

A novel chitosan-peptide system for cartilage tissue engineering with adipose-derived stromal cells

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ABSTRACT

The natural healing process of cartilage injuries often fails to fully restore the tissue's biological and mechanical functions. Cartilage grafts are costly and require surgical intervention, often associated with complications such as intraoperative infection and rejection by the recipient due to ischemia. Novel tissue engineering technologies aim to ideally fill the cartilage defect to prevent disease progression or regenerate damaged tissue. Despite many studies on designing biocompatible composites to stimulate chondrogenesis, only few focus on peptides and carriers that promote stem cell proliferation or differentiation to promote healing. Our research aimed to design a carbohydrate chitosan-based biomaterial to stimulate stem cells into the chondrogenesis pathway. Our strategy was to combine chitosan with a novel peptide (UG28) that sequence was based on the copin protein. The construct stimulated human adipose-derived stem cells (AD-SCs) cells to undergo chondrogenic differentiation. Chitosan 75/500 allows AD-SCs to grow and has no harmful effects on the cells. The combination of UG28 peptide with the chitosan composite offers promising properties for cell differentiation, indicating its potential for clinical applications in cartilage regeneration.

1. Introduction

Articular cartilage damages may result from different conditions such as inflammation, trauma and degeneration. According to the World Health Organization (WHO) in 2019, nearly 528 million people worldwide suffered from osteoarthritis, with a sharp increase in incidence of almost 113 % since 1990. The disease mostly affects people over the age of 55, with women comprising 60 % of the affected patients. Degeneration mainly affects the knee joints, followed by the hip and hand joints. In addition, factors such as aging and overweight exert a strong effect on the cartilaginous system, and cause an increase in the number

of sufferers. It can also affect young people, athletes, and people after injury limiting motor function for a long time [1].

Cartilage repair and regeneration remain complex processes, and although extensive research has been conducted, the unique properties of cartilage (e.g. lack of blood vessels and nerves), limit its ability to self-repair.

Currently available treatments for osteoarthritis include non-pharmacological (exercise, physical therapy), pharmacological (oral medication, injections), and surgical interventions (autologous chondrocyte implantation, microfracture, total knee arthroplasty) [2].

The main limitation of interventional techniques or physical therapy

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(e.g. exercise and weight-bearing) recommended to promote cartilage repair by inducing the growth of new cartilage and improving joint function, are the poor long-term clinical effect.

However, these methods are often only moderately effective, offering primarily short-term relief and limited long-term benefits. Intra-articular injections, which temporarily alleviate pain and improve joint function but are limited by factors such as lymphatic clearance, synovial vessel absorption, protease degradation, and the short half-life of glucocorticoids within the joint. Long-term use of certain intra-articular injections, like corticosteroids, also raises concerns about cytotoxicity to cartilage tissue, as seen in some animal studies, though this has not been conclusively demonstrated in large human studies [3].

Tissue engineering and regenerative medicine offer promising alternatives through the development of biomaterials and bioactive substances for regeneration and replacement.

Stimuli-responsive scaffolds capable of accurately detecting and responding to cartilage damage (e.g. 3D printed and bionic cartilage scaffolds) that provide mechanical support for chondrocytes and extracellular matrix are being developed.

Stem cells, biologically active compounds, extracellular vesicles (EVs), and organoids are also used to stimulate chondrocyte proliferation and differentiation. However, the use of all of them requires some improvements. In the case of stem cells, the optimal source of cells, the ideal duration of treatment, and the optimal method of stem cell transplantation need to be assessed. The protocol for use of extracellular vesicles (EVs) needs to be researched and optimized, as the selection of appropriate carriers and biomaterials, and the control of vesicle release and targeted delivery [4].

The literature shows that there is still little effect of using natural materials (biomaterial, biocompatible, biodegradable, non-toxic that can be in direct contact with body tissues), in trauma orthopedic or craniofacial surgery to regenerate cartilage damage [5].

Many studies describe natural biopolymers extracted from brown alginate [6], fungal cell wall chitin, insect silk [7] or waste crustaceans' shells (chitosan [8,9]), marine crustacean shells, from ECM elastin, hyaluronic acid [9], blood fibrin [10], bovine skin and tendons, collagen from marine sponges [11,12] and wood, bacterial cellulose [13]. Various forms of biomaterials such as hydrogels, xerogels, membranes, films, foams, sponges, powders, nanofibres [12,14] microspheres, bio-films, composites, scaffolds can replace future bio-dressings.

Chitosan, which is known for its biocompatibility, antibacterial [15, 16] anti-inflammatory, antifungal, and anticancer properties has a great potential in medicine [17–20]. Due to its cationic nature, it has controlled drug release properties, and its hydrophilicity gives it the ability to fill lesions of any shape [21–23]. It acts as a carrier of compounds in the wound healing process [24] and resembles living tissue with various physical and chemical properties [25]. The viscoelasticity of chitosan hydrogels allows load transfer to chondrocytes, ensuring their survival and controlling chondrogenic differentiation [26].

A novel approach of cartilage reconstruction is the use of adipose derived stem cells AD-SCs to differentiate into chondrocytes [27]. Stimulation of cells with peptides increases proliferation and differentiation on a biodegradable substrate, that suggests promising regenerative strategy in cartilage defects.

Despite extensive efforts, no current treatment reliably restores cartilage structure and function. Our research investigates an innovative solution by combining a pro-chondrogenic peptide with chitosan and stem cells to treat cartilage injuries.

Mesenchymal adipose tissue cells have a great potential for proliferation and differentiation into various cell lineages, including cartilage and bone [28–30]. Adipose stromal cells with their easy availability and usually low invasiveness in harvesting, offer substantial potential for tissue engineering [30]. Compared to other mesenchymal stem cells like BM-MSCs, AD-MSC show higher proliferation rate, and larger cell population after isolation [7].

Recently studies has shown that peptides have ability to stimulate

chondrogenesis, as they mimic the functions of cartilage ECM and chondroinductive growth factors [31]. Among the many examples of peptides with above properties is Copin family (CPNE) proteins fragments. CPNE depend on intracellular Ca²⁺ levels, that translocates to the plasma membrane, binds to phospholipids, that are highly conserved and are found in a variety of eukaryotes [32]. Copins have two C2 domains and a Willebrand domain A. The C2 domains (CA2 and C2B), are recognized in protein kinase C and mediate active interaction with calcium [33]. Copin 7 shows osteogenic potential on MSCs [34–36]. CPNE7 is a regulator of odontoblast formation and differentiation. Peptide derivatives of CPNE7 may serve as therapeutic agents in the future and provide an alternative to macromolecular proteins [11,35].

The aim of our study was to create a new composite biomaterial based on chitosan and a stimulating peptide (fragment of CPNE protein) for cartilage regeneration. Our research has shown that such a biomaterial is effective in pre-clinical testing and safe at the cellular level.

2. Material and methods

2.1. AD-SCs isolation

Adipose-derived stromal cells (AD-SCs) were isolated from subcutaneous adipose tissue obtained from elective/routine surgical operations obtained from patients admitted at the Medical University Hospital of Gdańsk: Department of Surgical Oncology and Department of Plastic Surgery. Used tissues for cells isolation were a medical waste, which routinely undergoes utilization. Samples collection was approved by the Independent Bioethics Commission for Research of the Medical University of Gdańsk (consent no. NKBBN/672/2019, NKBBN/672–65/2023).

AD-SCs isolation was carried out based on standard protocol [37]. Cells were routinely cultured in DMEM LG (Dulbecco's Modified Eagle Medium Low Glucose - with 1 mg/ml of glucose), supplemented with 10 % Fetal Bovine Serum and antibiotics (penicillin/streptomycin), at 37 °C, 5 % CO₂. The culture medium was changed every 2–3 days.

2.2. Peptide synthesis and purification

Peptides UG28 (LEAVNPKYKQKRR) and CPLG-UG28 (CPLGLEAVNPKYKQKRR) were synthesised by solid phase peptide synthesis using peptide synthesiser Liberty Blue (CEM Corporation) and general conditions of solid-phase synthesis. Synthesis was performed on a ProTide resin (0.2 mmol/g), using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with the following side chain protected amino acid derivatives: Fmoc-Pro-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH. Peptides were cleaved from the resin for 4 h using a mixture of 88 % TFA, 5 % fenol, 5 % deionized water and 2 % triisopropylsilane (10 ml/1 g of resin at room temperature for 4 h). After filtration of the exhausted resin, solutions were concentrated in vacuum, and the residue was triturated with Et₂O. The precipitated peptide was centrifuged for 15 min, 4000 rpm, followed by decantation of the ether phase from the crude peptide (process was repeated three times). After evaporation of Et₂O, the peptide was dissolved in H₂O and lyophilized.

