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RESEARCH ARTICLE

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Inducing chondrogenic differentiation in ATDC5 cells using a three-dimensional hydrogel with GAG-mimicking properties

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Abstract

This study aims to develop a method using a three-dimensional hydrogel that mimics glycosaminoglycans to accelerate the development of cartilage cells. The hydrogel contains a specific glycosaminoglycan-like peptide sequence with the potential to enhance the effectiveness of chondrogenic differentiation and provide a more efficient approach. In the study, ATDC5 cells were cultured within a synthetic scaffold incorporating peptide amphiphile (PA) nanofibers designed to emulate the structure of glycosaminoglycans in a three-dimensional format for tissue engineering applications. Cellular characterizations were conducted to induce chondrogenic differentiation. ATDC5 cells cultured on GAG-mimicking peptide nanofibers expressed cartilage-specific extracellular matrix components statistically significantly over a 14-day period compared to cells cultured on TCP without insulin induction. Amphiphilic peptide nanofibers offer a valuable approach to replicate glycosaminoglycan properties and support chondrogenic differentiation in ATDC5 cells without the need for growth factors or external stimuli. This approach holds substantial potential for clinical applications in cartilage tissue engineering.

Keywords: 3D culture; ATDC5 cell; cartilage tissue engineering; GAG-mimicking peptide amphiphile nanofiber;, in vitro chondrogenic differentiation

1. Introduction

The capacity of cartilage tissue to regenerate is restricted because to the absence of blood vessels and nerve supply. Joint degradation can result from acute accidents or degenerative disorders like osteoarthritis. Many approaches, such as tissue engineering, gene therapy, and stem cell therapy, have been studied to support cartilage tissue healing. Scaffold materials, which can be formed from a range of materials including polymers, ceramics, and naturally present extracellular matrix components, are essential to cartilage tissue engineering. These scaffolds facilitate the in vitro culture of cells such as chondrocytes or stem cells, hence promoting the development of

functional cartilage tissue [3–4].

Chondrogenic differentiation is the process by which cells differentiate into cartilage; tissue engineering is particularly interested in this process because it may be applied to repair damaged cartilage. To mimic the environment of natural cartilage more accurately, researchers grew cells in three-dimensional (3D) hydrogels. By transmitting mechanical signals, hydrogels with distinct stiffness and elasticity properties promote chondrogenic growth [5–6]. Peptide-based hydrogels, such as peptide amphiphile nanofibers (PANs), have been investigated for tissue engineering applications; one such application is in progenitor cell chondrogenic development. PANs contain certain peptide groups that are similar to the extracellular matrix of cartilage tissue. One example of this is the induction of chondrogenic cell development in ATDC5 cells with the application of a PAN containing the RGDS peptide sequence present in multiple ECM proteins [7]. One promising strategy for promoting chondrogenic growth would be the application of a hydrogel made of certain peptides, such as sulfated glycosaminoglycans. The structure and activities of naturally occurring glycosaminoglycans in the extracellular matrix of cartilage tissue can be mimicked by a peptide sequence. Chondrogenic differentiation and cartilage tissue regeneration can be enhanced by the application of growth factors, peptide sequences, and appropriate mechanical stimulation [8–10]. In our study we aim to induce chondroprogenitor ATDC5 cells through peptide amphiphile nanofibers without the requirement for external stimulation.

2. Experimentals

2.1. Materials

Lauric acid and DIEA had been supplied by Merck, whereas amino acids and HBTU were obtained from NovaBiochem for the synthesis of PA. All the cell consumables had been supplied by Nest Scientific USA Inc. and Biological Industries (Kibbutz Beit Haemek), and ATDC5 cells were contributed.

2.2. Synthesis of PAs

Three PA molecules (E-PA, K-PA, and GAG-PA) were synthesized using solid-phase Fmoc chemistry. Different resin molecules were employed for each PA molecule. The synthesis steps were executed as detailed in the literature [11]. Following synthesis, the PA molecules underwent purification via an HPLC system. Both PAs were synthesized achieving a purity exceeding 95%. The evaluation of purity was conducted based on the amide content, as determined by absorbance measurements at 220 nm. A common benchmark is that peptide purity, particularly for bioactive peptides, should be above 95% when used in biological assays to ensure that the observed effects are due to the peptide itself and not impurities. An Agilent LC-MS system was utilized for ascertaining the molecular mass and purity of the PAs. Purity was assessed at 220 nm relative to peptide content. One percent solutions of all PA molecules were prepared in a 1:1 (v/v) ratio. The synthesized nanofibers were dried in ethanol at increasing concentrations for the initial ten minutes and subsequently dried using a Tourismis Autosamdri critical dryer.

