation rate of them compare with scaffolds without surface modification. So that, biodegradation in 0.1 mM concentration of alkaline solution, revealed 2-fold faster degradation of PCL-PGS scaffolds compared with untreated scaffolds (Sant et al., 2013).

3.5 Biocompatibility

For investigation of cell viability and cell proliferation, MTT assay was used. As shown in Fig. 3, cell viability on TCP and UPCL scaffold was lower than treated scaffolds. HPCL scaffold have higher incretion in comparison with PHPCL group.

At the first day, all groups of scaffolds had a little more cell viability in comparison with TCP. By passing the time this difference became higher and the impact of using scaffold for cell proliferation became more apparent. According to the fig., using two types of surface modification simultaneously doesn't lead to higher cell viability and cell proliferation. This may be because excessive hydrophilicity of the scaffold's surface results in cell non-adhesion, thus compromising cell survival. So, it seems that HPCL group is a better choice for cell attachment, viability and proliferation.

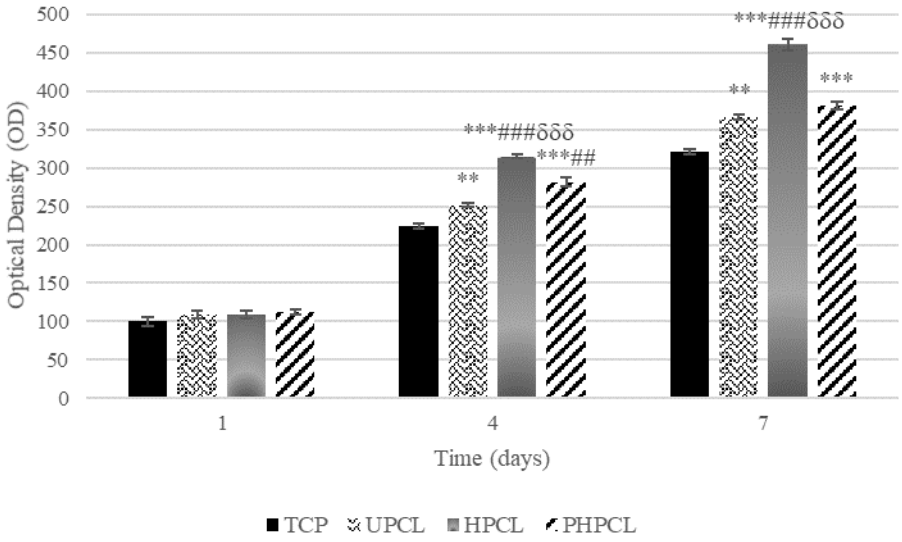

Fig. 3: MTT analysis of scaffolds during 7 days. It can be seen that surface treatment had positive effect on cell viability and cell proliferation. Moreover, HPCL group had higher OD in comparing with PHPCL group. Difference between TCP and other groups is significant at P<0.01 (**) and P<0.001 (***), difference between UPCL and other groups is significant at P<0.001 (###), and difference between HPCL and PHPCL is significant at P<0.001 (δδδ).

Similar studies with chondrocyte cells culturing on PCL scaffolds showed that using surface modification (both hydrolysis and plasma) have suitable effects on cell proliferation after 21 days (Janvikul et al., 2013). Also, Park et al. soaked PCL scaffolds in 1 N NaOH for an hour at room temperature and after preparation of scaffolds for cell seeding, osteoblast cells were cultured on scaffolds with and without surface modification. The results showed that treated PCL scaffolds, in comparison with un-treated ones, had higher cell viability after 28 days. This tendency was because of the enhanced surface wettability and hydrophilicity of the

surface-hydrolyzed fibrous scaffolds are main contributors to induce the effective cell-scaffold interaction as well as cell growth (Park et al., 2007).

3.6 Morphology and cell adhesion

In order to investigation of cell morphology and adhesion on the surface of different groups of scaffolds, SEM images were prepared after 60 days. No cell was observed in UPCL and PHPCL (Fig. 3). As it can be seen in Fig. 5, not only cells keep their hexagonal (Fig. 5a) and stretched morphology after 60 days, but also microvilli on their surface is obvious in HPCL scaffolds (Fig. 5d). It means that high hydrophobicity of UPCL scaffolds, caused cell death. Additionally, using two kinds of surface treatment simultaneously, leads to high hydrophilicity of the surface that not allowed cells to adhere well. These findings were observed and proved in all three replications for all groups and only one of SEM images of groups are shown in Fig. 4 and Fig. 5.

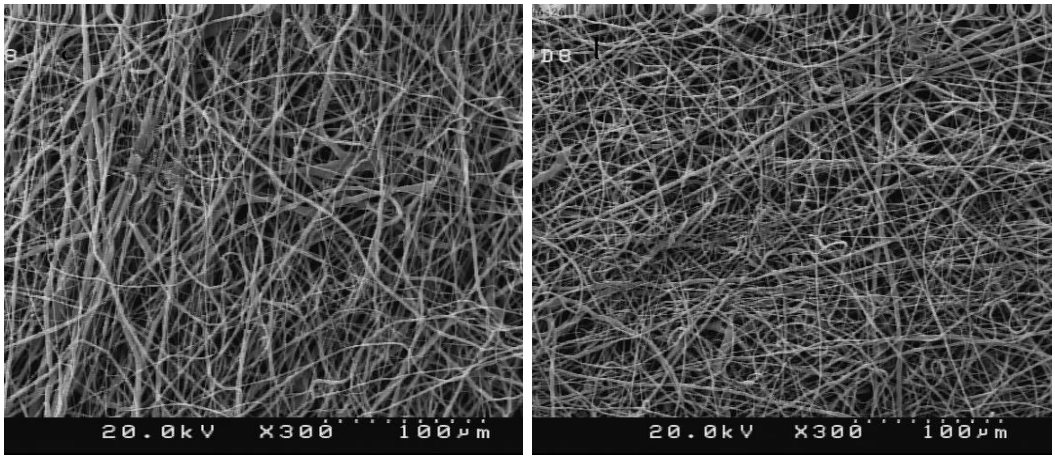

Fig. 4: No cell was seen in UPCL (a) and PHPCL (b) after 60 days by SEM. It seems that both groups are not good choice for cell proliferation and making a layer with the aim of AMD treatment.