Purification of the crude peptides were carried out by using RP-HPLC on a Kromasil C8 semipreparative(250 mm×10 mm, 5 µm) column. A linear gradient from 5 % B to 50 % B in 120 min was used. The aqueous system (A) consisted of 0.1 % (v/v) TFA solution in water, whereas the organic phase (B) was 80 % acetonitrile in water, containing 0.1 % (v/v) TFA. Purification was monitored by UV absorption at a wavelength of 223 nm. The purity of the peptide was verified by LC-ESI-IT-TOF/MS (Shimadzu, Shimpol), and by using RP-HPLC with a Kromasil C8 analytical column (250 mm×4.6 mm, 5 µm), where a gradient of 5–100 % B in A in 60 min was employed, with A and B as described above.

2.3. Functionalization of chitosan with CPLG-UG28 peptide

In the first stage, the chitosan was covalently functionalized with a maleimidoglycine linker, in line with the procedure described in [patent application no. P.443979 and P.442878, Patent Office of the Republic of Poland]. After the removal of the unreacted substrates, the chitosan-maleimidoglycine scaffold underwent drying under a vacuum. Subsequently, CPLG-UG28 peptide terminated with N-terminal cysteine amino acid was deposited, by Michael reaction between -SH group of cysteine and maleimide moiety, previously attached to chitosan (chitosan-maleimidoglycine). An aqueous solution of chitosan-maleimidoglycine- CPLG-UG28 peptide was stirred for 24 h at room temperature at pH 8.2 in inert gas. Final product chitosan - maleimidoglycine- CPLG-UG28 peptide was filtered with gooch G3 and dried under vacuum for 1 h. The degree of chitosan deposition by the CPLG-UG28 peptide was ca. 10 %.

2.4. Chitosan hydrogel membrane

To achieve a chitosan solution with a peptide concentration of 10 µg/ml, two types of chitosan, one with and the other without the peptide attached, were blended using a mass ratio of 69:1. A chitosan solution in hydroxyacetic acid was prepared following the methodology developed by Gorczyca [38], with minor modifications. Initially, a 1.5 % solution of chitosan in 0.1 M hydroxyacetic acid (Merck, Germany) was prepared by dissolving the polymer in the acid solution using a mechanical stirrer at 300 rpm (Heidolph Instruments GmbH & Co. KG, Kelheim, Germany, model RA 2020). Then, 0.5 M NaOH solution, (Gliwice, Poland) was gradually added to the chitosan solution to ensure complete precipitation of chitosan, aiming for a solution pH of about 9–10. This led to the complete precipitation of chitosan in microcrystalline form. The precipitated chitosan was filtered using a reduced-pressure filtration system and rinsed with distilled water (Milli-Q® IQ 7005 Water Purification System, Millipore, USA), until the pH of the rinse water stabilized at 7.0. In the final step, the precipitated chitosan was weighed and resuspended in distilled water to reach a concentration of 0.7 %. The suspension was homogenized at 10000 rpm for 3 minutes using Silent Crusher M (Heidolph Instruments), and then saturated with CO₂ (Linde Gaz Polska Sp. z o. o.). The saturation process consisted of continuous mechanical stirring using a gas saturation stirrer (BIOMIXBMX-10, Gdansk, Poland) until the chitosan was completely dissolved.

To prepare chitosan xerogel samples, the hydrogel solutions produced by the above method were dispensed into twelve wells of a multi-well plate, each to a depth of 15 mm. These samples were then frozen at –80°C and freeze-dried (at a pressure of 0.94 mbar, with the condenser temperature at –80°C and the shelf temperature maintained at 50°C). Samples were stored in a dry, airtight container in a cool environment before measurement.

Chitosan hydrogel membranes were prepared in an analogous manner with additional steps. After saturation, the chitosan solutions were poured into flat molds 90 mm in diameter to a height of 10 mm. The samples were then incubated at 25°C for 48 hours. Under these conditions, CO₂ is released as a decomposition product of unstable carbonic acid. As the system approaches equilibrium, the pH of the solution increases, leading to gelation of the chitosan - a transition from the sol state to the gel state. Once the phase transition was achieved, 20-mm-diameter samples were excised and placed in phosphate-buffered saline (Sigma-Aldrich PBS).

2.5. Proliferation assay (XTT)

The XTT assay (Cell Proliferation Kit II; Sigma Aldrich) measured AD-SCs viability through/by mitochondrial activity after treatment with UG28 peptide. 5000 cells per well (96-well plates) were seeded in DMEM LG medium supplemented with 10 % of FBS and antibiotics (penicillin/streptomycin). After cells resting (24 hrs), the media were

exchanged for a serum-free DMEM LG with different peptide concentrations (0,01; 0,1; 1; 10; 25; 50, and 100 µg/ml) to establish the most effective one. The AD-SCs were incubated with peptide for 48 and 72 hours. Thereafter, XTT reagent was added, the absorbance was read at OD 450 nm, after 4 hrs incubation, at 37 °C and the presence of 5 % CO₂. Cells proliferation was compared to the non-treated AD-SCs (C – control; established as 100 %), additionally, AD-SCs cultured with 10 % FBS were a positive control.

Fluorescence staining was carried out using a 1ul Calcein-AM solution and propidium iodide mixed with PBS at 1ul per 1 ml. The cells stained on chitosan membranes were observed under a fluorescence microscope (OLYMPUS) emitting green and red fluorescence.

2.6. Cytotoxicity assay (LDH)

The cytotoxicity of the UG28 peptide on AD-SCs was measured by the lactate dehydrogenase (LDH) activity in cell culture supernatants. AD-SCs were seeded in 96-well plates, analogical to proliferation assay (XTT). Cells cultured in a serum-free DMEM LG were stimulated by UG28 in the highest concentrations (50, 100, and 150 µg/ml). After 48 incubation hours, supernatants were collected for LDH analysis, which was performed according to the manufacturer's instructions. Cell death was compared with the non-treated control and the positive control (with maximum LDH release; 100 % cytotoxicity) induced by Triton X-100 detergent.

2.7. Migration assay (scratch assay)

The effect of UG28 on AD-SCs migration after 24 hrs was assessed using silicone culture inserts (Ibidi). 20`000 cells were seeded in each insert for 24 hrs in DMEM LG medium supplemented with 10 % FBS and antibiotics. Then cells` proliferation was blocked by adding mitomycin C (5 µg/ml) for 2 hrs, and the medium was changed into serum-free with peptide in two, selected concentrations (10, and 25 µg/ml). Migration was analyzed after 24 hrs. The cells were fixed with 3.7 % paraformaldehyde, and stained with 0.05 % crystal violet, and the migratory effect was measured with measurement of unoccupied area between cells.

2.8. Collagen production

The collagen production under UG28 influence was assessed by Sirius Red (Sigma Aldrich) staining. Cells were seeded into 96-well plates, 3`000 cells per well, and cultured in DMEM LG for 14 days. After the weeks, cells were fixed with Bouin's solution, and stained with Direct Red 80 (Sirius Red) for 1 hour with gentle shaking. In the next step, there was hydrochloric acid rinsing and finally eluted with sodium hydroxide. The measurements were taken at OD 550 nm.

2.9. Immunogenicity

The immunogenicity potential of the UG28 peptide was checked on human peripheral blood mononuclear cells (PBMC), isolated from 'buffy coats' using a ficoll density gradient. After erythrocyte lysis and cell counting, the PBMCs were seeded onto a 24-well culture plate (1 mln cells/1 ml of RPMI 1640 (P/S, 10 % FBS)/ well). To allow cells to adapt to the culture conditions there was cell resting for the next 24 hrs. Next PBMC was stimulated by UG28 peptide in a final concentration of 10 µg/ml, and incubated for the next 24 hrs under controlled conditions (37 °C, 5 % CO₂). The unstimulated cells constituted a negative control, whereas cells stimulated with lipopolysaccharide (LPS; 1 µg/ml) were a positive control. After incubation, the PBMCs were collected, washed with PBS, and stained with monoclonal, fluorochrome-labeled antibodies (anti-CD3, CD4, CD8, CD25, CD69, CD71, and HLA-DR). Following 30 min of incubation at RT in the dark, the cells were analysed using a flow cytometer Cytoflex (Beckman Coulter). Statistical

significance was checked with the Mann-Whitney U test ($p < 0,05$) using STATISTICA software and graphs were prepared with GraphPad Prism 5 software.

2.10. Basophil activation test (BAT)

Basophil activation test (BAT) was performed with the use Allergy Kit, Cellular Analysis of Allergy, (Beckman Coulter, A17116). Blood was taken from five healthy volunteers, and collected in EDTA tubes, and the test was carried out within the next 2–3 hrs of blood sampling. Each sample of 100 μ l blood was incubated with 20 μ l of the UG28 peptide (10 and 25 μ g/ml) or chitosan, and stained using 20 μ l of CD3, CD203c, and CRTH2 antibodies mixture. Then, the samples were incubated for 15 min at 37 °C in a water bath, lysed, washed a few times, and analyzed using a Cytoflex cytometer (Beckman Coulter). For each patient sample, negative and two positive (anti-IgE and fMLP) controls were prepared. The cut-off values for the analyzed allergen were estimated as 5 % of the negative stimulation control sample.