2.3. Formation of 3-Dimensional peptide hydrogels and cellular viability

To generate 3D peptide hydrogels, E-, K-, and GAG-PA solutions were combined in HEPES buffer at a concentration of 10 nM and a volume of 100 µl. A micro mass culture technique was employed to facilitate chondrogenic differentiation, simulating the three-dimensional cellular interactions occurring during embryogenesis. The cell solution was prepared using K-PA and mixed with 10 nM PA solutions (E- or GAG-PA) in cell culture wells, resulting in a cell-hydrogel composite. In this study, $5x10^6$ cells were used per 100 µl of hydrogel, consistent with acceptable ranges established in prior research. Following 3D cell culture formation, cells were incubated for two weeks in a growth media, with media changes occurring every 48 hours. Cellular viability was assessed for

three days using an MTT kit from Roche (catalogue number 11465007001), in accordance with the kit's manual. MTT reagent (0.5 mg/ml) was added to each well. Plates were kept in the dark for four hours before the addition of a solubilization solution to dissolve the formazan crystals. The absorbance was quantified at a wavelength of 570 nm utilizing a BioTek Synergy H1 microplate reader.

2.4. Observing the production of glycosaminoglycans

Post 14-day incubation, hydrogels were fixed in 4% paraformaldehyde for 48 hours at 4°C, dehydrated in ethanol, and cleared in xylene. Resulting samples were sectioned into 5 µm slices using a microtome (Leica RM2125 RTS). Safranin-O and Fast Green were employed for glycosaminoglycan visualization, while Mayer's Hematoxylin and Eosin were used for general staining. Immunohistochemical staining was performed on slides using an antigen retriever (Sigma, C9999) to expose the epitopes. Slides were subsequently blocked and incubated overnight with primary antibodies for Collagen II (Abcam, ab34712). After thorough washing, a secondary antibody (Merck, AP124C) was applied to detect primary antibodies. Finally, cells were washed and analyzed under a fluorescence microscope (ZEISS LSM 510).

2.5. Statistical analyses

All variables were analyzed by calculating their mean and standard error (SEM). A non-parametric Suduki comparison test was employed to evaluate differences between experimental groups. Statistical significance was determined based on p-values less than 0.05. All calculations were performed using GraphPad Prism (San Diego, CA, USA).

3. Results and discussion

The synthesis of both peptide amphiphiles (PAs) resulted in a purity greater than 95%. Purity assessments were performed by quantifying the amide bond content, utilizing absorbance readings at the 220 nm wavelength. It is widely recognized in bioactive peptide research that a purity threshold of 95% is critical for biological assays, as it ensures that the peptides' biological activity is not compromised by contaminating substances. In this study, two distinct 3-dimensional hydrogel environments were generated for culturing ATDC5 cells. The first hydrogel, termed GAG-H, was synthesized by mixing GAG-PA and K-PA at neutral pH and room temperature. The hydrogel designated as Cntrl-H was developed by mixing E-PA with K-PA in a similar set of conditions (refer to Figure 1. Our research introduces peptide frameworks designed to imitate natural glycosaminoglycans (GAGs). This imitation is achieved by producing extended nanofibers adorned with repetitive chemical groups such as carboxylate, hydroxyl, and sulfonate. The GAG-PA peptide used in our study encompasses all these group types, effectively mimicking the characteristics of sulfonated glycosaminoglycans. We've named this peptide focusing on its sulfonate component to distinguish it from other peptides in our research. Scanning electron microscopy revealed that the resultant nanofiber structure closely resembled the natural extracellular matrix in terms of fibril width and porosity (Figure 1d). The purity and efficacy of the synthesized peptides were confirmed through liquid chromatography and mass spectrometry (Figures 1b and 1c). To initiate nanofiber formation, K-PA, a positively charged peptide amphiphile, was employed. The nomenclature of these molecules is based on the charged amino acid residue present at the C-terminus (see Figure 1).

