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Effects of polyacrylamide hydrogel used in the treatment of osteoarthritis on mesenchymal stem cells and human osteoblasts

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Ethics Committee Approval
The study was approved by the Istanbul Medipol University Noninterventional Clinical Research Ethics Committee (number: E-10840098-604.01.01-19391) Date: 03/07/2020.
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

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No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Adipose-derived mesenchymal stem cells (AD-MSCs) have gained popularity for use in treating osteoarthritis (OA) although their long-term effects remain unsatisfactory for treating the end stages of OA. However, clinically injectable polyacrylamide hydrogels (PAHGs) remain in the joint indefinitely, which could make them ideal candidates as AD-MSC carriers. Our aim was to evaluate whether combinations of PAHG and AD-MSCs have positive effects on cell viability, thereby the potential of this combination prior to clinical use in OA treatment in addition to the effects of PAHG on human osteoblasts (HOBs).
Methods: Cell lines of AD-MSCs of canine origin and HOBs were culture-expanded and seeded in 96-well plates (0.5 x 10^5 cells/well). The PAHG substrate at doses of 2, 6, 10, 20, or 40 µL per 200 µL of sample were added to the wells, and their effects were compared to the positive and negative control groups and among substrate doses for each cell line. The experiments were repeated three times, and cell viability was studied using tetrazolium (MTT) method.
Results: Cell viability in all dose groups was significantly greater than that of their negative control groups for both cell lines ($P < 0.001$). Among the different dose groups, significant dose-dependent viability increases were only observed for the HOB cell line ($P < 0.001$). The PAHG substrate was not lethal to AD-MSCs or HOBs up to the maximum assayed doses and had positive effects on the viability of these cell lines, including slight increases in proliferation.
Conclusion: Combination of PAHG with AD-MSCs may have positive long-term effects for OA treatment. However, further trials are needed.

Keywords: Adipose-derived mesenchymal stem cells, Cell viability, Human osteoblasts, Polyacrylamide hydrogels, Osteoarthritis

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Introduction

Osteoarthritis (OA) is a highly prevalent joint disease worldwide [1]. OA is characterized by degeneration and loss of articular cartilage in association with changes in the underlying subchondral bone [2]. OA is not currently a curable disease because its pathophysiology and mechanism of progression remain incompletely understood. Therefore, the goal of OA treatment is simply to reduce pain and inflammation to allow a patient to maintain a better quality of life. The therapeutic spectrum ranges from conservative treatments (such as weight loss, physical therapy, biomechanical interventions, and intra-articular injections) to joint replacement surgery [1, 3].

Recently, adipose-derived mesenchymal stem cell (AD-MSC) therapy has increased in popularity for the treatment of OA and emerged as an intriguing therapy option because of their immunomodulatory behavior and potential to differentiate into chondrocytes and osteoblasts [4-6].

Polyacrylamide hydrogels (PAHGs) are fully synthetic, nontoxic, highly biocompatible, biostable filling materials that are commonly used in aesthetic and urologic interventions [7–9]. Because of their resistance to biodegradation, injectable PAHGs are used as long-term visco-supplementation agents in the treatment of OA, especially in Russia and Asian countries [10]. Furthermore, PAHGs have been widely researched *in vitro* as scaffolds of various stiffnesses to study the differentiation pathways of stem cells based on the mechano-transduction properties of the medium [11]. However, no *in vitro* or *in vivo* trials have been described in which the effects of clinically injectable PAHGs on mesenchymal stem cells (MSCs) and human osteoblasts (HOBs) have been evaluated together.

In the present study, we hypothesized that injectable PAHGs are good agents to promote AD-MSC survival and can facilitate the proliferation of AD-MSCs and human osteoblasts such that a PAHG and AD-MSC mixture appeared to be a good candidate for the treatment of OA. The study also aimed to establish the scientific basis of this treatment for further clinical trials.

Materials and methods

Ethics committee approval

Our experiments were approved by Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (number: E-10840098-604.01.01-19391) on 03/07/2020.