2.11. Analysis of differentiation

For the analysis of differentiative potential AD-SCs were seeded into 96-well plates and cultured for 14 days in appropriate differentiating media stimulating cell differentiation into adipocytes, osteocytes, and chondrocytes. After two weeks cells were fixed with 4 % paraformaldehyde and stained with Oil red (adipocytes), or Alizarin red (osteocytes). Additional quantitative analysis was performed. Oil-red stained adipocytes were incubated with isopropanol to eluate the stain (absorbance read at 490 nm). Alizarin red stained osteocytes were incubated in the first step with 10 % hydrochloric acid and next in 10 % ammonia water (absorbance read at 450 nm). Differentiated chondrocyte cells were detached with trypsin and washed away. A mixture of papain proteinase (Papain Extraction Reagent), EDTA, cysteine HCL, sodium acetate, and PBS was added to the cells. The pellets were incubated at 65°C for 3 hours in a thermoblock. Sulfated GAGs after digestion were quantified using the Blyscan Sulfated Glycosaminoglycan Assay Kit, according to the manufacturer's instructions. The absorbance of the stained supernatant was read at 656 nm.

2.12. Quantitative PCR analysis of chondrogenesis markers

In the first step RNA was isolated following protocol (REF), total RNA was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH) and then cDNA was multiplied with Light Cycler 480 SYBR Green I Master (Roche Diagnostics GmbH). Data analysis was performed using Light Cycler 480 Software (Roche Diagnostics). The $2^{-\Delta\Delta CT}$ method was utilized to analyze the data. The following primers were applied in the study: *SOX9* (F: TACCG-CACTTGCACAAC, R: TCTCGCTCTCGTTCAGAAGTC), *COMP* (F: CCAAGTGGGCTACATCAGG, R: GTCCAAGACCAGTTGCTG), *MMP13* (F: GTTCGGCCACTCCTTAGGTC, R: AGCATAAAGTGGCTTTTGCCG). The *RFLP13* gene (F: CAACCGGATGAACACCAAC, R: TGTGGGGCAG-CATACCCTC) was selected as the reference gene.

2.13. Histological analysis

For histological evaluation of cartilage structure formation, cells at passage 2, $0,5 \times 10^6$ in number, were cultured for 6 weeks in chondrogenesis differentiation medium and an appropriate amount of UG28 peptide was added, final concentration 10ug/ml. The control was cells simultaneously cultured without the peptide. Afterwards, cells were fixed in 4 % PFA for 24 h, and processed for paraffin embedding. Blocks were cut into 5um sections, which were stained with Alcian blue, and the presence of glycosaminoglycans was assessed.

2.14. Analysis by TEM (transmission electron microscopy)

AD SCs cells of $0,08 \times 10^6$ were seeded into a well of a 96-well plate. They were stimulated with UG28 peptide at a concentration of 10 μ g/ml, changing the medium every two days and adding the appropriate pre-solubilized amount of peptide. Cells were differentiated with chondrogenesis medium for a period of 14 days. After this time, samples were placed in PBS solution, washed and flooded with 2.5 % glutaraldehyde fixative, at 4 °C for 24 hours. Sucrose cacodylate buffer was washed with fixative, 3 \times 5 minutes each, and contrasted in 1 % osmium tetroxide at 4 °C for 60 minutes. The samples were dehydrated in increasing series of alcohol and propylene oxide. They were then soaked in Poly/Bed® 812 epoxy resin and embedded in gelatin capsules. They were left to polymerize for about 2 weeks in a 37 °C hothouse. After this time, ultra-thin Sections (2–5 nm) of the preparation were cut. The slides were viewed in a JOEL type JEM 1200EXII transmission electron microscope and photographed with a Samsung Electronics camera.

Cells seeded on a chitosan membrane with covalently attached UG28 peptide at a concentration of 10 μ g/ml were cultured in 24-well plates at $0,3 \times 10^6$, differentiated with chondrogenesis medium for 14 days. After culture, the procedure above was followed.

2.15. Statistical analysis

Data represent the mean \pm SEM of four independent experiments (4 replicates in each). Comparisons between groups were made using the Mann-Whitney U test. Statistical significance was determined assuming a two-tailed distribution. Statistically significant results were denoted by * $p < 0,05$. Statistical analysis was performed Statistica 13.3, and for the graphical presentation of the results, the program GraphPad Prism 5.0.

3. Results

3.1. Confirmation AD-SCs phenotype according to ISCT

Confirmation of mesenchymal cell phenotype was checked according to the International Society of Cell and Gene therapy guidelines [39,40]. Mesenchymal stem cells should be plastic adherent, and show the ability to differentiate into adipocytes, osteocytes, and chondrocytes (Fig. S1). They should exhibit the primary stem cell markers CD105, CD90, CD73 in the absence of CD45, CD31, CD19 (Fig. S2).

3.2. Peptide design

Based on literature data [35], a peptide (CDP4), which is a fragment of the CPNE7 protein, was selected (Table 1). Literature data indicate that the CDP4 peptide has excellent pro-regenerative properties in *in vitro* studies. The protein fragment includes amino acid residues 340–350 with the sequence VNPKYKQKRR (Val-Asn-Pro-Lys--Tyr-Lys-Gln-Lys-Arg-Arg). In a further step of our work, we wanted to covalently attach this peptide to chitosan. The covalent attachment of the peptide should guarantee the long-term release of the peptide from the polymeric scaffold (chitosan) into the environment. The release of the peptide into the environment was to be ensured by the presence in

Table 1

The amino acid sequences of the designed peptides. The sequence specific to the MMP-7 enzyme has been marked in red. The cysteine residue, which participates in the Michaelis reaction with the maleimide linker via the SH group, has been highlighted in bold font.

Name	Amino acid sequence
CDP4	V ³⁴⁰ N PKYKQKRR ³⁵⁰
UG28	LEAVNPKYKQKRR
CPLG-UG28	CPLG↓LEAVNPKYKQKRR

the peptide sequence of the PLGJLEA amino acid sequence sensitive to the metalloproteinase type VII (MMP-7), where the symbol "↓" denotes the enzymatic cutting site [lit]. Therefore, considering the activity of the MMP-7 enzyme, a peptide with the active peptide sequence of CDP4 including the cut fragment (the sequence on the right side of the arrow) i.e. LEAVNPKYKQKRR should be released into the environment. Therefore, the UG28 peptide was synthesized and subjected to biological studies. This peptide proved to have very good pro-regenerative properties.

In a further step of the work, a peptide with the sequence CPLGLEAVNPKYKQKRR (CPLG-UG28) containing a cysteine residue at the N-terminus to facilitate the attachment of the peptide to maleimidochitosan was also synthesized, followed by a fragment specific for the MMP-7 metalloproteinase (PLGLEA) and the active peptide sequence UG28. The attachment of the peptide to chitosan was performed by forming a covalent bond between the thiol group of the peptide and chitosan (Michael reaction), using a maleimidoglycine linker. The polymer thus obtained was subjected to biological tests. The polymer thus obtained was used to form a membrane, which was subjected to biological testing.

3.3. Proliferation assay

To determine cell proliferation, the XTT colorimetric assay was used to analyse the viability of AD-SCs cells after stimulation with UG28 peptide, relative to controls after 48 and 72 h. Cells were exposed to peptide derivative in a concentration range from 0.01 to 150 µg/ml.

In mesenchymal cell viability studies, the UG28 peptide showed a pro-proliferative effect. It increased the viability value by approximately 35 % at concentrations of 10 and 25 µg/ml at two-time points after 48 and 72 h, as well as a concentration of 50 µg/ml after 72 h stimulation with the peptide. A slight proliferative effect was seen for concentrations of 1 and 100 µg/ml after 48 h of culture with the peptide. The results showed a level of statistical significance.

3.4. Cytotoxicity assay

A method of assessing the cytotoxicity of compounds, and peptide based on the LDH colourimetric assay measuring the activity of lactate dehydrogenase (LDH), which is released into the environment, i.e. into the filtrate, medium, after cell membrane damage. The cytotoxicity of the peptide derivatives was tested at the three highest concentrations of 50, 100, and 150 µg/ml after 48 h.

Lactate dehydrogenase levels in the culture medium of the cells after stimulation with the peptides showed no cytotoxicity in the experiment. In the case of cells treated with TRITON X 100 (positive control), a statistically significant increase in dehydrogenase was observed.

3.5. Migration assay

A scratch overgrowth assay in a 24-hour culture was used to assess cell migration under the influence of a specific peptide concentration. The images taken of the scratch overgrowth area after incubation were then analysed.

