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The effect of polycaprolactone/graphene oxide electrospun scaffolds on the neurogenic behavior of adipose stem cells

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ABSTRACT

Stem cell destiny can be controlled with scaffold biomaterials in tissue engineering and regenerative medicine. This study aimed to investigate the neuronal differentiation potential of human adipose tissue-derived mesenchymal stem cells in graphene nanofiber matrix in vitro. Stem cell isolation was performed from adipose tissue taken from human by mechanical and enzymatic methods. The differentiation potential was examined after incubation of adipose stem cells in normal medium and neural differentiation medium, on graphene oxide (GO) and polycaprolactone (PCL) composite scaffolds produced by electrospinning technique. In vitro studies indicated that the presence of GO in PCL scaffold increases an effect on cell attachment, proliferation, infiltration into the scaffold, and neuronal differentiation. Also, unlike subcutaneous tissue, it has been shown immunohistochemically that mesenchymal stem cells derived from epidural adipose tissue tend to differentiate into oligodendrocytes.

1. Introduction

The central nervous system has limited regenerative capabilities in response to degenerative diseases and traumatic injuries. Stem cell-based regenerative applications have shown great promise, however low survival rate upon transplantation is still an obstacle for studies to the regeneration of neuronal tissues [1]. Mesenchymal stem cells (MSCs) differentiation capability into cell types of all three germ layers allows the utilization of MSCs as a replacement therapeutic source for various diseases, and especially neurogenic property and immune-modulatory effects make them an option for neurological disorders [2]. Adipose-derived stem cells (ADSCs) were introduced in 2001 by Zuk et al. [3] as an abundant alternative source of stem cells isolated from adipose tissue, morphologically, and phenotypically similar to MSCs. A wide range of natural and synthetic biomaterial scaffolds have been developed that mimic the cellular microenvironment to prevent diminished viability of stem cells after transplantation into the damaged area, promote cellular proliferation, and provide rapid, easy, and precise implantation of stem cells. In tissue engineering, scaffolds are used not only as extracellular matrix template for transplantation, but also they induce differentiation of stem cells toward the desired type of cells with an appropriate composition, mechanical and physicochemical properties [4–7]. Recently, graphene and its derivatives, which are frequently used in tissue engineering due to their advantageous chemical, electrical, and mechanical properties, have been shown to guide the differentiation of stem cells into neuronal cells [1,8].

Graphene is a 2-dimensional material consisting of a single layer of carbon atoms arranged in a hexagonal honeycomb lattice [9]. Graphene has several outstanding properties [10,11], and graphene-family nanomaterials such as graphene oxide (GO) and reduced graphene oxide (rGO) in particular are defined as new biocompatible materials for a wide range of biomedical applications [9,12]. Graphene is a biomaterial used to stimulate the regeneration of excitable cells such as nerve and muscle cells, thanks to its superior electrical properties. This biomaterial is promising for an electro-scaffold design that can provide electrical stimulation [8,13]. Reports published in recent years have clearly shown that graphene and related substrates are excellent nanoplatforms to support the adhesion, proliferation, and differentiation of various stem cells [8,14–16]. Polycaprolactone (PCL), a plain synthetic biodegradable aliphatic polyester, has a formable structure in different forms,

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excellent thermal stability, and Food and Drug Administration (FDA) approval and is a cheap biomaterial that can be mixed with other biomaterials [17–20]. Nanofiber structures created with PCL have a large surface area, high porosity, and small pore diameter, and in this way, they stimulate optimal cell growth by mimicking natural extracellular morphology [21].

In this study, we aimed to examine the neuronal differentiation potential of ADSCs, which were isolated from human subcutaneous and spinal epidural adipose tissues, in vitro on nanofiber scaffold produced from GO and PCL.

2. Materials and methods

2.1. Preparation and characterization of scaffolds

GO was synthesized using a modified Hummers method reported by a previous study [22]. 10 wt% PCL was dissolved in a mixture of DMF/THF 1:1 (v/v) and magnetically stirred until a homogenous solution was obtained. The GO in a mixture of DMF/THF 1:1 (v/v) dispersion was added into the PCL solution to give a PCL + GO mixture with the GO concentration of 0.1 wt% and sonicated for at least 30 min with an ultrasonic homogenizer to uniformly disperse GO particles. The solutions were placed into a 10 ml plastic syringe with the 20-gauge needle. The electrospinning process was performed at a 12 cm needle to collector distance, an applied voltage 14–18 kV and a flow rate of 1.5–3 ml h^{-1}. The fiber diameter and surface morphology of electrospun scaffolds were studied by scanning electron microscopy (EVO MA-10). The fiber diameter was measured using Image-J software.

