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In Vitro Effects of Hyaluronic Acid on Equine Mesenchymal Stem Cells in Osteoarthritis Research

Bachelor's Thesis

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submitted by

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<u>Declaration of Academic Integrity</u>

Hereby, I declare that I have composed the presented thesis independently on my own and without any other resources than the ones indicated. All thoughts taken directly or indirectly from external sources are properly denoted as such.

This thesis has neither been previously submitted to another authority nor has it been published yet.

Vienna, 14.06.2024 Johanna Halbertschlager

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Abstract

Mesenchymal stem cells are, due to their potential in cartilage regeneration and their immunomodulatory functions, of a great interest in osteoarthritis research and therapeutic applications. Osteoarthritis is a painful condition leading to a loss of articular cartilage, inflammation, and changes in synovial fluid composition, such as reduction of hyaluronic acid molecular weight. Hyaluronic acid, a key component of synovial fluid, interacts with cells via its receptor, CD44, and has differing effects on cells depending on its molecular weight. Intra-articular injection of hyaluronic acid, and/or mesenchymal stem cells, is an effective treatment for osteoarthritis. This study investigates how inflammatory conditions and hyaluronic acid molecular weight affects equine mesenchymal stem cells.

Adipose-derived equine mesenchymal stem cells were treated with TNFα and IL-1β to simulate the onset of inflammatory conditions and simultaneously conditioned with high (> 950 kDa), and low molecular weight (15-40 kDa) hyaluronic acid, to determine if hyaluronic acid modifies effects caused by the cytokines. Effect of hyaluronic acid without cytokine induction was also explored. After six days of conditioning, cell viability was measured and CD44 expression was quantified with flow cytometry and visualised via immunocytofluorescence. Morphology of mesenchymal stem cells was visualised via phase-contrast microscopy.

Hyaluronic acid conditioning had either no, or negative effects on viability of cytokine non-induced cells compared to the unconditioned control. Cytokine induction had a positive effect on cell viability compared to the unconditioned control. This effect was enhanced by conditioning with low molecular weight hyaluronic acid. CD44 was highly expressed across all groups and hyaluronic acid conditioning did not affect CD44 expression in induced and non-induced groups.

The presented study brought a novel finding of the effect of hyaluronic acid on cell viability during the onset of inflammatory conditions, which can serve as a basis for further studies.

Zusammenfassung

Mesenchymale Stammzellen sind aufgrund ihres Potenzials für die Knorpelregeneration und ihrer immunmodulatorischen Funktionen von großem Interesse für die Osteoarthritisforschung und therapeutische Anwendungen. Osteoarthrose ist eine schmerzhafte Erkrankung, die zu einem Verlust von Gelenkknorpel, Entzündungen und Veränderungen in der Zusammensetzung der Synovialflüssigkeit führt, z. B. zu einer Verringerung des Molekulargewichts der Hyaluronsäure. Hyaluronsäure, ein wichtiger Bestandteil der Synovialflüssigkeit, interagiert mit Zellen über CD44 und hat, je nach Molekulargewicht, unterschiedliche Wirkungen auf die Zellen. Die intraartikuläre Injektion von Hyaluronsäure und/oder mesenchymalen Stammzellen ist eine wirksame Behandlung für Osteoarthrose. In dieser Studie wird untersucht, wie Entzündung, und das Molekulargewicht der Hyaluronsäure, equine mesenchymalen Stammzellen beeinflusst.

Aus Fettgewebe gewonnene equine mesenchymale Stammzellen wurden mit TNFα und IL-1β behandelt, um den Beginn von Entzündungszuständen zu simulieren, und gleichzeitig mit Hyaluronsäure mit hohem (> 950 kDa) und niedrigem Molekulargewicht (15-40 kDa) behandelt, um festzustellen, ob Hyaluronsäure die durch die Zytokine verursachten Effekte modifiziert. Die Wirkung von Hyaluronsäure ohne Zytokininduktion wurde ebenfalls untersucht. Nach einer sechstägigen Konditionierung wurde die Lebensfähigkeit der Zellen gemessen und die CD44-Expression mittels Durchflusszytometrie quantifiziert und durch Immunfluoreszenz sichtbar gemacht. Die Morphologie der mesenchymalen Stammzellen wurde durch Phasenkontrastmikroskopie visualisiert.