The peptide had a small effect on cell migration at a concentration of 10 µg/ml, about 10 % compared to the control, the result reached a statistically significant level. At a concentration of 25 µg/ml, the peptide-induced cells to move by about 40 %, no statistically significant differences were obtained.

3.6. Collagen production

After stimulation with UG 28 peptide at a concentration of 10 µg/ml, an increased level of collagen synthesis by adipose tissue mesenchymal cells up to approximately 14 % was noted, a result that was statistically significant compared to the control. At a concentration of 25 µg/ml, cells

induced with the peptide showed no increase in collagen synthesis, no statistically significant differences. Cells were cultured for 14 days.

3.7. Immunogenicity assay

CD25/CD69 early activation antigens and CD71/HLA-DR late activation antigens do not show increased levels of activated human Th and Tc lymphocytes relative to UG28 peptide and chitosan.

3.8. Basophil assay

A basophil activation test performed on blood samples from five volunteers showed in four patients the absence of allergenic potential of the UG28 peptide at both concentrations, while a slight basophil degranulation effect above a threshold value of 5 % was seen in one patient.

3.9. Analysis of differentiation

The graph shows AD-SCs cells treated with MesenPro (non-differentiating) medium as control (C), cells cultured in Chondro differentiation medium as a control for cells treated with differentiation medium with UG28 peptide. Cells were cultured for 14 days.

After stimulation with UG28 peptide at a concentration of 10 µg/ml, an increase in glycosaminoglycan synthesis by adipose tissue mesenchymal cells by 30 % compared to the control without the peptide was observed, the result was statistically significant.

3.10. Analysis of gene transcripts

Diagnostics for Mycoplasma fermentans infection did not reveal the presence of Mycoplasma fermentans DNA in the samples tested.

Transcript analysis of SOX9, COMP and MMP13 genes, showed significantly low levels of COMP and MMP13 expression after 14 days of culturing AD-SCs cells in a differentiation medium with added UG28 peptide at a concentration of 10 µg/ml. SOX 9 gene expression remained at the same level of amplification efficiency $\Delta\Delta C_t$ as control (n=4).

3.11. Histological analysis

The histological preparation in image 9 shows differentiated chondrocytes (Fig. 9C, D) together with the extracellular matrix stained with Alcian blue. From the outer part of the clustering of cells, a fibrous 'membrane' resembling connective tissue is visible just near the surface.

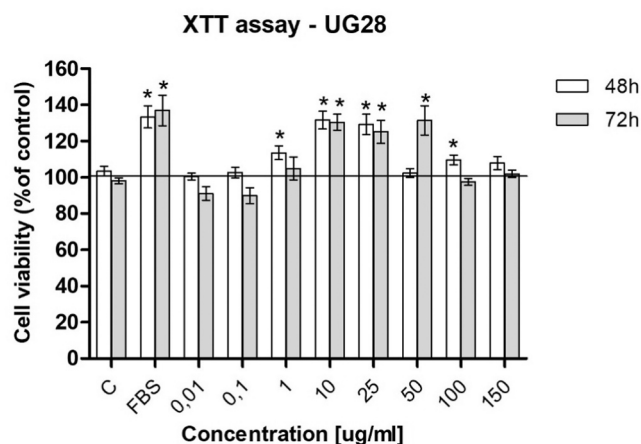


Fig. 1. Effect of UG28 peptide on the number of viable AD-SCs cells after 48 and 72 h incubation. Graph shows mean SEM± from 4 independent experiments (4 replicates in each, n=16), *- statistically significant differences compared to control, Whitney's U-Mann test, p<0.05.

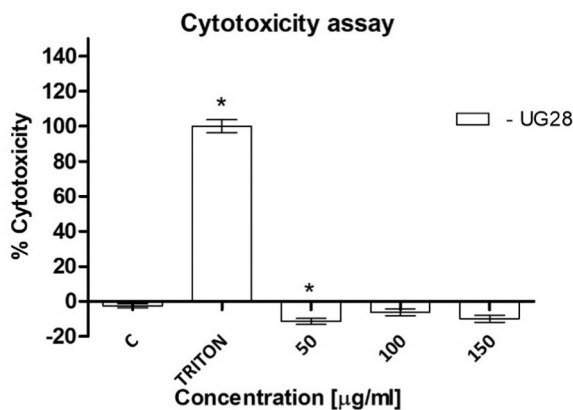


Fig. 2. Analysis of the cytotoxicity of UG28 peptide against AD-SCs cells after 48 h incubation. Graph shows mean SEM \pm from 4 independent experiments (4 replicates in each, n=16), *- statistically significant differences compared to control, Whitney's U-Mann test, p<0.05.

Fig. 9D shows the chondrocyte cells after mitotic division, which do not diverge but form groups of two to five cells banded by a 'capsule'. The chondrocytes formed lie in a common niche, the cavity, resembling the structure of the isogenic group of vitreous cartilage.

3.12. Analysis by TEM

Photographs of transmission electron microscopy slides show the structure of chitosan Fig. 10A and chondrocytes differentiated in 14-day culture of control Fig. 10B and UG28 peptide-treated chondrocytes at a concentration of 10µg/ml Fig. 10C.

Fig. 10B. Ultrastructure of AD-SCs stem cell after 14 days of culture in chondrogenic medium. Image taken by electron microscopy (TEM), X12,000. L - liposome, G - glycoproteins, V - vacuoles, N - nucleus. When examined by transmission electron microscopy, multiple liposomal vesicles (L), a nucleus with euchromatin (N) with a U-shaped nuclear membrane structure, multiple vacuoles (V) merging into one space, several granules of glycoproteins (G) in the cytoplasm, and no visible extracellular matrix elements are visible. Fig. 10C image of cells treated with chondrogenesis differentiation medium supplemented with UG28

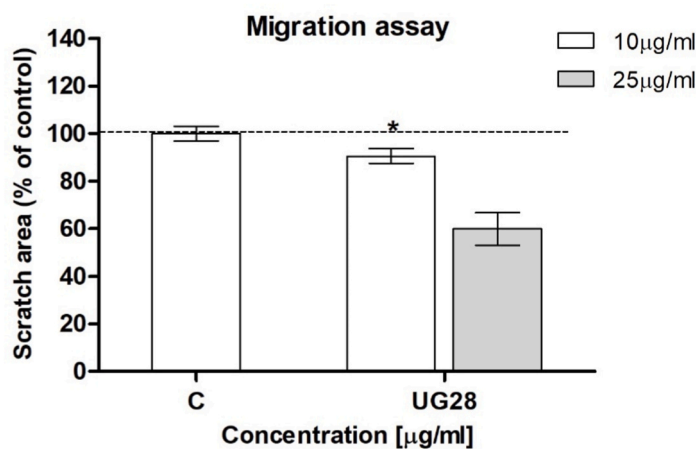


Fig. 3. Effect of the peptide on migration towards AD-SCs cells. Effect of UG28 peptide, at two concentrations, on the migration of AD-SCs after 24 h of incubation. The graph shows mean \pm SEM of 5 independent experiments (each in 4 replicates). *statistically significant differences - overgrowth of the scratch area reduces the bar value resulting from AD-SCs migration compared to control-C, U-Mann-Whitney test, p<0.05. The images show an example of cells overgrowing a crack. (A) control, (B)- stimulation with UG28 peptide at a concentration of 10 µg/ml, (C) stimulation with UG28 peptide at a concentration of 25 µg/ml.

peptide.

Cells in culture with the peptide were characterized by the presence of large Golgi vesicles (AG) just above the nuclear membrane. Fig. 11E. Numerous exocytic vesicles (SV) are seen in the cytoplasm just below the cell membrane. Fig. 11D, open to the extracellular space, originating from the Golgi apparatus (AG), releasing its contents into the extracellular space (X), most likely consisting of proteoglycans and tropocollagen. Active and dense rough endoplasmic reticulum (rER) with multiple ribosomes visible Fig. 11B, C indicates increased protein synthesis. Concentrations of ribosomes (r) and mitochondria (M) are also visible in the cytoplasm. Fig. 11A. In the extracellular matrix of Fig. 11D and F, characteristic fibers with inserts that build collagen I and II (C) and many protein granules that play an important role in tissue differentiation.

3.13. Live/dead staining imaging

AD-SCs cells stained with calcein and propidium iodide in control culture without chitosan media and on 75/500 chitosan membranes after 24 h, 48 h, and 72 h. Images were taken by fluorescence microscopy, 100X

The study showed that chitosan membranes with peptide do not produce a significant decrease in viability of AD-SCs stem cells, in any of the three time measurements against the control without membrane.