2.2. Isolation, phenotypic characterization and differentiation potential of human subcutaneous and epidural derived adipose stem cells (ADSCs)

Human subcutaneous and epidural adipose tissue was obtained from a lumbar spinal operation performed at Marmara University Pendik Education And Research Hospital (Istanbul). Previously the Human Research Ethics Committee of Marmara University approved (protocol number: 09.2018.240) this study following in accordance with the Declaration of Helsinki. Patients were asked to fill informed consent form before planned lumbar spinal surgical operation. Adipose tissue specimens were washed twice with phosphate-buffered saline (PBS) to remove extra blood, small vessels, and connective tissues. After digestion with 0.075% collagenase type II (Biochrom), under agitation for 60 min at 37 °C, filtration and centrifugation at 600 g were performed, and the stromal vascular fraction (SVF) obtained. The supernatant was then discarded, and the cell pellet was resuspended in DMEM medium (Invitrogen,), supplemented with 10% fetal bovine serum (FBS; Invitrogen,), and 1% antibiotic/antimycotic (Invitrogen,). Culture medium was changed 48 h after initial plating and every 3 days after that. Subcutaneous ADSCs were selected by plastic adherence and harvested (P0) 5 days after isolation at 90% confluence.

Isolated subcutaneous and epidural derived adipose stem cells (ADSCs) were characterized using cell surface markers by flow cytometry analysis. Briefly, 1×10^5 cultured subcutaneous ADSCs at 3rd subculture resuspended in buffer (3% BSA (bovine serum albumin) in PBS (phosphate buffered saline)) and then appropriate fluorescein isothiocyanate (FITC), phycoerythrin (PE), and PECy5 conjugated primary antibodies for 30 min at 4 °C. CD34-PE and CD45-PECy5 as a negative control, CD90-FITC and CD105-FITC as positive control and isotypic control were used and afterwards, labelled epidural ADSCs were analyzed on a FACs Calibur Flow Cytometer (BD Biosciences).

2.3. Cell viability (MTT)

PCL and PCL + GO composite scaffolds were sterilized by UV light irradiation for 3 h and placed at the bottom of 96-well cell culture plates. Subcutaneous and epidural ADSCs suspension ($5x10^3$ cells/well) seeded scaffolds were cultured for 1, 4, and 7 days at 37 °C in a humidified incubator with 5% CO_2 (NuAire). At the same time, monolayer cell cultures were incubated with the same number of cells in 150 μl as a control. To investigate cytotoxicity at a given time point, MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Glentham Life Sciences) cytotoxicity assay method was used according to manufacturer's protocol. The absorbance value of the cytotoxicity test was measured at 570 nm wavelength (690 nm as Ref. value) in ELISA reader (Enspire, Perkin Elmer). The assay was studied 3 times, and an average of results was considered as the final result.

2.4. Neuronal differentiation on composite scaffolds

According to the cell viability procedure, the media were changed one day after the cells were seeded onto the scaffolds in 96 well plates. DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, a normal growth medium, was added to the control group wells. For neural differentiation group, cells were incubated differentiation medium (DMEM supplemented with 1% penicillin/streptomycin, 0.5 mM isobutylmethylxanthine, 10 ng/ml brain-derived neurotrophic factor (BDNF), 10 ng/ml epidermal growth factor (EGF), 10 ng/ml basic- fibroblast growth factor (BFGF), and 20% neural stem cell proliferation supplement) for 10 days.

2.5. Cell staining for fluorescent microscopy and confocal microscopy

Nuclei were stained with 4 ', 6-diamidino-2-phenylindole (DAPI) (ProLong Gold antifade reagent with DAPI, Invitrogen, CA, USA) (0.2 μg / mL). The cell suspension ($5x10^3$ cells/well) were incubated on each type scaffolds in 96 well plates with standard cell seeding procedure. After 1, 4, and 7 days, the growth medium was discarded, and scaffolds were washed with 100 μl pre-warmed PBS three times. Following, the cells were initially fixed with 4% formaldehyde (Sigma) at room temperature for 15 min, washed, and incubated with 0,1% Triton X-100 (Merck) to increase permeability for 10 min. Further 1/5000 DAPI (Invitrogen) was added on each sample and kept for 5 min at room temperature to stain the nucleus of the cells. Afterwards, DAPI solution was discarded, the scaffold was placed between the slide and coverslip and results were observed and captured under Florescence inverted microscope (Leica).

Neurogenic differentiation on biomaterials was performed by immunocytochemical staining with neuronal markers such as nestin, MAP2, GFAP, and CNPase in confocal microscopy (Zeiss LSM 700). After 10 days of culture by neuronal differentiation induction, 96 well plate wells were washed with PBS solution and cells were fixed with 4% formaldehyde in PBS for 15 min. After that, cells were washed twice with PBS for 10 min and permeabilized with 0.5% Triton X-100 (20 min), followed by three washes with PBS alone. After washing, it was blocked with PBS containing 4% bovine serum albumin (BSA) for 20 min. Then cells were incubated overnight at 4 °C with primary antibodies (anti-nestin, anti-GFAP, anti-CNPase, and anti-microtubule-associated protein-2 (MAP2) (1: 200, ABclonal, MA, USA)). Following incubation with primary antibodies, goat anti-rabbit DyLight 594 (1: 500; Thermo™) was incubated secondary antibodies for 1 h at room temperature.