Die Konditionierung mit Hyaluronsäure hatte entweder keine oder negative Auswirkungen auf die Lebensfähigkeit von Zellen ohne Zytokininduktion im Vergleich zur unkonditionierten Kontrolle. Die Zytokininduktion hatte einen positiven Effekt auf die Lebensfähigkeit der Zellen im Vergleich zur unkonditionierten Kontrolle. Dieser Effekt wurde durch die Konditionierung mit niedermolekularer Hyaluronsäure verstärkt. CD44 wurde in allen Gruppen in hohem Maße exprimiert, und die Konditionierung mit Hyaluronsäure hatte keinen Einfluss auf die CD44-Expression in den induzierten und nicht-induzierten Gruppen.

Die vorgestellte Studie brachte eine neue Erkenntnis über die Wirkung von Hyaluronsäure auf die Lebensfähigkeit von Zellen während des Beginns von Entzündungszuständen, die als Grundlage für weitere Studien dienen kann.

Table of contents

List of tables and figures

List of Abbreviations

CD44	Cluster of Differentiation-44
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
eAD-MSC	Equine Adipose Derived Mesenchymal Stem Cell
FBS	Fetal Bovine Serum
HA	Hyaluronic Acid
HMWHA	High Molecular Weight Hyaluronic Acid
IA	Intra-articular
IL	Interleukin
INF	Interferon
LMWHA	Low Molecular Weight Hyaluronic Acid
MSC	Mesenchymal Stem Cell
OA	Osteoarthritis/Osteoarthritic
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
SF	Synovial Fluid
TGF-β	Transforming Growth Factor-β
TNFα	Tumour Necrosis Factor α

1 Introduction

1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) may be defined as multipotent, non-hematopoietic stem cells (Mishra et al. 2020). They are best known for the potential to differentiate into cells of the mesodermal lineage, which includes cells such as adipocytes, osteocytes, and chondrocytes. Research has also uncovered their capability to differentiate into cells of ectodermal lineage such as neurocytes, and endodermal lineage cells such as hepatocytes (Gugjoo et al. 2020, Mishra et al. 2020). MSCs can be isolated from bone marrow, umbilical cord tissue, adipose tissue, skin and many other sources (Song and Jorgensen 2022). Small numbers of MSCs can be obtained from synovium or even the synovial fluid (SF) of healthy joints (Morito et al. 2008). The numbers of MSCs in the SF increases significantly after ligament injury (Morito et al. 2008), and in arthropathies, especially in osteoarthritis (OA) (Jones et al. 2004). MSCs are in focus of biomedical research due to their regenerative and immunomodulatory functions. Bone marrow derived MSCs and adipose tissue derived MSCs are most commonly used in research and clinical applications due their availability (Song and Jorgensen 2022). Intra-articular (IA) application of MSCs may be a promising stem cell based treatment option for OA (Ho et al. 2022, Satué et al. 2019). Exact mechanisms of MSCs regenerative functions are largely unknown, especially in OA, but it is discussed that it includes secretion of growth factors, cytokines and other soluble factors, and the potential of MSCs to differentiate and self-renew (Satué et al. 2019, Song and Jorgensen 2022).