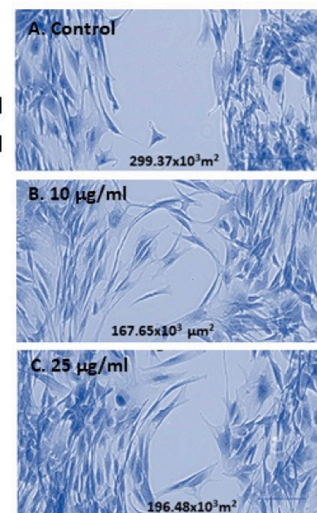
3.14. TEM analysis - AD-SCs in ChondroStem Pro medium on chitosan membrane with CPLGUG28-10 µg/ml

See Fig. 13

4. Discussion

Cartilage regeneration is a slow and difficult process mainly due to the lack of vessels and nerves in the cartilage structure, leading to challenging environment in the supply of growth factors, oxygen, nutrients and exogenous biochemical mediators. Therefore, many studies are focusing on the use of cell therapies in advanced arthritis lesions [41]. While most of the studies administered stem cells intrathecally to the site of cartilage damage [42–44], only a few of them focus on implantation of scaffolds or matrices with mesenchymal cells.

In our study, we investigated the effect of a novel UG28 peptide and



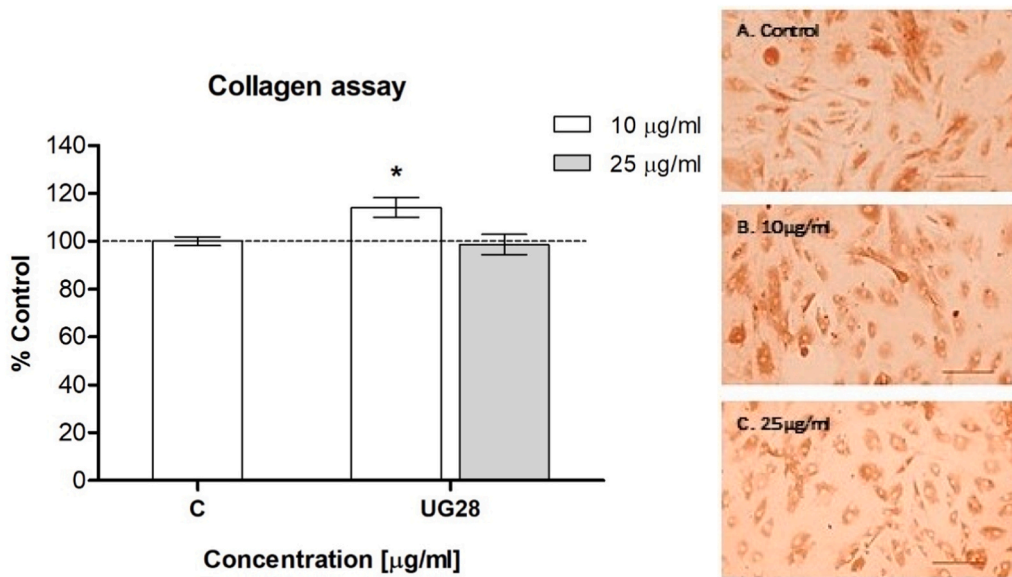


Fig. 4. Effect of UG28 peptide at a concentration of 10µg/ml on collagen synthesis by AD-SCs. (A) control, (B)- stimulation with UG28 peptide at a concentration of 10 µg/ml, (C) stimulation with UG28 peptide at a concentration of 25 µg/ml.

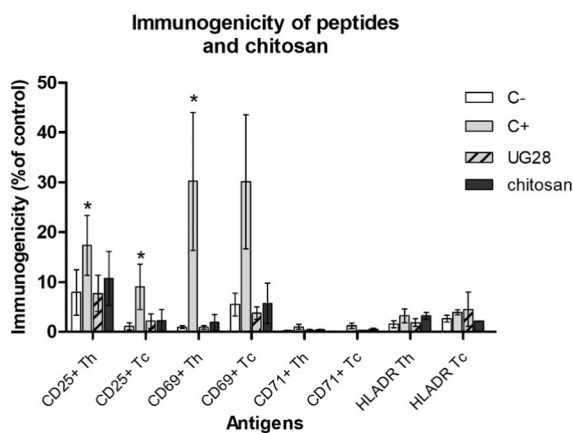


Fig. 5. Analysis of immunogenicity markers on Th -helper and Tc- cytotoxicity against UG28 peptide derivative, at a concentration of 10 µg/ml and chitosan. Graph shows mean ±SEM of 4. Independent experiments (4 replicates in each, n=16), *- statistically significant differences compared to control, Whitney’s U-Mann test, p<0,05.

chitosan substrates on the biological activity and differentiation of adipose tissue stem cells. We demonstrated its stimulating chondrogenic properties on human adipose tissue-derived mesenchymal cells.

Based on our team’s research [45–47] a wide range of concentrations (0.01–150 µg/ml) of the tested compound was selected for proliferation assays. Testing cytotoxicity on human cells is essential when evaluating potential compounds or future drugs, as certain concentrations may prove toxic. It is known that they can be toxic to cells at selected concentrations, so the focus was on the highest concentrations of the peptide (50–150 µg/ml), as the results of the proliferation assay of lower concentrations showed a high proliferative value. Following these results, two concentrations (10 and 25 µg/ml) with the best proliferation rates were selected and used in the migration and collagen synthesis assays. As it is reasonable to use lower drug concentrations, cell differentiation analyses of AD-SCs were conducted at a concentration of 10 µg/ml of UG28 peptide, which allowed a higher chondrogenesis result compared to the control.

In the first steps, we evaluated the proliferative potential and

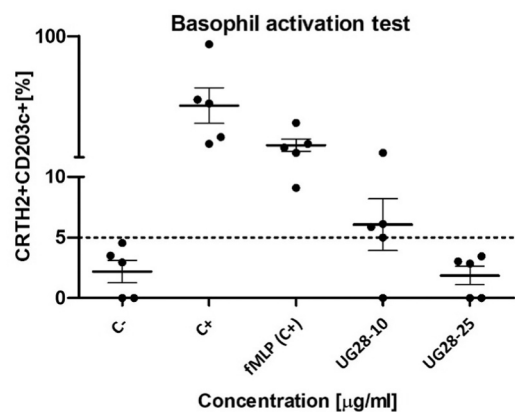


Fig. 6. Activation of basophils after 15 minutes of incubation with CDP peptide derivatives. Values represent the percentage of activated CRTH2+CD203c+ basophils for individual patients (n=5) and the median. Threshold value for allergic effect ≥ 5 %.

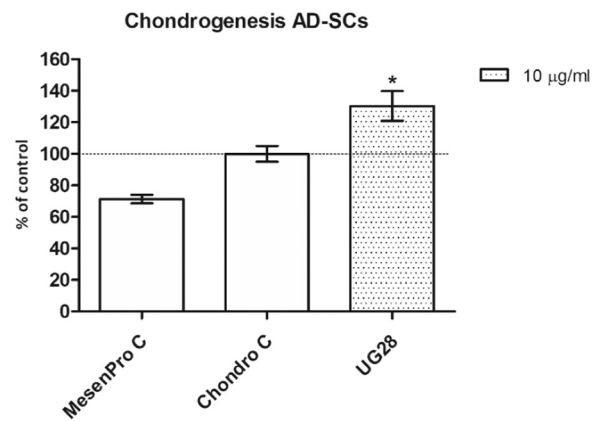


Fig. 7. Differentiation of adipose tissue mesenchymal cells with UG28 peptide at a concentration of 10 µg/ml. Graph shows mean SEM± from 5 independent experiments (4 replicates in each, n=20), *- statistically significant differences compared to control, Whitney’s U-Mann test, p<0.05.

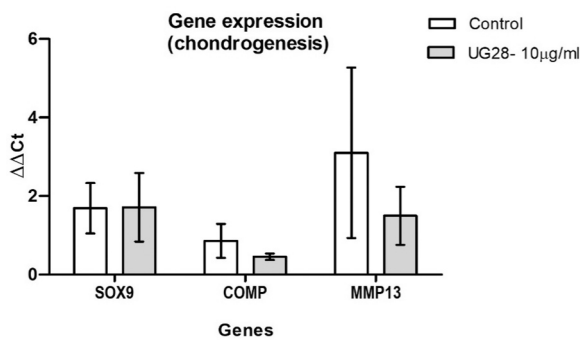


Fig. 8. Evaluation of gene expression on AD-SCs cells undergoing differentiation towards chondrogenesis after the addition of UG28 peptide. Determination of specific markers for chondrogenesis by qPCR for 4th patients. Data obtained from RT-PCR was normalized to the reference gene RFLP13. No statistical significance.

cytotoxicity of the peptide to determine the optimal concentration of the peptide. We observed the highest viability after the 2nd and 3rd day of culture at peptide concentrations of 10 and 25 µg/ml (Fig. 1). Similar observations were made against BMP2 stimulation, bone marrow-derived MSC cells [48] and adipose tissue-derived MSCs stimulated with BMP6 [49].