2.6. Scanning electron microscopy (SEM) analysis

Morphological analyzes of composite scaffolds were characterized using a scanning electron microscopy (SEM, EVO MA-10, Zeiss, Oberkochen, Germany). After 1and 7 days of incubation, samples were fixed with 2.5% glutaraldehyde (Sigma, St. Louis, MO, USA) for 2 h and then dehydrated through serial dilutions of ethanol (30%, 50%, 70%, 90%, and 99%). The as-fixed samples were examined using SEM analysis to study cell adhesion. The samples were sputter-coated with gold (SC 7620 model, Quorum Technologies, Sacramento, CA, USA) for 90 s and

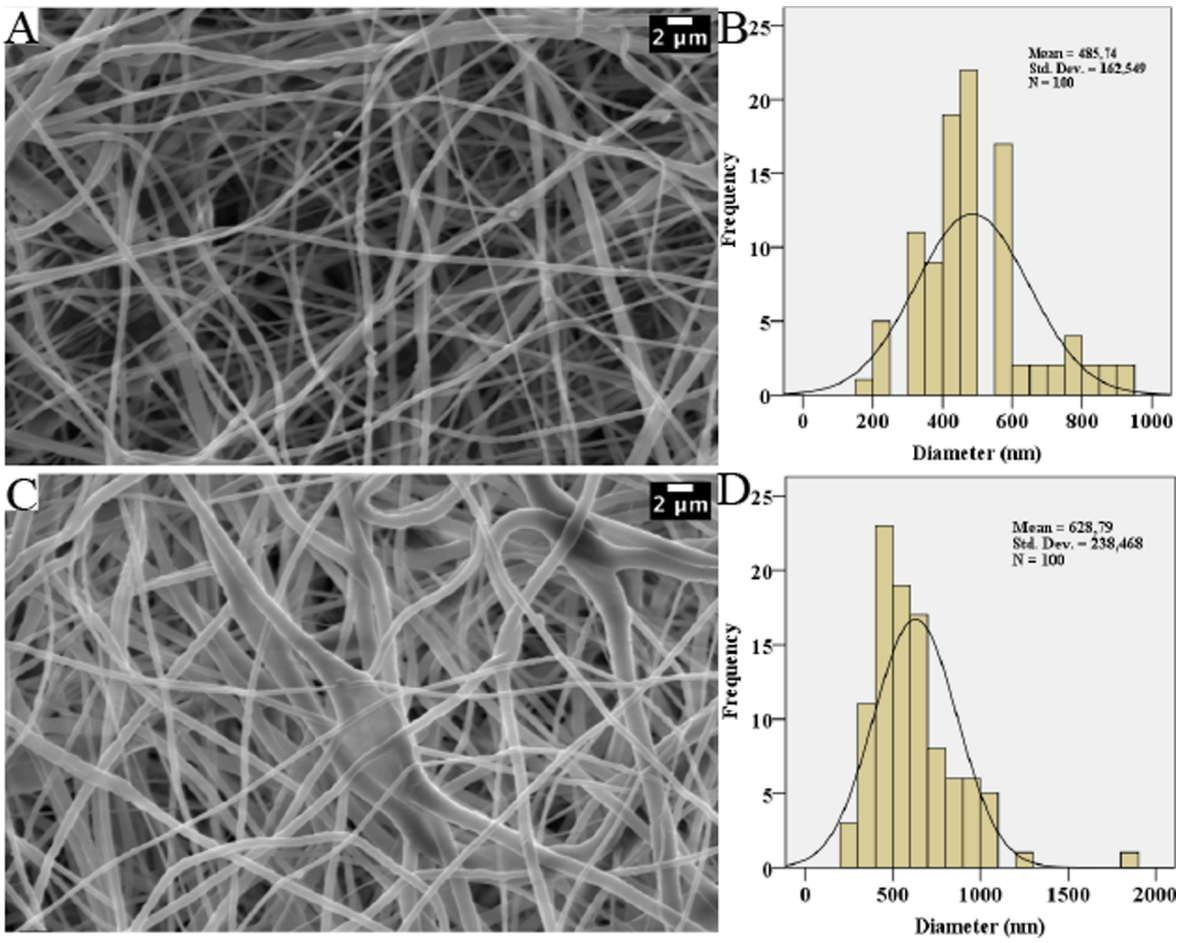

Fig. 1. SEM images PCL (A) and PCL + GO (C). Fiber diameter distribution of PCL (B) and PCL + GO (D).

observed by SEM (EVO MA-10, Zeiss, Oberkochen, Germany) with an accelerating voltage of 10 kV.

2.7. Statistical analysis

The data were normally distributed and were expressed as M ± SD of the mean. Differences between the groups were determined by one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A P-value of<0.05 has been accepted to be statistically significant.