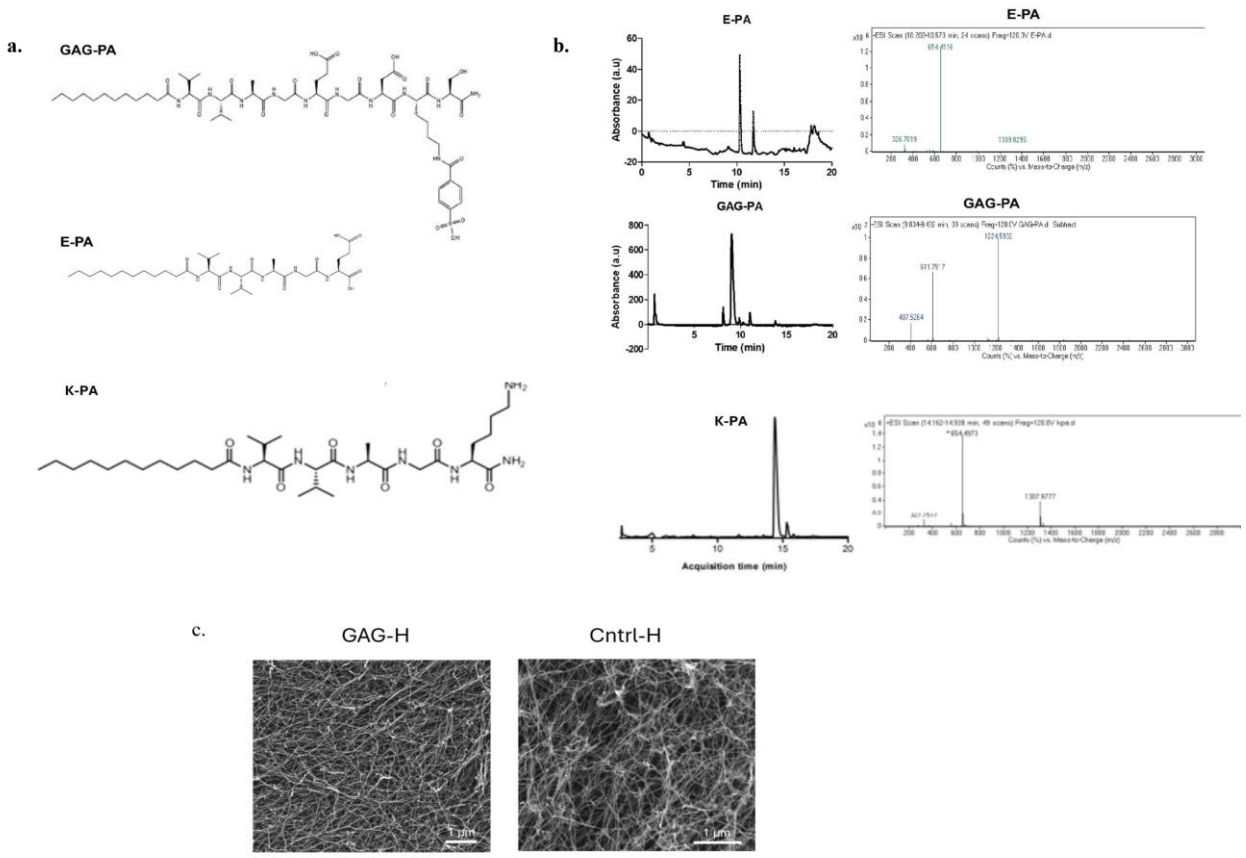

Fig. 1. (a) Chemical representation of PA molecules used in the study. (b) Liquid Chromatogram and (c) Mass Spectroscopy results of PAs. (d) Scanning Electron Microscopy images of PA nanofibers.