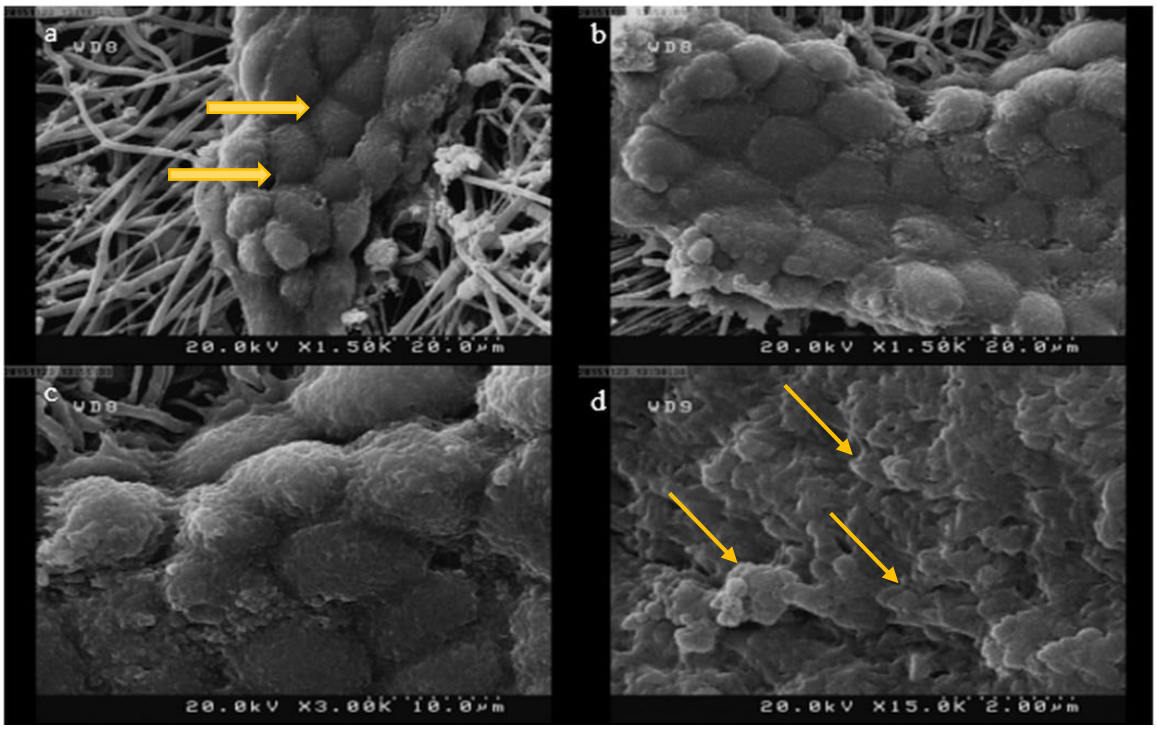

Fig. 5: a,b) Cell morphology and formation of RPE layer on HPCL scaffold. As shown by two arrows in (a), hESC-RPE cells have hexagonal morphology. Additionally, cell layer formation can be seen in (b). c, d) Microvilli of cells with different scales (10 µm and 2 µm, respectively) on HPCL scaffolds interestingly are able to be seen by three arrows in (d) in larger scale rather than (c).

The effect of surface treatment (air plasma treated for 2 min at a power of 80 W) of commercially available polyurethanes was investigated by Williams et al. Morphological assessment of cells demonstrated that on the untreated substrates the cells which adhered to the surface were not well spread. While, the cells on treated surfaces were well spread (Williams et al., 2005). Tezcaner et al. studied the effect of different situation of plasma radiation (100 W, 10 min and 100 W, 20 min) on cell culture of bed. During the first 4 h in culture, the cells that were just weakly attached to the beds had a round shape. They observed an improvement in the adhesion of RPE cells onto poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) films after surface modification. Additionally, the percent of spread cells within a population was highest in 100 W, 10 min plasma treated films (Tezcaner et al., 2003).

4 Conclusion

Electrospun PCL scaffolds were optimized with fuzzy model for the first time in order to reach minimum diameter. During incorporation of scaffold for AMD treatment, cell adhesion and proliferation are the most important parameters. To enhance the mentioned factors, surface treatment of PCL was performed. Water uptake, porosity and biodegradability of treated scaffolds were increased significantly. Culturing hESC-RPE cells on all groups of scaffolds had different results. MTT analysis showed that UPCL had no significant difference with TCP. While treated scaffolds had better cell viability and proliferation during

a week. Additionally, SEM images of cells on scaffolds, showed that HPCL was the most suitable scaffold for cell proliferation after 60 days. The main reason we did not observe any cell on PHPCL is its high hydrophilicity of this scaffold which leads to lower cell adhesion and cell viability. Based on the results, it seems that HPCL scaffolds can be a good choice for RPE cell attachment and proliferation which can be used in AMD treatment.

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