3. Results and discussion

PCL and PCL + GO scaffolds were fabricated by electrospinning method. Fig. 1A and 1C represent the SEM images of PCL and PCL + GO scaffolds, respectively. PCL and PCL + GO fiber diameter were estimated to be 485 ± 162 nm (Fig. 1B) and 628 ± 238 nm (Fig. 1D), respectively. Fiber morphology and arrangement influence cell morphology and adhesion and cell activity [23,24]. Several researchers have studied the link between the electrospun fiber diameter and the cell behavior, but have not yet reached a clear consensus. NIH 3 T3 fibroblasts cells seeded on polycaprolactone scaffolds with fiber diameters of ~ 428 nm were found to support significantly increased cell proliferation and cell spreading [24]. Hippocampus-derived adult neural stem cells (rNSCs) cultured on lignin-coated polyethersulfone (PES) fibers with diameters of ~ 283 nm showed increased proliferation and spreading compared to cells cultured on larger fibers (749-nm and 1452-nm) [23].

The mesenchymal nature of the isolated subcutaneous and epidural adipose stem cells was confirmed before creating the scaffolds. Flow cytometry analysis resulted that subcutaneous ADSCs expressed a low percentage of CD 34 and CD45. In contrast, the typical surface markers of stem cells, CD90 and CD105 were highly expressed and differentiated three different lineages in subcutaneous and epidural ADSCs, showing to meet the standards established by the International Society for Cellular Therapy (Supplementary Fig. 1) [25].

Neural, mesenchymal, embryonic, and induced pluripotent stem cells were used as stem cells in cellular-based neuroregeneration studies. However, there are obstacles in front of the clinical uses of the embryonic stem cells due to the long time to obtain, the induced pluripotent stem cells due to the risk of tumorigenesis [2,26], and the neuronal stem cells due to technical and ethical difficulties [27]. On the contrary, MSCs stand out with some advantages: their ability to differentiate into neuronal cells by transdifferentiation, being autogenous or allogenated, easy to obtain, and supporting regeneration by paracrine factors and immunomodulation [28]. In particular, ADSCs formed the basis of our study, since they can be received more efficiently and in larger amounts than other MSCs [29].

A very recent study has also shown that due to its anatomical proximity, the neuronal differentiation capability of epidural ADSCs is more than ADSCs isolated from other anatomical regions [30]. So, we have also used epidural ADSCs for this reason and also subcutaneous ADSCs due to their easy acquisition. According to the criteria proposed by ISCT [31], we proved that the cells isolated from the epidural and subcutaneous adipose tissues were ADSCs by determining the surface markers of CD90(+), CD105(+), CD34(-), and CD45(-) in flow cytometry of the cells shown to differentiate into different cell lines (adipogenic, osteogenic, and neuronal) upon adhering to the plastic surface.

Nanofiber scaffolds produced by electrospinning are used in neural tissue engineering due to their advantageous properties such as their

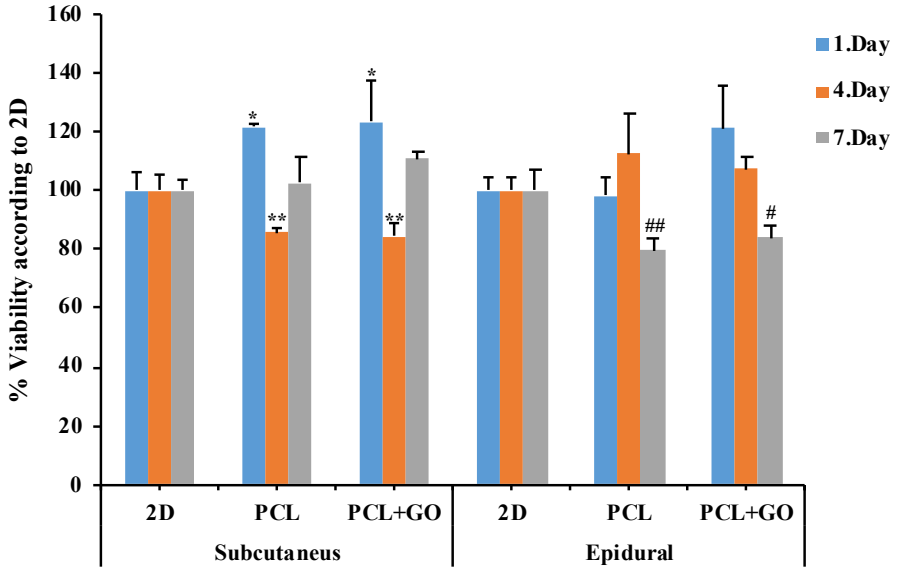

Fig. 2. Percentage of cell viability at 1, 4, and 7 days after culturing onto 2D (control), PCL and PCL + GO scaffolds, *p < 0,05, **p < 0,01versus subcutaneus 2D; $^{\#}$p < 0,05, $^{\#\#}$p < 0,01 versus epidural 2D. Data are the mean ± S.E.; n = 3; ANOVA, Tukey–Kramer multiple comparison test.