Initial cellular viability assessments were conducted on ATDC5 cells cultured in GAG-H and Cntrl-H hydrogels for a period of 3 days. The findings indicated that the hydrogels did not adversely affect ATDC5 cells in contrast to those cultivated on tissue culture plastic (TCP) in a micro mass culture setting (Figure 2a). 3D culture systems are instrumental in simulating the extracellular environment, thereby enhancing cellular interactions, growth factor deposition, directed differentiation, and the maintenance of differentiated states [12]. After 14 days of static culturing, ATDC5 cells formed distinct clusters within the hydrogels, suggesting cell-cell interactions and aggregation (Figures 2b and 2c). Hematoxylin and Eosin staining corroborated the presence of cellular aggregates in both hydrogels on day 14 (Figures 2d, e, f, and g). Notably, this aggregate formation occurred without the addition of insulin, a key inducer for chondrogenic differentiation in ATDC5 cells. Furthermore, the cells synthesized chondrogenic extracellular matrix (ECM), as evidenced by the deposition of glycosaminoglycans and the expression of Collagen II.

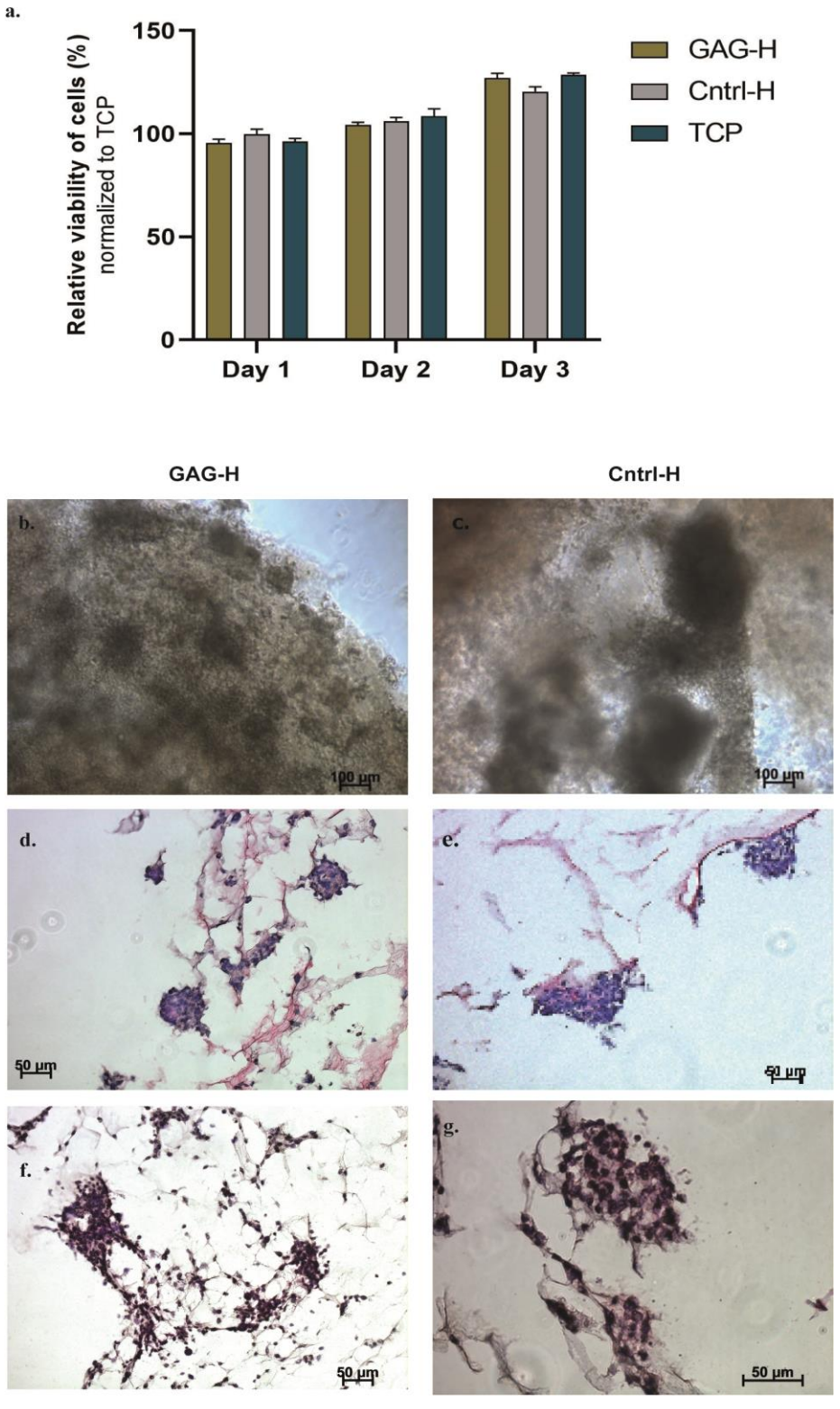
a.
Relative viability of cells (%)
normalized to TCP
150
100
50
0
Day 1
Day 2
Day 3
GAG-H
Cntrl-H
TCP
GAG-H
Cntrl-H
b.
c.
100 µm
100 µm
d.
e.
50 µm
50 µm
f.
g.
50 µm
50 µm