Another significant function of MSCs is immunomodulation. It is important to note that the anti-inflammatory or pro-inflammatory actions of MSCs can vary depending on the microenvironment. As an example, at low level stimulation with tumour necrosis factor α (TNFα) and interferon (IFN)-γ, MSCs may exhibit pro-inflammatory activity by producing cytokines and chemokines that act on effector T-cells. Conversely, environments with high concentrations of cytokines like IFN-γ and TNFα seem to promote the immunosuppressive properties of MSCs. This suggests the requirement of stimulation with pro-inflammatory cytokines for activation of immunosuppressive functions of MSCs (Carrade et al. 2012, Mishra et al. 2020).

Secretion of prostaglandin E2 (PGE2) has been shown to play a role in the immunosuppressive action of MSCs (Fan et al. 2012, Mishra et al. 2020, Song and Jorgensen 2022). MSCs were found to secrete PGE2 following interleukin (IL)-1β stimulation, which in turn was identified as a player in immunomodulation by acting on macrophages (Gray et al. 2015, Roth et al. 2022). Furthermore, PGE2 along with other paracrine factors released by MSCs, help stimulate activation of regulatory T-cells and suppression of effector T-cells (Mishra et al. 2020). Transforming growth factor-β (TGF-β) has also been proposed as an important part of the immunomodulating secretome of MSCs (Mariñas-Pardo et al. 2018).

1.2 Joint anatomy and synovial fluid composition

The basic structure of synovial joints consists of the articulating bones, whose ends are layered with hyaline articular cartilage, SF and a structure to contain the SF, most often the joint capsule (McIlwraith et al. 2016). The inner lining of the joint capsule is the synovial membrane consisting of vascular connective tissue (Goodrich and Nixon 2006). Cells lining the synovial membrane are termed synoviocytes, which have both secretory and phagocytic functions. Articular cartilage is made up of chondrocytes embedded in an extracellular matrix, which is mainly made composed of water, collagen and proteoglycans and also contains hyaluronic acid (HA) (McIlwraith et al. 2016). The fenestrated microvasculature of the synovial membrane allows for a certain permeability, allowing for production of SF, which, at its core, is a plasma dialysate, mixed with secretion products made by resident cells such as synoviocytes (Goodrich and Nixon 2006, McIlwraith et al. 2016). Some important components of SF include HA, proteoglycan-4, lubricin and surface active phospholipids (McIlwraith et al. 2016).

1.2.1 Hyaluronic acid

HA is abundant in SF and is crucial for its function (Zheng et al. 2023). It belongs to the glycosaminoglycan family of molecules. Being the simplest and the only unsulfated glycosaminoglycan, it is made up of linear chains of β(1->4) linked disaccharides of β(1->3) linked D-glucuronic acid and N-acetyl-D-glucosamine (Šimek et al. 2020).

High molecular weight hyaluronic acid (HMWHA) is a greatly hydrophilic, viscoelastic substance. These properties are responsible for the macromolecular effects of HMWHA (Garantziotis and Savani 2019). In combination with other components in SF, mainly lubricin and proteoglycan-4, HMWHA ensures lubrication and minimisation of cartilage surface wear in healthy joints (Watkins et al. 2021, Zheng et al. 2023). HA is thought to be the backbone of brush-like nanofibers with lubricin and lipid side chains, which are present in SF, as well as bound to the cartilage surface, forming a lubrication layer (Zheng et al. 2023).

By interacting with cells, HA plays an important role in mediating inflammation, cell and tissue development, cell proliferation and cell migration. HA interacts with cells on a molecular level via receptors. One important HA receptor is cluster of differentiation-44 (CD44), which is so far the most studied HA receptor (Garantziotis and Savani 2019). CD44 is a glycoprotein acting as a non-kinase receptor for HA, and is common in many cell types (Garantziotis and Savani 2019, Lesley and Hyman 1998), including MSCs (Paebst et al. 2014). The CD44 receptor has been shown to affect cell motility, cell growth, proliferation, inflammatory processes and lymphocyte homing (Garantziotis and Savani 2019). Notably, cellular response to HA is discussed to be dependant on molecular weight. For example, HMWHA has been shown to inhibit angiogenesis (Lu et al. 2023, Slevin et al. 2007), whereas HA fragments and low molecular weight hyaluronic acid (LMWHA) enhance proliferation of endothelial cells and angiogenesis (Lu et al. 2023, Slevin et al. 2007, West et al. 1985). Additionally, HMWHA may have anti-inflammatory properties, while LMWHA may act pro-inflammatory (Lu et al. 2023, Petrey and La Motte 2014). When binding with HMWHA, clustering of CD44 receptors is observed due to the high number of binding sites of HMWHA for CD44. LMWHA or HA fragments exhibit fewer binding sites for CD44 and do not cause clustering of CD44. This is thought to be responsible for the differing cellular responses (Lu et al. 2023).