Safety analysis of UG28 peptide on human immune cells showed minimal activation of lymphocytes and basophils *in vitro*. (Figs. 5, 6).

Previous studies conducted at our group [50] have shown that peptides obtained by chemical synthesis can induce basophil activation significantly more frequent in patients with allergies compared to healthy volunteers.

Collagen synthesis is a critical factor in extracellular matrix formation, accounting for as much as 70 % of articular cartilage [51]. Quantitative analysis of collagen synthesis in our experiments with UG28 peptide showed an increase in the production of this protein at low concentrations of the peptide (Fig. 4), just as supplementation with TGF β1,2,3 increased the deposition of type II collagen and proteoglycans in the ECM [52–54]. In TEM studies, we confirmed the deposition of collagen proteins in the form of fibrils (Fig. 11D, F).

Cell migration in a healing wound is an important process affecting the speed of defect regeneration, so we examined AD-SCs in the scratch assay. Cells migrated slower in low concentration of the peptide, and in high concentration the result was higher but did not reach statistical significance (Fig. 3). Xia Y., studied the migration of BM-MSCs cells after IGF-1 stimulation, noted that the migration ability depends on the concentration of the stimulatory molecule, which activates the ERK1/2 signaling pathway via IGFR without activating p38. Perhaps the concentration of UG 28, was too low to activate signaling pathways faster, this would need to be further investigated [55].

Histologically, after 6 weeks of cell differentiation under the influence of the peptide, clusters of cells with isogenic niches (Fig. 9), typical of cartilaginous vitreous tissue, were visible [56]. The accumulation of cells indicated a variety of changes in AD-SCs under the influence of the peptide, the outer layer of pellet cells formed a structure similar to the

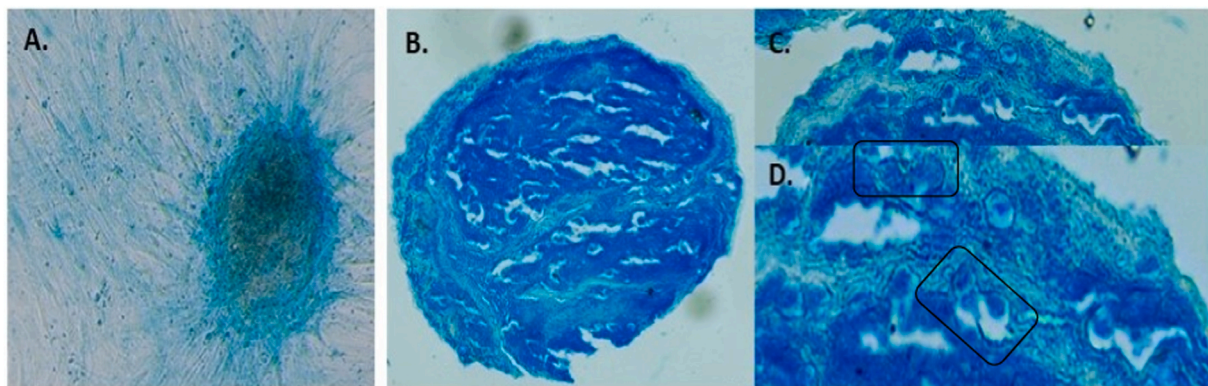


Fig. 9. Image of Alcian blue stained AD-SCs cells after 6 weeks of culture in differentiation medium with UG28 peptide at a concentration of 10 µg/ml. Density of AD-SCs cells differentiated towards chondrogenesis after stimulation with UG28 peptide at a concentration of 10 µg/ml after 6 weeks of culture. Photo (A) shows AD-SCs cells treated with MesenPro non-differentiating medium, magnification 100X (B) magnification 100X, shows embryonic cartilage tissue with differentiated histological tissue. (C) magnification 200X. (D) shows isogenic groups with chondrocytes, at 400X magnification. The photograph was taken with a Leica inverted light microscope.

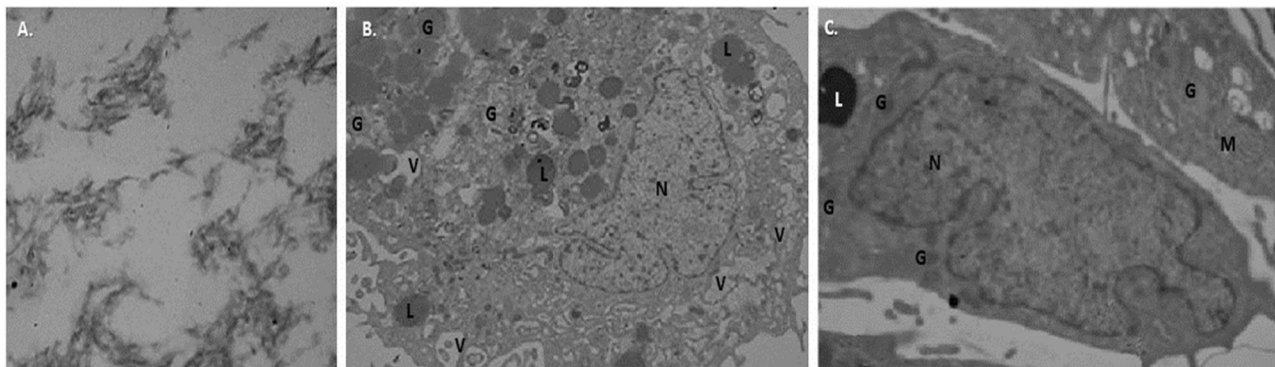


Fig. 10. (A) Shows an image of chitosan in TEM, without cells. (B) Cell treated with ChondroStem Pro differentiation medium, (C) Cells treated with differentiation medium with UG28 peptide at a concentration of 10 µg/ml.