Fig. 2. (a) Cell viability on days 1, 2, and 3. (b-c) Comparison of ATDC5 cell behavior in GAG-H and Cntrl-H hydrogels on Day 14. Panels (b-c) display light microscope images of the cells, while panels (d-g) depict aggregate formation using Hematoxylin and Eosin staining in 3D cultures.

Significantly, these outcomes were attained without employing any external inducers, indicating the potential clinical relevance of this approach. Furthermore, the 3D culture system facilitated the formation of aggregates and the synthesis of cartilage-specific proteins, suggesting that the hydrogel environment is conducive to the differentiation of ATDC5 cells into chondrocytes (Figure 3 c-f). Figure 3g shows that the GAG-H gel formulation had a higher GAG/DNA ratio (12.2 ug/ug) and more distinguishable cell aggregates than the Cntrl-H group (8.2 ug/ug). This suggests that the charge and functional groups of the GAG-H hydrogel may play a role in promoting cell-cell interactions and aggregation in terms of chondrogenic differentiation. The analysis of GAG deposition showed faint staining after treatment with chondroitinase (Figure 3a, b), confirming that GAG synthesis occurred in the cells' environment. Overall, these results demonstrate the potential of peptide nanofiber hydrogels as a 3D culture system for differentiating ATDC5 cells into chondrocytes without employing external growth factors or inducers.