Cell lines and cultivation

In our present study, canine AD-MSCs (cAD-MSCs) and a human osteoblast (HOB) cell line were used. HOB cells were obtained from PromoCell, Heidelberg, Germany (C-12720), while AD-MSCs of canine origin were purchased from Tekgen Health Services Company, İstanbul, Turkey (VetStem-D009/40) on January 24, 2020. Human Osteoblast Growth Medium (Merck- 417-500) supplemented with 10% fetal bovine serum (Sigma Aldrich-F7524) and a 1% antibiotic–antimycotic (Sigma Aldrich- A5955) solution was used to expand the HOB cell line. Low glucose Dulbecco's Modified Eagle's Medium (DMEM) from Biowest-L0060 supplemented with 15% fetal bovine serum (Sigma Aldrich-F7524), 2% L-glutamine (Sigma Aldrich- G6392), and 1% antibiotic–antimycotic solution (Sigma Aldrich A5955) was used to expand the cAD-MSCs. Frozen vials of cells were removed from a nitrogen tank and heated at 37 °C for 1 to 2 min after which the liquid suspensions of cells were transferred into a sterile tube and brought to a final volume of 5 ml with appropriate media. Subsequently, the cell suspensions were centrifuged at 1500 rpm for 5 min after which the supernatant was removed, and the cell pellet was suspended in 1 ml of medium before being transferred to T25 flasks containing 4 ml of medium. Subsequently, the cells in the flask were placed in a 37 °C incubator under an atmosphere with 5% CO_2 for cultivation.

Characterization of AD-MSCs

Stem cells should highly express CD105, CD146, CD73 surface antigens and low levels of anti-HLA-DR, CD19, and CD25 surface antigens. The flow cytometry results were obtained from the company from which the cells were provided and is presented in the results section of the present study.

Demonstration of AD-MSC nuclear morphology by DAPI staining

Cells cultured in T-25 flasks were removed by trypsinization and enumerated before being seeded in 8-well chambers (Ibidi- 80841**)** at 3,000 cells per well. The medium was aspirated the following day after the cells reached confluence and then washed once with phosphate-buffered saline ([PBS] Sigma Aldrich-BSS-1006). Subsequently, the cells were fixed in 10% neutral buffered formalin for 5 min and then rinsed briefly with PBS before undergoing permeabilization with 0.5% Triton X-100 for 10 min. After permeabilization, the cells were washed once with PBS for 5 mins, covered with a 300 nM 4'-6-diamidino-phenylindole (DAPI) staining solution and then incubated for 5 min while being protected from light. The staining solution was then removed, and the cells were washed three times with PBS. Images were recorded with a Zeiss Lsm 800 confocal microscope at a laser excitation of 405 nm.

Preparation of synthetic implant doses based on polyacrylamide hydrogel

Noltrex™ (Bioform, Moskow, Russia) was selected as the PAHG substrate. Noltrex™ is a fully synthetic, three-dimensional (3D), cross-linked polyacrylamide hydrogel that is available in a volume of 2.5 ml. Furthermore, Noltrex™ has a high viscosity and consists of 4.0% ± 1.5% cross-linked polyacrylamide, 96.0% ± 1.5% purified water, and 0.001%–0.0025% silver ions. Stock concentrations were prepared in a 1:1 ratio by mixing 500 µL of Noltrex™ with 500 µL of the medium used for cell culture cells.

Cell viability assays

Following aspiration of the cultured cell medium in T75 flasks, the cells were washed with sterile PBS after which 1 ml of trypsin/ethylenediaminetetraacetic acid (EDTA) from Sigma Aldrich (T4049) was added, and after 3 min in the incubator, the cells were removed. After a 5 min centrifugation step at 1500 rpm, the supernatant was discarded, and the cells were resuspended in medium. A Scepter Automated Cell Counter was used for cell counting. Cells were seeded into 96-well plates (0.5×10^5 cells per well) and cultivated for 24 h until reaching confluence. Subsequently, the medium was aspirated from the wells.

Each dose of Noltrex™ (2, 6, 10, 20, 40, and µL/200 µL of sample) was added to the AD-MSC and HOBs with an equal amount of medium and incubated for 24 h. All samples were assayed in triplicate during the incubation period (24 h). Dose-free and Triton X-treated groups were used as the positive and negative control groups, respectively.