No clear definition of LMWHA and HMWHA regarding the molecular weight could be found in the literature. For example, one paper defined HMWHA to have a molecular weight greater than 500 kDa (Snetkov et al. 2020), while another named a minimum of 1000 kDa (Meszaros et al. 2020).

1.3 Osteoarthritis

OA is the most common joint disease in horses as well as humans (Burk, Badylak et al. 2013). Factors that contribute to OA are joint injury and physical overload of joints resulting in disruption of joint homeostasis and inflammation, kickstarting OA disease processes (Baccarin et al. 2022).

OA is a painful condition which causes gradual loss of cartilage. Moreover, OA leads to remodelling of the subchondral bone (Jang et al. 2021), osteophyte formation and synovial inflammation of varying degrees (Zheng et al. 2023). Early in the disease progression, increased friction of the cartilage layers is caused by the loss of lubricating effects of SF. Consequently, chondral debris is formed, leading to inflammation and secretion of proteolytic enzymes, which leads to a cycle of cartilage degradation (Zheng et al. 2023).

In OA SF, the concentration (Fuller et al. 2001, Zheng et al. 2023), as well as the molecular weight (Fasanello et al. 2021, Goodrich and Nixon 2006, Zheng et al. 2023) of HA tend to be reduced. Fasanello et al. measured the molecular weight distribution of HA in equine healthy and OA SF and found the medians of the mean molecular weight to be 1520 kDa in healthy, and 1260 kDa in OA SF (Fasanello et al. 2021). Degradation of HA in OA is thought to be caused by the inflammatory processes in the joint. Chemokines, cytokines, reactive oxygen species and other by-products of inflammation may lead to HA being degraded. Additionally, enzymes released from damaged synoviocytes contribute to the degradation of SF HA (Goodrich and Nixon 2006, Lu et al. 2023). Enzymes that take part in HA degradation are hyaluronidases, which, alongside CD44, facilitate extracellular and intracellular HA degradation. CD44 binds HA to the cell surface, where extracellular degradation can take place. It then assists in endocytosis of HA, after which intracellular degradation starts (Zheng et al. 2023). As already described, research has also shown that LMWHA then possesses pro-inflammatory characteristics, as opposed to anti-inflammatory effects of HMWHA (Lu et al. 2023, Petrey and La Motte 2014). Moreover, degradation of HA contributes to the loss of lubricating ability of SF, accelerating disease progression (Zheng et al. 2023).

Inflammatory cytokines secreted by immune cells such as macrophages play a major role in pathogenesis and progression of OA. IL-1β, TNFα, as well as IL-6, IL-15, IL-17 and IL-18 have been identified as some of the most important cytokines associated with OA disease processes in human studies (Wojdasiewicz et al. 2014). IL-1 family cytokines stimulate the expression of matrix metalloproteases in OA, contributing to cartilage degradation. In murine models, inhibition or genetic ablation of IL-1β has been shown to be protective against the development of OA (Troeberg and Nagase 2012).