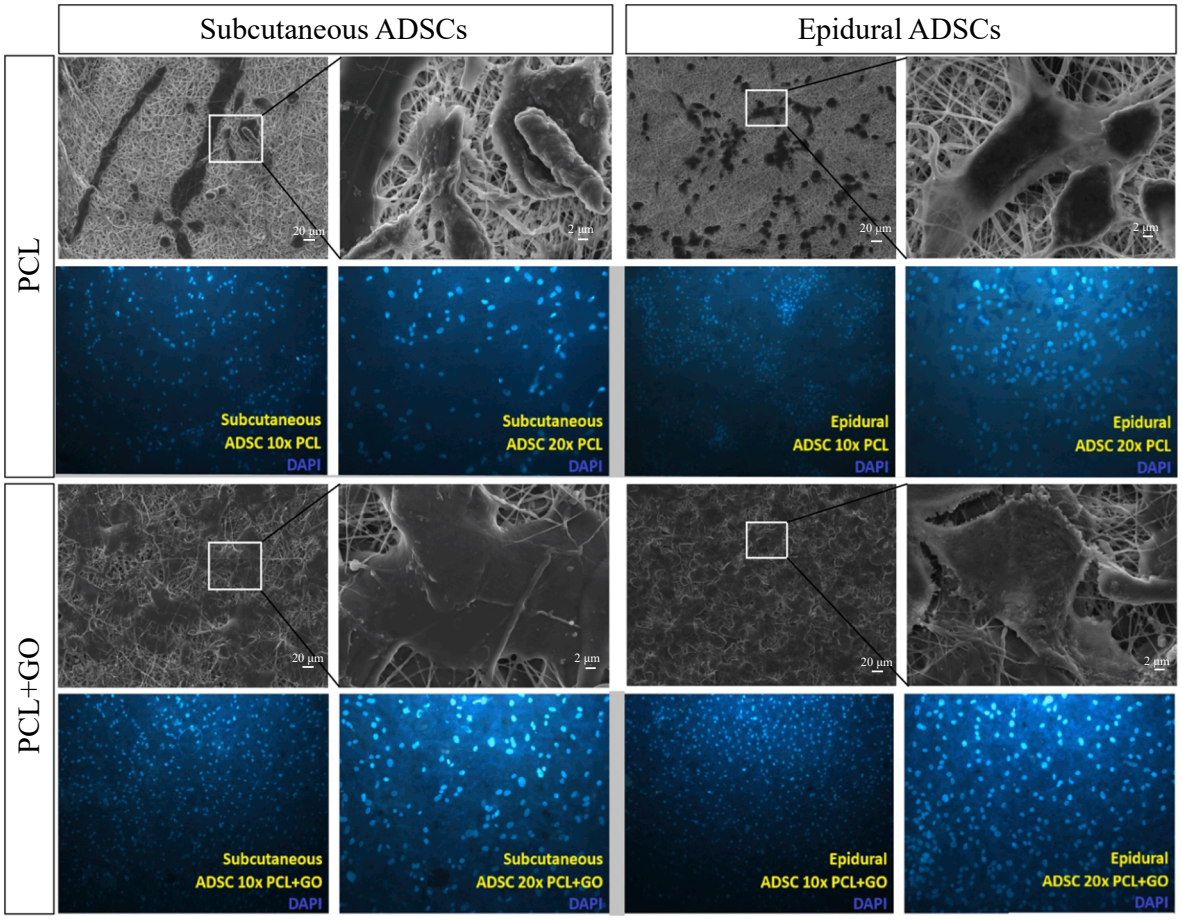

Fig. 3. SEM and Immunofluorescence images after DAPI staining of subcutaneous and epidural ADSCs on PCL and PCL + GO scaffolds, after 1 day incubation.

high degree of porosity, large surface area, and structural resemblance to the extracellular matrix [20,32]. PCL, which can also be used in clinical applications, is used in the production of these nanofiber scaffolds and these scaffolds are coated with GO. The GO-based biomaterials have more positive contributions to neuronal differentiation on ADSCs than rGO [14,29]. Also, in a study conducted on neural stem cells, selectively differentiation into oligodendrocytes was higher in scaffolds coated with GO at a concentration of 1 mg/ml [1]. In line with this information, the same scaffolds with the same concentration of GO were used in our study. We have also shown that these two biomaterials do