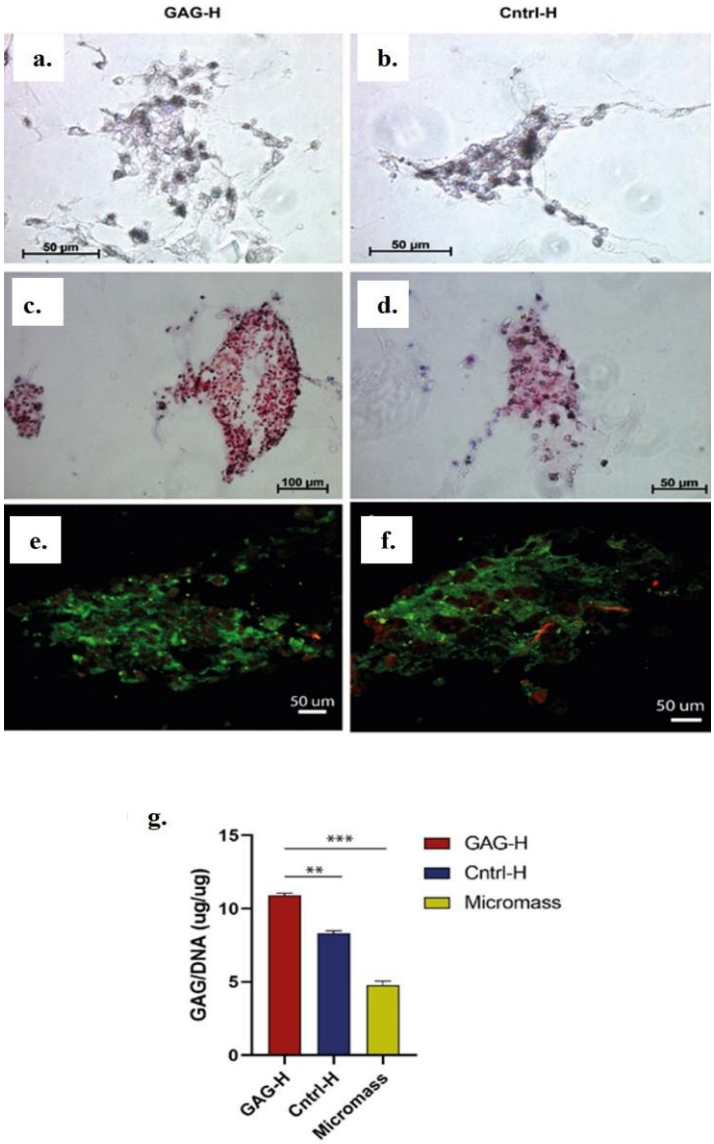

Fig. 3. Characterization of ATDC5 cell behavior in 3D GAG-H and Cntrl-H hydrogels on Day 14. Panels (a-b) and (c-d) show Safranin-O staining with and without chondroitinase treatment, respectively. Panels (e-f) illustrate collagen II staining, (Cy3) and cell nuclei (DAPI-3). (g) GAG/DNA (ug/ug)

The study presented here provides a valuable contribution to the field of tissue engineering by demonstrating the potential of nanofibers that mimic glycosaminoglycans to promote the chondrogenic differentiation of ATDC5 cells. The development of new biomaterials will heavily focus on ECM GAGs, which play a role in various biological functions, including embryonic development, infection, inflammation, wound healing, and cancer. The content and composition of ECM GAGs vary depending on the tissue, age, and pathology [13].

As demonstrated in this study, using synthetic GAG analogues offers a promising approach to address limitations of natural GAGs in hydrogels, such as limited availability, structural heterogeneity, batch-to-batch variability, and

immunogenicity. Several studies focusing on the development of GAG-based hydrogels have examined the use of GAG analogs in tissue engineering. Zhang et al., for instance, used hydrogel to encapsulate chondrocytes for cartilage regeneration, mimicking synthetic hyaluronic acid [14]. Even so, through the manufacture of chondroitin sulfate-mimetic hydrogels, Liu et al. were able to promote chondrogenic development in human mesenchymal stem cells [15]. Kim et al. were mainly looking at how mesenchymal stem cells' chondrogenic development in glycosaminoglycan-based hydrogels was influenced by their type and degree of sulfation. They noticed that the pattern and degree of sulfation had a substantial impact on the synthesis of extracellular matrix and the chondrogenic differentiation of stem cells [16]. Based on these findings, functional groups in GAG-based hydrogels need to be carefully designed and controlled to optimize their ability to promote chondrogenic development. Our study highlights the importance of sulfate/sulfonate groups in a peptide hydrogel as it increases GAG synthesis by ATDC5 cells. Sulfate groups can be incorporated into hydrogels and promote cell differentiation. Recently, Huang et al. conducted a study that fully sulfated sodium cellulose sulfate (NaCS) could be used as a scaffold for cartilage repair. NaCS increases chondrogenesis in human mesenchymal stem cells, which means that cellulose sulfate can be used as a scaffolding material in cartilage tissue engineering [18].

Our study reveals that these nanofibers effectively imitate the functions and properties of glycosaminoglycans. Through integrating amino acid sequences with carboxylate, hydroxyl, and sulfonate groups, we engineered a hydrogel system that fosters chondrogenic differentiation in ATDC5 cells, eliminating the need for external growth factors. Notably, the GAG-H hydrogel with sulfonate surpasses the CNTR-H hydrogel in enhancing chondrogenic cell differentiation. This innovation leads to the development of diverse, multifunctional GAG-like materials, providing a promising alternative to natural GAGs in tissue engineering applications.

4. Conclusion

Our research results demonstrate that adding sulfate/sulfonate groups to peptide hydrogels can increase GAG production and provide a useful substitute for natural GAG products in tissue engineering. Through the development of a hydrogel system that mimics the characteristics of naturally occurring GAGs, we have demonstrated the encouraging potential of these artificial analogs in promoting cell differentiation and extracellular matrix synthesis.

Ethical Approval

Ethical approval for this study was not required. Secondary cell line cultures were utilized in this investigation.

Author Contribution

S.Y. organized and performed all experiments and wrote the manuscript.

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