At the end of the incubation period, the medium-PAHG mixtures were aspirated away from the cells after which the tetrazolium (MTT) agent (Roche-11465007001) was added at a ratio of 1:20 (MTT agent: total medium) and incubated for 3 to 4 h (37 °C, 5% CO_2). Following the incubation period, the MTT was aspirated, the solvent dimethyl sulfoxide (DMSO) was added at a ratio of 1: 1 (medium: DMSO), and the samples were incubated in an orbital mixer for 1 h in darkness. Subsequently, the absorbance values of the samples were measured with a spectrophotometer (SpectramaxI3) at 570 nm to assess any resulting color changes.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0. One-way analysis of variance (ANOVA) was used to compare the assayed parameters among the dose groups (2, 6, 10, 20, and 40 µL/200 µL) and positive and negative control groups. An overall *P*-value of < 0.05 was considered to indicate a significant difference. When an overall significance was observed, a pairwise post hoc test was performed using Tukey's multiple comparisons test.

Results

Characterization of AD-MSCs

The flow cytometry results showed that the surface markers CD105, CD146, and CD73 were highly expressed at rates of 73.46%, 82.62%, and 62.72%, respectively (Figure 1). In contrast, anti-HLA-DR, CD25, and CD19 showed significantly lower expression rates of 17.58%, 12.64%, and 19.43%, respectively (Figure 2).

Figure 1: Flow cytometry analysis of positive surface markers and percentage rates

CD105-PE				CD146-FITC				CD73-PE			
Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total
UL	9	0.10	0.09	UL	131	1.45	1.31	UL	16	0.18	0.16
UR	7346	81.52	73.46	UR	8262	91.69	82.62	UR	6272	70.90	62.72
LL	152	1.63	1.52	LL	305	3.38	3.05	LL	354	4.00	3.54
LR	1504	1669	15.04	LR	313	3.47	3.13	LR	2204	24.92	22.04

PE: Phycoeryritrin, FITC: Fluorescein isothyocyanate

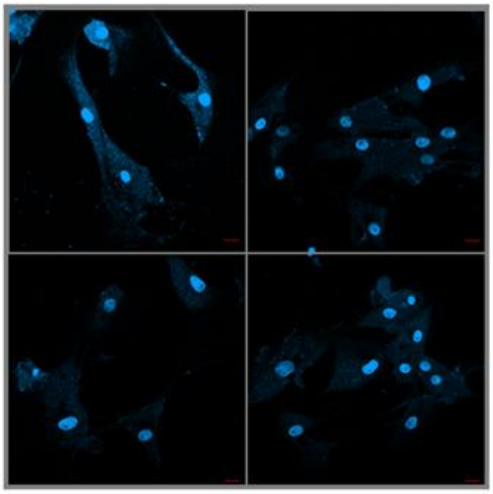
Figure 2: Flow cytometry analysis of negative surface markers and percentage rates.

CD19-PerCP				CD25-APC				Anti-HLA-DR-PE			
Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total
UL	5518	62.38	55.18	UL	5946	67.22	59.46	UL	6007	67.24	60.07
UR	1943	21.96	19.43	UR	1264	14.29	12.64	UR	1758	19.68	17.58
LL	1373	15.52	13.73	LL	1588	17.95	15.88	LL	11.63	13.02	11.63
LR	12	0.14	0.12	LR	48	0.54	0.48	LR	6	0.07	0.06

APC: Allophycocyanin, PE: Phycoeryritrin, PerCP: Peridinin-Chlorophyll

Cell nuclei were stained with DAPI, and images from different areas in the cell were taken with a Zeiss Lsm 800 confocal microscope to assess cell and nuclear morphology (Figure 3).

Figure 3: Nuclear staining images of AD-MSCs (20× magnification)



Cell viability assays

Both cell lines were incubated for 24 h with PAHG at doses of 2, 6, 10, 20, or 40 µL/200 µL per sample. Wells containing only medium were used as the positive control group, whereas Triton X-treated wells served as the negative control group. The normalized values of the triplicate results are shown in Tables 1 and 2, and graphs created based on statistical results are shown in Figures 4 and 5.