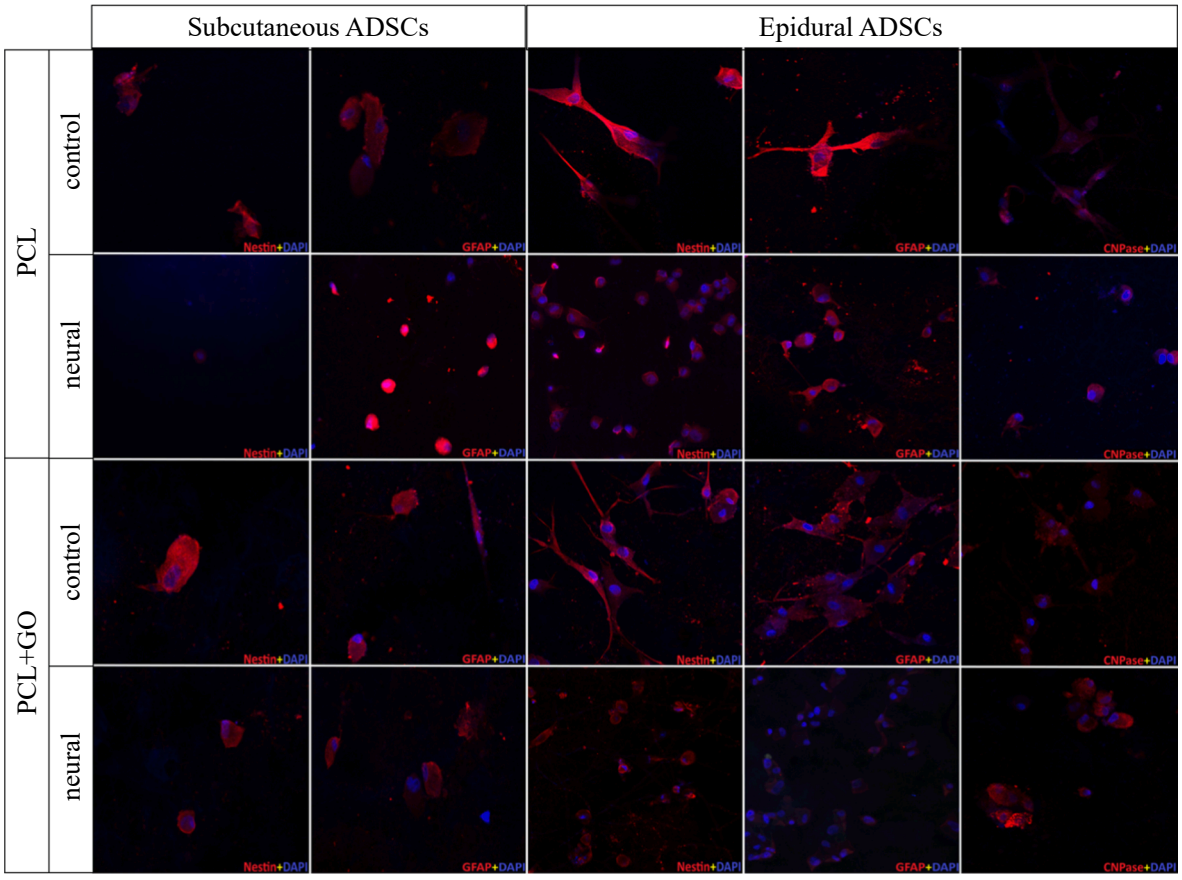

Fig. 4. Immunofluorescence staining images of subcutaneous and epidural ADSCs at 10 days of culture on PCL and PCL + GO scaffold with control and neural differentiation medium. Nestin (red), GFAP (red), CNPase (red) and DAPI (blue) staining (20x magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not cause chemical changes on each other during blending. In this study, subcutaneous and epidural ADSCs were cultured on 2D, PCL and PCL + GO scaffolds for 1, 4, and 7 days. Cell viability analysis was performed using MTT assay after the incubation periods. As shown in Fig. 2, after 1 and 7 days of epidural ADSCs, the cells on PCL + GO scaffold showed a faster proliferation rate than the cells on PCL scaffold. The results showed no statistically significant difference between subcutaneous ADSCs viability of different scaffolds; on the other hand, the