1.4 Intra-articular injection of hyaluronic acid or mesenchymal stem cells as treatment options for osteoarthritis

1.4.1 Intra-articular hyaluronic acid supplementation

IA injection of HA aims to supplement possibly degraded HA in the joint. This is thought to aid in increasing SF viscoelasticity and lubricating ability. Furthermore, anti-inflammatory effects, inhibition of macrophage chemotaxis, reduced prostaglandin secretion, reduction of lymphocyte migration and proliferation as well as scavenging of reactive oxygen species have been reported as possible effects of IA HA (Goodrich and Nixon 2006, Lu et al. 2023). Research generally suggests an advantage of using HMWHA for IA injection, possibly due to anti-inflammatory effects and better viscoelastic properties (Lu et al. 2023).

Despite IA HA being commonly used to manage OA in human (Migliorini et al. 2021) and equine patients (Johnston et al. 2020), the efficacy of IA HA remains controversial. Meta-analyses performed on human clinical trials have found no or only small clinically significant differences in IA HA and placebo groups (Colen et al. 2012, Migliorini et al. 2021). In contrast, a review by Maheu et al. discusses a moderate reduction in symptoms after IA HA injection combined with a favourable benefit/risk ratio in human knee OA (Maheu et al. 2019). Gigante and Callegari also described a reduction in pain after treatment of human patients while risk of the procedure was found to be minimal (Gigante and Callegari 2011).

1.4.2 Intra-articular injection of mesenchymal stem cells

MSCs have immunomodulatory functions and potential to induce tissue regeneration. These properties make IA injection of MSCs a compelling treatment option for OA. Cartilage repair, reduction of pain and better athletic performance have been reported following IA injection of MSCs. As MSCs seem to engraft only temporarily into cartilage, paracrine and immunomodulatory functions may be the main mechanisms which lead to cartilage repair (Jammes et al. 2023, Satué et al. 2019).

Satué et al. reported IA injection of MSCs to be a safe and effective way to promote cartilage regeneration in a rat model. Potential to migrate to cartilage injury sites was observed. Some injected MSCs were detected in the cartilage lesions a week and a month after injection. However, two and six months after treatment, no injected MSCs were detected in the synovial cavity or cartilage defects. Nonetheless, effective cartilage regeneration was seen in MSC-injected groups. Nearly normal hyaline cartilage was observed six months after treatment at cartilage lesion sites. Interaction between MSCs and macrophages was observed, and a slight reduction of IL-1β expression in cartilage defect sites was seen (Satué et al. 2019). Mariñas-Pardo et al. described a reduction in lameness after IA injection of allogeneic equine adipose-derived mesenchymal stem cells (eAD-MSC) and highlighted the role of TGF-β in immunomodulation and protection against cartilage degeneration (Mariñas-Pardo et al. 2018). Positive outcomes and no adverse events were reported in a study conducted in human OA patients. Compared to IA HA, study participants receiving IA bone marrow-derived-MSCs experienced a significant reduction in pain and an increased quality of life (Ho et al. 2022).

A combination of HA and MSC injection is of interest for OA treatment, as artificially changing HA molecular weight or concentration in joints could modify MSC behaviour and treatment outcomes. In a study using a canine cartilage defect model, combination of IA injection of MSCs and HMWHA was found to promote new cartilage formation significantly better than IA HA or saline injections (Li et al. 2018). Similar results were reported in a study done in rabbits (Chiang et al. 2016). Very little information could be found on the performance of combined treatment in comparison to IA MSC injection alone, though one study in guinea pigs saw significant cartilage regeneration only when MSCs were injected in combination with HA (MW = 800 kDa) (Sato et al. 2012). Baboolal et al. describe a strong connection between HA contained in SF and the adhesive properties of canine MSCs to cartilage, which may be crucial to their function. HMWHA with a molecular weight of over 900 kDa in SF was linked to a decrease in the adhesive ability of MSCs to cartilage *in vitro* as well as *in vivo* by HMWHA forming a pericellular coat of HA (Baboolal et al. 2016). However, Gómez-Aristizábal et al. found HMWHA to support the immunomodulatory and anti-inflammatory functions of MSCs, suggesting a more potent effect of MSCs in combination with HMWHA (Gómez-Aristizábal et al. 2016). Further investigation is needed to better understand the effects of SF or exogenous HA on injected MSCs.