Figure 4: Statistical graph of the absorbance values of adipose derived stem cells showing dose-dependent viability.

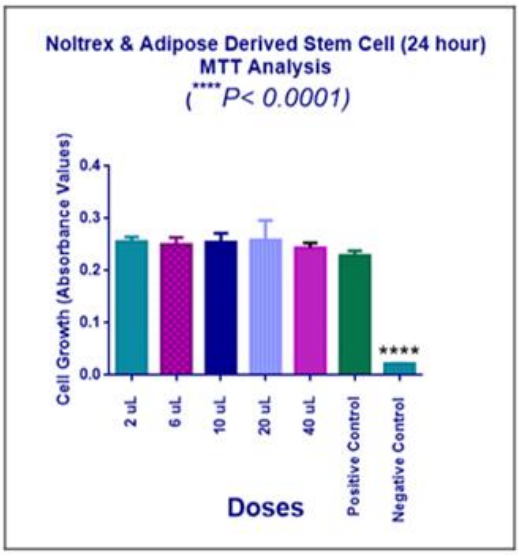
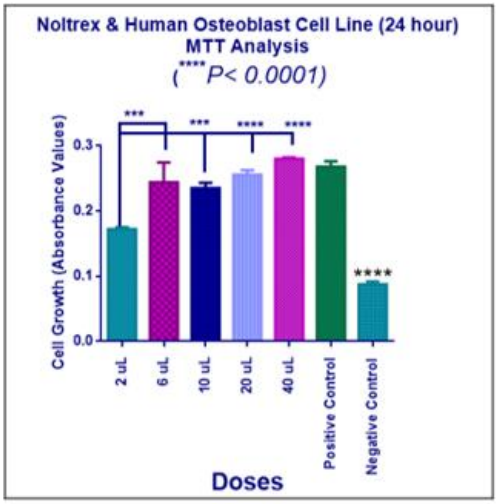


Figure 5: Statistical graph of absorbance values of human osteoblast cells showing dose-dependent viability



The MTT method, which is often the preferred technique, was used for viability analysis [12]. The MTT results for cAD-MSCs showed an obvious and significant difference when all dose groups were compared with the negative control group ($P < 0.001$). No significant differences were observed when the dose groups (2–40 µL/200 µL) were compared among themselves (Figure 4). In addition, the viability results of all dose groups were higher than that of the positive control group, even if no significant difference was observed. Therefore, the results clearly showed that the PAHG substrate Noltrex™ did not produce any negative effects on cAD-MSCs up to a dose of 40 µL / 200 µL of sample but did lead to an increase in cell proliferation (Table 1).

Table 1: Absorbance values shown as optical density (OD) unit of triplicate experiment showing the dose-dependent viability of adipose-derived stem cells and their mean values using one-way analysis of variance (ANOVA)0

Dose	First Experiment	Second Experiment	Third Experiment	Mean Values (±SEM)
2 µL/200 µL	0.2667	0.2661	0.2389	0.2559±0.01[a]
6 µL/200 µL	0.2297	0.2748	0.2461	0.2502±0.01[a]
10 µL/200 µL	0.2214	0.2756	0.2678	0.2549±0.01[a]
20 µL/200 µL	0.2238	0.3338	0.2181	0.2585±0.01[a]
40 µL/200 µL	0.2603	0.2429	0.2274	0.2435±0.01[a]
Positive Control	0.238	0.2381	0.2113	0.2291±0.01
Negative Control	0.0237	0.206	0.020	0.0221±0.01