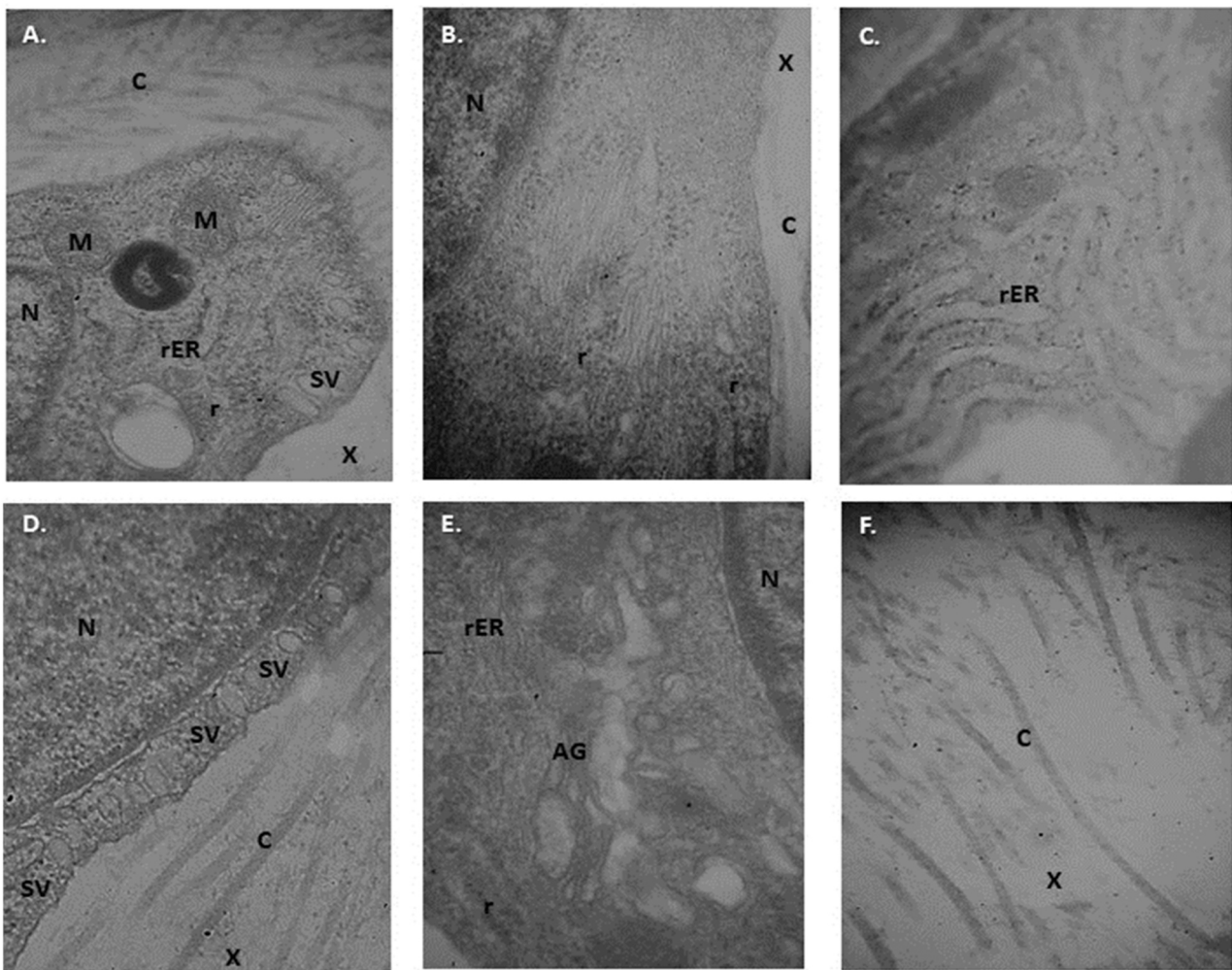


Fig. 11. Structural elements of a cell differentiated in ChondroStem Pro medium after 14 days of culture with UG28 peptide at a concentration of 10 $\mu\text{g}/\text{ml}$. Elements of the cell structure differentiated in Chondro medium after the addition of the UG28 peptide at a concentration of 10 $\mu\text{g}/\text{ml}$. (A) shows a cell with visible mitochondria and secretory vesicles, (B) - visible actin filaments with ribosomes, (C) - rough endoplasmic reticulum, (D) - active exocytic vesicles, (E) - large Golgi apparatus, (F) - collagen fibers with extracellular matrix. AG - Golgi apparatus, rER - rough endoplasmic reticulum, SV - exocytic vesicles, r-ribosomes, C - collagen fibers, M - mitochondrion, N - nucleus, X - extracellular matrix, magnification 50,000X.

cartilaginous, looser lighter stained, the interior of the pellet stained darker in the Alcian Blue test for glycosaminoglycans (Fig. 9B), as in Zuliani's studies under the influence of IGF1 on amniotic fluid stem cells [57].

In our quantitative studies involving the peptide, we noticed an increased in glycosaminoglycan synthesis compared to controls without the peptide (Fig. 7). Literature findings also indicate that MSCs stimulated with other compounds, e.g. $\text{IFN}\gamma$ (interferon γ), BMP, TGF exhibit enhanced differentiation capacity into chondrocytes [58]. It was also proved that pretreatment with $\text{TNF}\alpha$ modulates SOX11 and β catenin levels, increases canonical WNT signaling in MSCs, stabilizing SOXC expression increasing chondrogenic potential.

Since the regulator of chondrogenesis is the transcription factor SOX9, which induces its own transcription, in addition to the transcription of Sox5 and Sox6, forming the Sox Trio [59,60], which stimulates the overexpression of Col2a1 (collagen- α 1, type II), Acan (ang. Aggrecan) [59,61,62] it seemed reasonable to analyze the expression of the SOX9 gene, as well as the oligomeric cartilage matrix protein COMP and MMP13. Transcriptomic studies of RT PCR reactions showed slightly increased expression for the SOX9 gene (Fig. 8), identical results were obtained by Huang Y, after 14 days of culture. In experiments on chitosan Huang Y., notes a much higher expression of the SOX9 gene at 14 days, and a decrease at day 28 [63]. The authors suggest that the

decreased values may be the reason for the differentiated cells of AD-SCs and increase the expression of late-stage chondrogenesis markers such as COL2A1 and ACAN [64]. The expression of COMP genes in our experiments correlated with a low Huang Y score after 14 days, this may signal the regulation of chondrocyte metabolism into a negative feedback loop and intensive joint matrix synthesis [63,65,66]

Transmission electron microscopy showed that stromal cells on the chitosan construct exhibiting features indicative of chondrocyte differentiation. Visible on TEM images, cells cultured in ChondroStemPro medium with peptide were characterized by the presence of large Golgi apparatus vesicles located just above the nuclear membrane (Fig. 11E). Numerous exocytic vesicles visible in the cytoplasm beneath the cell membrane opened into the extracellular space most likely releasing proteoglycans and tropocollagen which in the matrix form characteristic fibers with inserts that build type II collagen (Fig. 11 D,F) [67] and many protein granules that play an important role in the differentiation of the extracellular matrix compartment (Fig. 11 D,F) [68]. An active and dense rough endoplasmic reticulum with many visible ribosomes indicates increased protein synthesis (Fig. 11C). Concentrations of ribosomes and mitochondria in the cytoplasm are also evident (Fig. 11A,B). These structures distinguish cells cultured in the directing medium alone, which have vacuoles, numerous liposomes and probably glycoproteins.

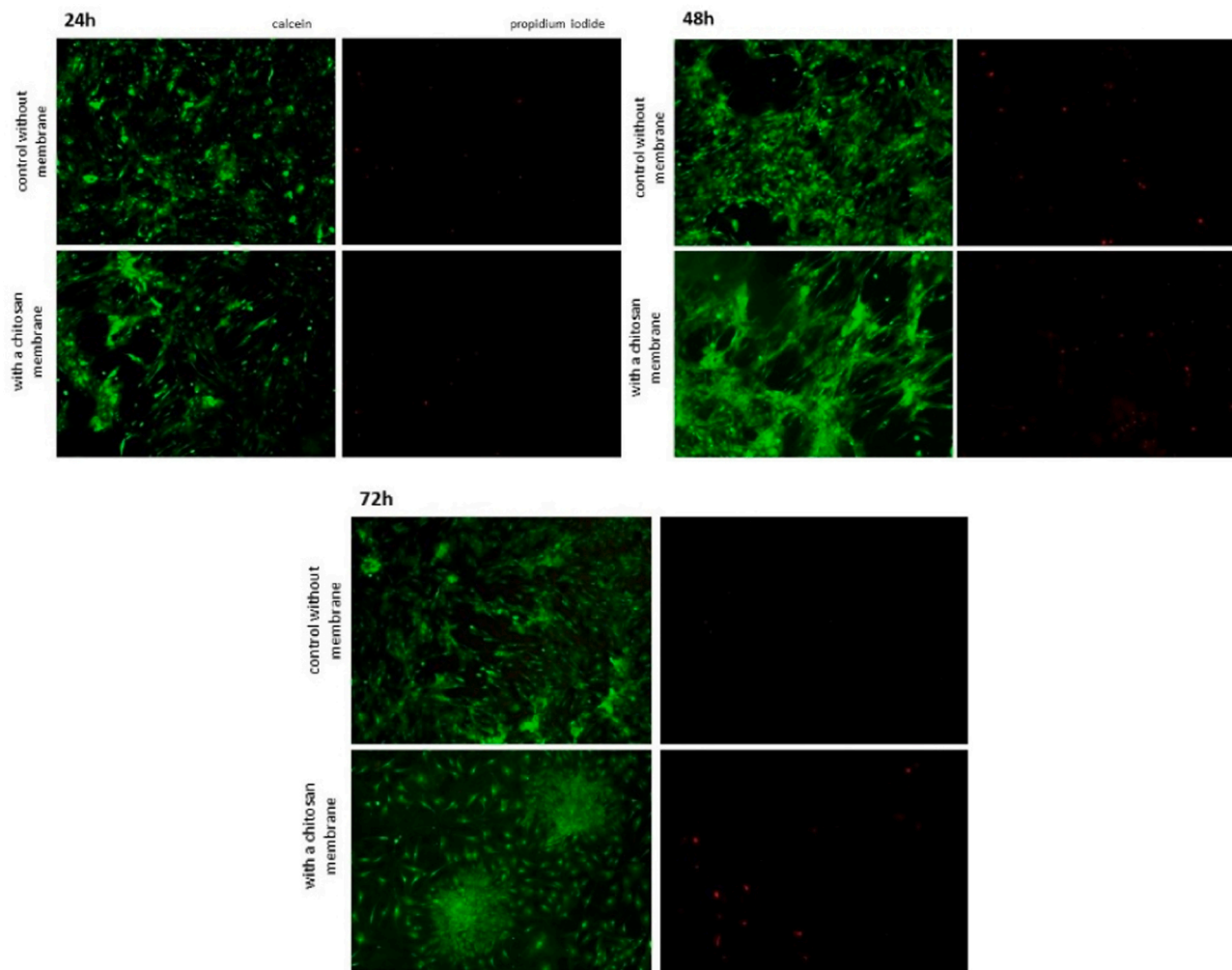


Fig. 12. Evaluation of the effect of chitosan membranes with covalently attached UG28 peptide (CPLUG28), at a concentration of 10 $\mu\text{g/ml}$, on the viability of AD-SCs at 24, 48, and 72 h.