As described, OA has a clear impact on the joint environment and SF composition, the most apparent being HA degradation leading to increased presence of LMWHA fragments (Goodrich and Nixon 2006, Lu et al. 2023, Zheng et al. 2023), as well as elevated levels of cytokines as a result of inflammatory processes in OA joints (Lu et al. 2023, Wojdasiewicz et al. 2014). As IA injection of MSCs is a promising treatment option for OA (Ho et al. 2022, Satué et al. 2019), the question, how inflammatory OA joint environment or possible HA supplementation affects MSCs, arises.

1.5 Hypotheses and aims

This thesis aims to explore the *in vitro* effect of different molecular weights of HA, HMWHA (> 950 kDa) or LMWHA (15-40 kDa), on eAD-MSCs viability and the expression of CD44 during the onset of inflammatory conditions, to see, if HA can modify the effects of cytokine induction. TNFα and IL-1β will be used to induce eAD-MSCs to mimic inflammatory conditions *in vitro*.

The following hypotheses will be tested:

1) Induction of eAD-MSCs with cytokines decreases cell viability compared to non-induced cells.
2) Treatment of eAD-MSCs with LMWHA, but not HMWHA, leads to an increase in cells viability and CD44 expression compared to untreated control.
3) Cytokine induction together with LMWHA, but not HMWHA, treatment causes elevation of eAD-MSCs viability and CD44 expression compared to cytokine induced control and cytokine non-induced control.

2 Material and methods

2.1 Study design

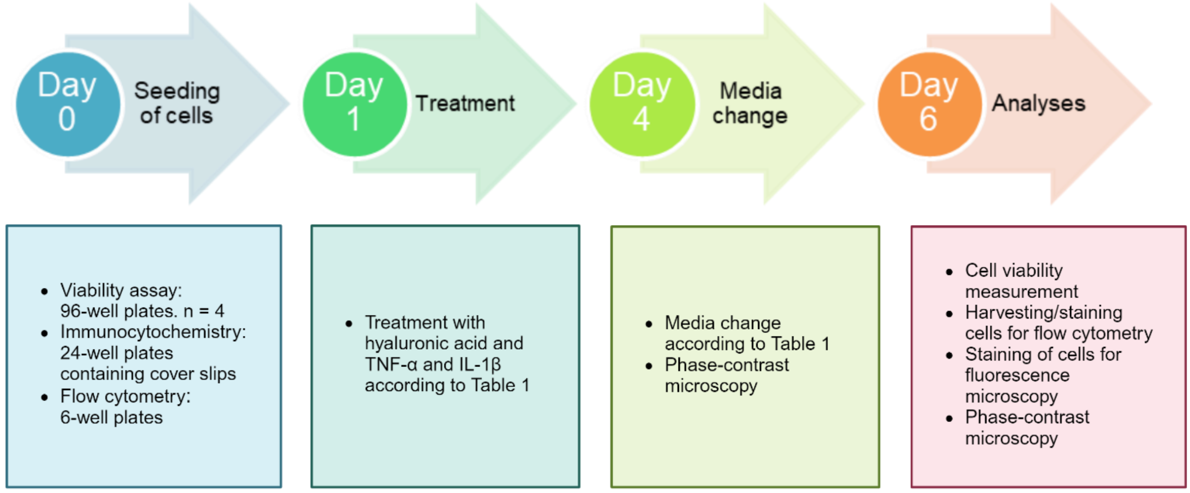

Figure 1. Timeline for experiments Created with BioRender.com

Figure 1 summarises the timeline for the experiments. All the methods described were repeated three times ("experiments 1-3") using different vials of stock cells from the same donor (three replicates). In case of the cell viability assay, four technical replicates were performed for each experimental group.