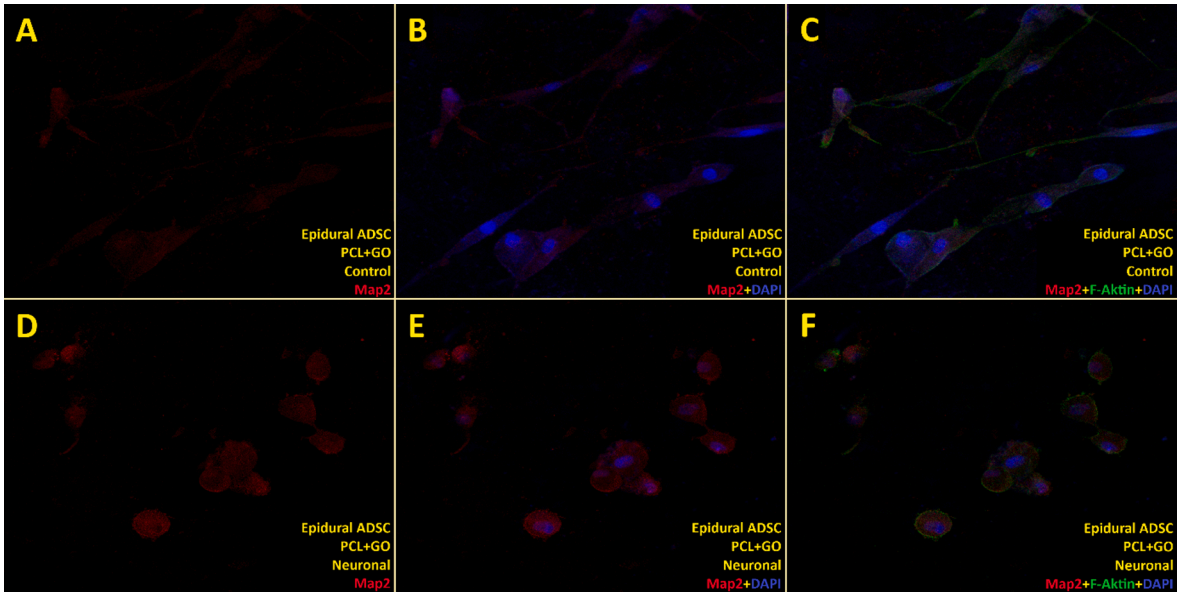

Fig. 5. Immunofluorescence images of control (A, B, C) and differentiation (D, E, F) groups of epidural ADSCs cultured in control and neural differentiation medium for 10 days on the PCL + GO scaffold. Map2 (red), DAPI (blue) and F-actin (green) staining images (20x magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

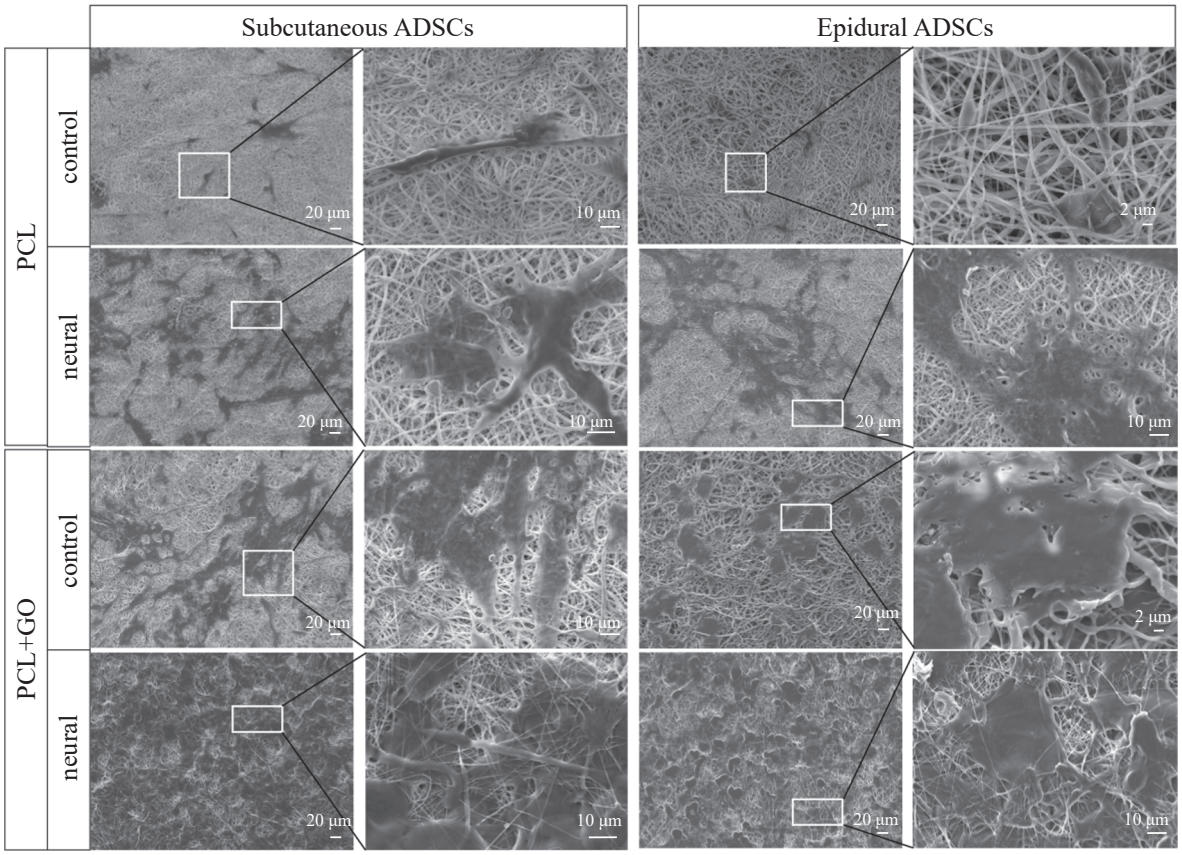

Fig. 6. SEM images of subcutaneous and epidural ADSCs at 7 days of culture on PCL and PCL + GO scaffold with control and neural differentiation medium.

proliferation rates on the scaffolds were increased when compared with the 2D. This finding is also correlated with studies in the literature showing that PCL [19] and GO [29] biomaterials are biocompatible.

In studies conducted with neural [1,8] and adipose stem cells [19,29] have been shown that the stem cells adhere to PCL and PCL + GO biomaterials, and this attachment increases with GO biomaterial. Supporting this, with DAPI immunohistochemistry staining and SEM imaging, it was clearly shown in Fig. 3 that ADSCs adhered to scaffolds after 7 days of incubation and compared to pure PCL scaffold, GO added PCL scaffold supported a more significant number of cell adhesions. The fact that the PCL is hydrophobic and the GO is hydrophilic due to the oxygen-containing groups (–COOH, –OH) on it may explain this dense cell attachment observed especially in SEM images.