a: $P < 0.001$ versus negative control group, SEM: Standard error of the mean

MTT analysis was also performed on HOBs using the same incubation time and the same doses. When the results of all doses were evaluated, significant differences were observed when compared with the negative control group ($P < 0.001$). No significant differences were observed when all dose groups were compared with the positive control group. When the 2 µL dose group was compared with the other dose groups, a significant difference in terms of increasing viability was observed (2 versus 6 µL/200 µL [$P < 0.001$], 2 versus 10 µL/200 µL [$P < 0.001$], 2 versus 20 µL/200 µL [$P < 0.001$], 2 versus 40 µL/200 µL [$P < 0.001$]), and significant differences at 6 and 10 µL versus 40 µL/200 µL ($P < 0.05$) comparisons ($P < 0.05$) were found (Figure 5). These results indicate that the PAHG substrate can cause an increase in cell viability to a certain extent. When the 40 µL dose was compared with the positive control group, it did not produce a negative effect on the viability of HOBs, similar to that observed for the cAD-MSCs although cell proliferation was increased even if it was not statistically significant (Table 2).

Table 2: Absorbance values as Optical Density (OD) unit of triplicate experiment showing the dose-dependent viability of human osteoblasts and their mean values using one-way ANOVA

Dose	First Experiment	Second Experiment	Third Experiment	Mean Values (±SEM)
2 µL/200 µL	0.169	0.175	0.173	0.1723±0.00[a]
6 µL/200 µL	0.267	0.256	0.210	0.24443±0.02[a,b]
10 µL/200 µL	0.245	0.231	0.230	0.2353±0.00[a,b]
20 µL/200 µL	0.260	0.260	0.248	0.2560±0.00[a,c]
40 µL/200 µL	0.279	0.278	0.283	0.2800±0.00[a,c,d,e]
Positive Control	0.271	0.275	0.259	0.2683±0.00
Negative Control	0.089	0.091	0.082	0.0873±0.00

a: $P<0.001$ versus Negative control group, b: $P < 0.001$ *versus* 2 µL/200 µL, c: $P < 0.001$ versus. 2 µL/200 µL, d: $P < 0.05$ versus 6 µL/200 µL. e: $P < 0.05$ versus 10 µL/200 µL, SEM: Standard error of the mean

Discussion

In this experimental *in vitro* study, it was observed that the clinically injectable PAHG substrate Noltrex™ did not kill cAD-MSCs and HOBs up to the maximum assayed doses when dose groups for each cell line were compared with the corresponding Triton X-treated negative control group. In addition, the PAHG substrate led to an increase in the proliferation of cAD-MSCs at all doses but only at the 40 µL dose for HOBs although these differences were not significant when compared with the positive control group. No differences were observed among the different cAD-MSC cell line dose groups. However, significant dose-dependent viability increases in HOB cell line (2 versus 6 µL/200 µL, 2 versus 10 µL/200 µL, 2 versus 20 µL/200 µL, 2 versus 40 µL/200 µL, 6 versus 40 µL/200 µL, and 10 versus 40 µL/200 µL) were found. These findings show that the clinically injectable PAHG substrate Noltrex™ can be combined with MSCs to provide slightly increased proliferation rates.

Although PAHGs are thought to be nontoxic at the cellular level, clinical side effects of PAHGs other than Noltrex™, particularly after aesthetic surgeries, have been reported. These side effects have been described very rarely as transient surgical side pains, hematomas, irregularities, gel deposition, asymmetry, tissue reactions, including infection, foreign body granuloma, edema, inflammation, tenderness, and sensitivity, and adverse effects, including gel migration and induration [8, 9].

However, the clinical side effects of PAHGs toward avascular joint tissues are limited. In a study involving 527 patients, Zar et al. [10] reported a post-Noltrex™ injection adverse effect profile as inflammable pain in 6.8% of patients at the injection area, joint pain in 9.3% of patients, and joint effusion in 0.05% of patients. They concluded that these adverse effects were resolved within three days using local cold compresses and acetaminophen treatments within, and joint aspiration or debridement was not required in any of the patients.