As reported by Huang Y., culturing AD-SCs cells on chitosan polymer, which shares structural similarities with glycosaminoglycans stimulate the formation of cartilaginous structures [63]. In our study, two forms of chitosan deacetylation (95 % and 75 %) were analyzed in the form of gel and lyophilzate (Figs. S3-S7), from which the concentration of deacetylation (75 %) and average molar mass of 500 emerged, which is associated with better viscosity, solubility, breaking strength and elasticity [69]. It also offers the possibility more effective flow of medium and distribution of extracellular matrix and occupying positions at different levels of the construct. The peptide in low concentration was covalently attached to chitosan. A chitosan membrane was formed from such a formulation. Fluorescence tests confirmed the high viability of AD-SCs stem cells (Fig. 12). At the same time, the hydrolytic environment of the medium did not cause degradation of the membrane structure even after 3 days of cell culture.

In the comparative chondrogenesis results of Huang Y., two biomaterials chitosan and chitosan/HA, the researchers showed the superiority of the mixed material over chitosan alone [63]. They supported such conclusions with high level of the CD44 glycoprotein on AD SCs, which is involved in matrix-cell interactions. HA is a ligand for CD44, which may promote the initiation of chondrogenesis and the differentiation of chondrocytes [70,71] In the following sentences, the authors conclude that chitosan or chitosan/HA have a limited capacity to differentiate chondroblasts up to 7 days and the addition of morphogens is an important issue of cell differentiation potential [63]. Combining

chitosan with a morphogen-acting peptide showed differentiated AD-SCs localized in isogenic niches (Fig. 13A, B), rER activity, increased numbers of ribosomes and mitochondria (Fig. 13C, D), in the ECM collagen and most likely glycoproteins (Fig. 13E, F). Chitosan provides a favorable environment for signal transduction in cartilage tissue regeneration.

Our studies have demonstrated the formation of cartilage tissue on a biodegradable chitosan membrane with an attached UG28 peptide (Fig. 13), which may contribute to faster regeneration of cartilage defects [63]. Such a formulation still requires preclinical and clinical studies but could be a promising biomaterial for plastic, aesthetic or sports medicine applications.

5. Conclusions

Our study demonstrates that chitosan membranes, combined with the UG28 peptide, effectively stimulate adipose-derived stem cells (AD-SCs) to produce extracellular matrix and differentiate into chondrocytes. This chitosan-UG28 composite shows considerable promise as a biomaterial for tissue engineering and regenerative medicine mainly for treating articular cartilage damage. Compared to conventional treatment, such as pharmacological approaches, physical therapy, or surgical interventions, this composite offers a regenerative strategy that targets the main cause of cartilage deterioration. Existing treatment options often provide limited long-term efficacy and focus primarily on pain

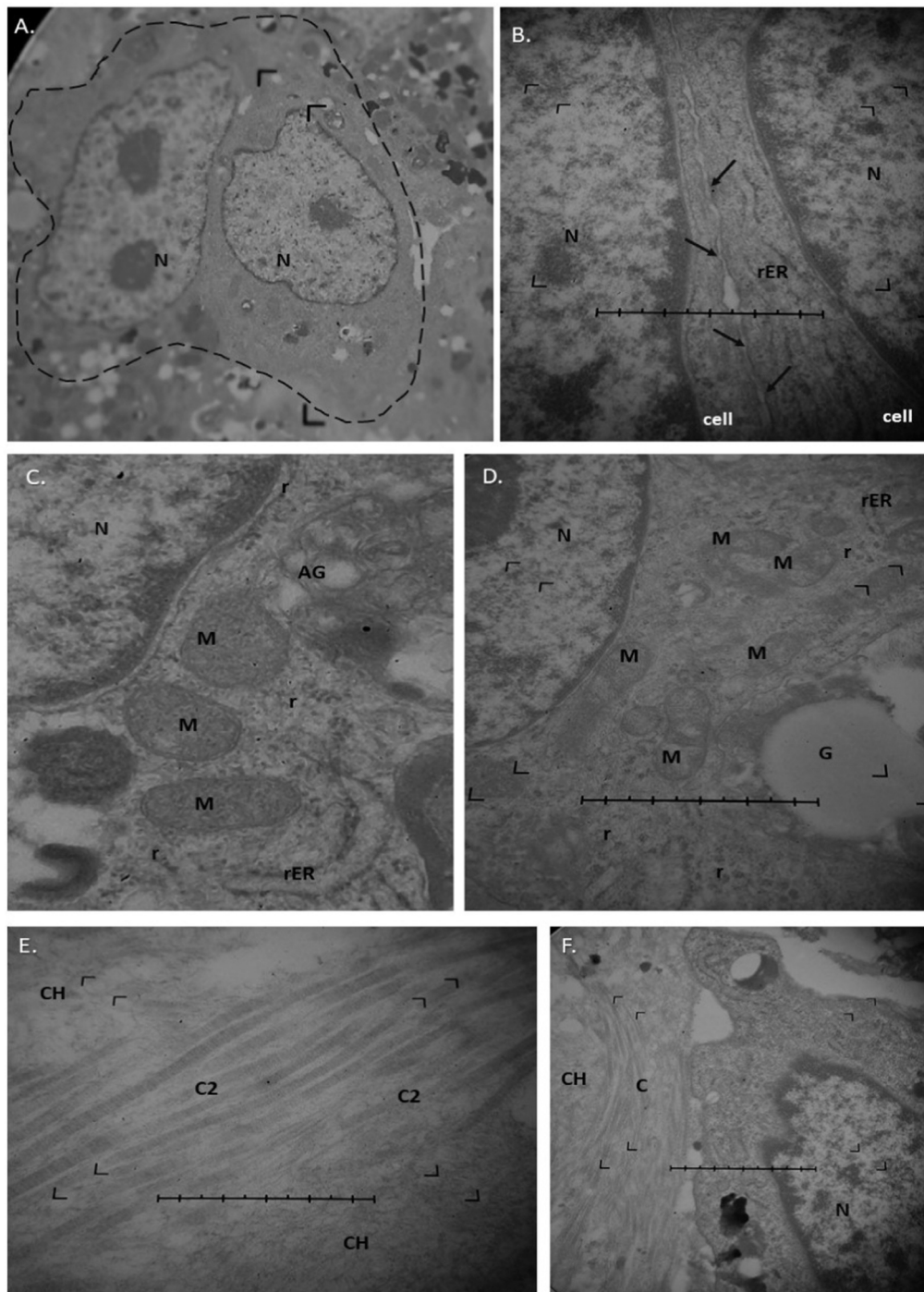


Fig. 13. Evaluation of the effect of chitosan membranes with covalently attached UG28 peptide, at a concentration of 10 $\mu\text{g/ml}$, on the differentiation of chondrogenic cells. AD-SCs cells after 14 days of culture in chondrogenic medium on chitosan membrane with UG28 peptide at a concentration of 10 $\mu\text{g/ml}$. (A) - loop shows isogenic niches, (B) - two cells in close proximity touching cell membranes (black arrows), (C) - chondroblast with numerous mitochondria and Golgi apparatus, magnification X50,000, (D) - chondroblast with mitochondria and probably glycoproteins. (E) - bands of type II collagen visible on chitosan membrane, (F) - chondroblast lying on chitosan membrane, magnification X25,000, (arrow indicates exocytic vesicle AG - Golgi apparatus, N - cell nucleus, rER - endoplasmic reticulum, C - collagen, C2 - type II collagen, CH - chitosan membrane, M - mitochondria, G - glycoprotein-like structures.

management or symptom reduction. In contrast, the chitosan-UG28 system offers a minimally invasive approach with the potential to not only restore cartilage structure but potentially also to improve joint function through enhanced chondrogenesis. Future research will focus on optimizing this composite for clinical application, assessing its long-term effects, and exploring its potential for broader use in other types of cartilage repair and regeneration.

CRediT authorship contribution statement

Jacek Zielinski: Resources. **Karolina Kondej:** Resources. **Adrianna Banach-Kopec:** Methodology. **Szymon Mania:** Methodology. **Aneta Skoniecka:** Visualization, Methodology. **Małgorzata Zawrzykraj:** Visualization, Methodology. **Sylvia Rodziewicz-Motowidło:** Writing – review & editing, Supervision, Funding acquisition. **Agata Tyminska:** Writing – review & editing, Writing – original draft, Visualization,

Methodology, Data curation, Conceptualization. **Michał Pikula**: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Natalia Karska**: Methodology. **Piotr M. Skowron**: Funding acquisition. **Robert Tylingo**: Supervision. **Katarzyna Gurzawska-Comis**: Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michał Pikula reports financial support and equipment, drugs, or supplies were provided by National Centre for Research and Development. Michał Pikula reports a relationship with Medical University of Gdansk that includes: employment. Michał Pikula has patent #National patent application (P.448148), Poland pending to patent office of the republic of Poland [WIPO ST 10/C PL448148]. If there are other authors, they 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. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2024.117683](https://doi.org/10.1016/j.biopha.2024.117683).

Data availability

Data will be made available on request.

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