In this study, we tested the neural differentiation potential of ADSCs on the PCL and PCL + GO scaffold by immunocytochemical staining with neural markers in the presence and absence of a neural induction medium. Neural intermediate filament proteins of nestin and GFAP, which are used immunohistochemically to show neuronal differentiation, were expressed in all groups in our study (Fig. 4). It is known that ADSCs can differentiate into neurons by transdifferentiation [33–35]. In characterization results, we have shown again that ADSCs can differentiate into neurons on scaffolds. It has been found in previous studies that PCL + GO materials direct ADSCs to neural differentiation without neural induction medium [9]. In contrast, the control group ADSCs, which were not applied neuronal differentiation process on PCL, were not stained with nestin in the study of Barbarisi et al. [19]. Since nestin protein is detected in many tissues, including adipose tissue, and other proliferation markers are also expressed simultaneously, previous studies are suggesting that it should be used as a proliferation marker rather than a neuronal marker [36].

Shah et al. [1] showed that neural stem cells selectively differentiate into oligodendrocytes on the PCL + GO scaffold, and this differentiation is due to overexpression of integrin-related intracellular signal molecules. After the intense cell attachment on the PCL + GO scaffold and some cells enter the neuronal differentiation path, some of this stem cell population turns into glia-like cells. Similar to the cultivation of differentiated cells in culture medium to promote differentiation, glia-like cells can induce other undifferentiated cells on the PCL + GO scaffold for neuronal differentiation and push cells that have begun neuronal differentiation into advanced neuronal differentiation [8]. As shown in Fig. 5, epidural ADSCs were stained on both scaffolds with CNPase, an enzyme associated with myelin, while on the PCL + GO scaffold they were stained with Map2, a protein associated with microtubules. The high CNPase staining of epidural ADSCs compared to subcutaneous ones suggests a tendency to differentiate in the direction of oligodendrocytes responsible for myelin production in the CNS. Also, staining of epidural ADSCs with Map2 may indicate that neuritic extensions will be relatively higher. We think that ADSCs will be more beneficial in re-establishing the functional electrical network and will support functional regeneration, in addition to the oligodendrocytic differentiation tendency. The fact that Map2 staining took place on the PCL + GO scaffold can be explained by the more adhesion and contact of the cells depending on the electrical properties of GO.

The SEM images show the cell morphology that incubated on the scaffolds with control and neural differentiation medium for 10 days (Fig. 6). SEM imaging allowed us to observe the penetration of the cytoskeleton of ADSCs incubated in both medium into PCL + GO scaffolds. These results demonstrated that the presence of GO in PCL improved cell growth, proliferation rate, cell adhesion, and the infiltration of the cells into the scaffold. The cells incubated on scaffolds showed cell–cell and cell-matrix interactions and made extensions. It can be possible that these connections in these cells leading to neuronal differentiation were synapses and the extensions were axonal extensions and dendrites. This morphological observation must be confirmed with

further tests. The bipolar and tripolar morphology in ADSCs kept in normal medium on the scaffold has been interpreted as neuronal differentiation [29]. However, the shrinkage and more rounded shape of the cells maintained in the neural differentiation environment due to the retraction of the cell body is the morphological changes that occur in the early stages of neuronal differentiation [37,38]. In our study, immunohistochemical staining showed that cells in all groups kept in neural differentiation medium were smaller and rounded compared to the control groups. We interpreted this as the retraction of the body of cells maintained in neural differentiation medium. We have also shown that the neural differentiation medium accelerates the neuronal differentiation of ADSCs on scaffolds. It is necessary to test whether the neuronal differentiation of the stem cells is functional and to clarify the mechanisms by which this differentiation on scaffolds occurs.

4. Conclusion

In conclusion, the PCL and PCL + GO nanofibers were successfully produced without any beads in the entire scaffolds by electrospinning method. ADSCs have morphologically and phenotypically neuronal differentiation potential on PCL and PCL + GO scaffolds. Also, the neuronal differentiation effect of the PCL scaffold on ADSCs is synergistically increased by the presence of GO. It was morphologically and immunocytochemically demonstrated that ADSCs taken from the epidural area had a higher neuronal differentiation potential than subcutaneous one. Epidural adipose tissue, which will be taken during decompression and stabilization operations, which are frequently performed in patients with spinal cord injury, can be used as a suitable source for stem cell research in these patients.

In the future, ADSCs isolated from the epidural space which are prone to oligodendrocytic differentiation can be implanted into the damaged area on the chemically, electrically, and mechanically advantageous PCL + GO scaffold for functional recovery of spinal cord injury.

Easy, precise and rapid isolation of stem cells, 3D implantation of stem cells to the scaffold and the surgical placement of this scaffold to the damaged spinal cord area are among the future challenges.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2022.111000.

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