In contrast, Tonbul et al. [13] reported severe foreign body reactions in a 64-year-old female patient after injection with Noltrex™, which eventually required surgical interventions to drain out and debride the hydrogel in both knees. They also concluded that they altered the course of her osteoarthritis by biologically causing intra- and extra-articular fibrosis, which may have subsequently altered the potential available treatments, such as arthroplasties and post-operative rehabilitation. In their case report, they applied intra-articular Noltrex™ immediately after arthroscopic intervention of the right knee. In our opinion, this intervention may have subsequently triggered an overactive immune response and led to serious foreign body reactions in both knees. Although this case was a clinical case report regarding Noltrex™, any safety concern must be taken seriously. Therefore, we evaluated the *in vitro* effects of the PAHG substrate Noltrex™ on cAD-MSCs and HOB cells before designing a clinical trial in combination with MSCs for use in the treatment of advanced stage human osteoarthritis in which subchondral bone is also impaired.

The exact mechanism of OA is still not fully understood due to the complex series of events leading to articular cartilage degeneration. Whether events in articular cartilage precede those in subchondral bone, are concomitant with them, or whether subchondral bone changes can actually lead to early cartilage damage remains unclear [14–16]. Therefore, simultaneous healing of both articular cartilage and subchondral bone is required for ideal regeneration in advanced OA. The best candidate should be the agent that can facilitate healing or regeneration of both tissues together.

Recently, AD-MSC injections have become popular for OA treatment due to the immunomodulatory behavior and regenerative capabilities of these cells [4]. However, findings from clinical trials show that stem cell injections alone do not appear to have a lasting effect on end-stage osteoarthritis [17–19]. These results suggest that the stem cells require a suitable matrix in which to reside, propagate, and effectively differentiate for a desirable recovery in cases of severe OA. In the search for such a matrix, the *in vitro* effects of the PAHG derivative Noltrex™ were evaluated before using it in combination with AD-MSCs in human clinical trials and assessing its effect on HOBs based on the resulting pathogenesis. To the best of our knowledge, no previous study has evaluated the effects of PAHGs on both cell lines together in this way.

On the clinicaltrials.gov website, searches using the terms “osteoarthritis” and “polyacrylamide hydrogel” together resulted in the identification of five trials registered as NCT03897686, NCT04045431, NCT04179552, NCT03067090, and NCT03060421. Trials NCT03897686, NCT04045431, and NCT03067090 appear to still be recruiting. However, five trials study polyacrylamide hydrogels alone.

In future trials, the *in vivo* effects of injectable PAHGs combined with MSCs on cell differentiation and tissue reactions as foreign body reactions should be investigated.

Although PAHGs have high biocompatibility, they interact with their surrounding host tissue and allow for gradual vessel ingrowth, which is followed by a modest macrophage reaction at the host tissue-gel border. Once the macrophages enter the gel, they transform into fibroblasts that connect and eventually form a vascular fibrous network. Consequently, PAHGs, remain in the tissue indefinitely as a viscoelastic deposit traversed by a network of fibrous strands [20].

However, in the presence of mesenchymal stem cells in a hydrogel, the immunoregulatory properties of PAHGs can cause macrophages to remain as anti-inflammatory M2 type cells, which can prevent or reduce foreign body reactions and result in a decrease in fibrous strands [21]. These properties could positively affect articular and subchondral bone tissue regeneration, which should be elucidated in future trials.

Last, in our experimental study, the effects of the clinically injectable PAHG substrate Noltrex™ on stem cell differentiation were not evaluated. Furthermore, commercially available AD-MSCs of canine rather than human origin were used. In addition, another PAHG agent clinically used in the treatment of OA is Arthrosamid™ (Contura International A/S, Søborg, Denmark) [22, 23]. However, as Arthrosamid™ was not commercially available in our country when this experiment was performed, it was not possible to study or compare both agents together. These points are viewed as the weaknesses of our present study.

Conclusion

OA is primarily a disease of articular cartilage and subchondral bone. Therefore, the regeneration of both tissues would ideally be performed simultaneously. MSCs are considered suitable candidates for use in the treatment of OA due to their regenerative potential. However, in the late stages of OA, these cells provide unsatisfactory long-term effectiveness. However, PAHGs remain in the joint indefinitely. In our present study, Noltrex™ were not lethal to cAD-MSCs or HOBs and rather led to induction of slightly increased proliferation rates, suggesting that their use in combination with AD-MSCs may have positive long-term effects in the treatment of OA. However, this possibility should be evaluated in further animal and human trials.

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