2.2 Preparation of culture media

Low glucose Dulbecco's Modified Eagle's Medium (DMEM) growth medium (Dulbecco's Modified Eagle's Medium - low glucose, Sigma-Aldrich, USA) supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco™ Fetal Bovine Serum, certified, heat inactivated, Thermo Fisher Scientific Inc., USA), 1 % Penicillin/Streptomycin (Gibco™ Penicillin-Streptomycin (10,000 U/mL), Thermo Fisher Scientific Inc, USA) and 2 % L-glutamine (L-Glutamine Solution 200 mM, Sigma-Aldrich, USA) was used for cell expansion and as a media basis for cell treatment.

2.3 Expansion of adipose derived equine mesenchymal stem cells

In the experiments, previously collected frozen eAD-MSCs were used. Cells of the same donor (passage 1) was used in all experiments. Cells were derived from post-mortem tissue collection from animals euthanised for different reasons than this study. The cells were isolated and frozen as a part of previous projects. The tissue collection and cell isolation was done according to a previously published protocol (Burk, Ribitsch et al. 2013).

Frozen cells were thawed, and 2 ml of medium was added to the cell suspension. Cells were centrifuged for 5 minutes at 300 g, washed in medium and centrifuged again. Cells were then seeded into T-75 culture flasks (VWR® 250mL Tissue Culture Flasks, Surface Treated, w/vent cap, Sterilized, VWR International, USA) with 10 ml of medium. The cells were passaged and passage 3 was used for experiments. Media changes were performed every two to three days during cell expansion. Standard culture conditions of 37 °C and 5 % CO_2 were used to incubate the cells.

2.4 Seeding and treatment of cells

To recover the cells from T-75 culture flasks, the medium was first removed, then phosphate buffered saline (PBS) (Dulbecco′s Phosphate Buffered Saline, Sigma-Aldrich, USA) was added to wash the cells. The PBS was removed and 5 ml of trypsin-EDTA (ROTI®Cell Trypsin/EDTA-Lösung (10x), Carl Roth GmbH + Co. KG, Germany) was added and incubated for 5 minutes, then 5 ml of medium was added to deactivate the trypsin. Afterwards, 5 ml of the cell suspension each was added to 15 ml centrifuge tubes (Greiner Bio-One GmbH, Austria). The tubes were centrifuged at 300 g for 10 minutes. The supernatant was discarded, and the pellets were each resuspended in 1 ml of medium. To count the cells, 10 µl of cell suspension was added to 10 µl of Trypan blue (Gibco™ Trypan Blue Solution, 0.4%, Thermo Fisher Scientific Inc, USA), of which 10 µl was pipetted onto a Bürker-Türk cell counting chamber. The Cells were counted and the number of cells/ml was calculated.

96-well plates (VWR® Multiwell Cell Culture Plates, VWR International, LLC, USA) were used for the viability assays and each well was seeded with 1000 cells according to the assay protocol provided by the manufacturer. For immunocytochemistry, cover glasses (Microscope cover glasses 16 mm Ø No. 1, Paul Marienfeld GmbH & Co. KG, Germany) placed in 24-well plates (VWR® Multiwell Cell Culture Plates, VWR International, LLC, USA) were used to grow the cells on, where each well was seeded with a density of 3000 cells/cm^2. Cells used for flow cytometry analysis were grown in 6-well plates (VWR® Multiwell Cell Culture Plates, VWR International, LLC, USA), with a seeding density of 3000 cells/cm^2.

After seeding (day 0), the cells were left to adhere overnight. On day 1, media were changed and the cells were cultivated in 6 different culture conditions. On day 4, media were changed again, although cytokines were no longer added, and culture media were only supplemented with HA. Table 1 summarizes supplements added to the culture media of each experimental group on day 1 and day 4. The cells were treated with conditioned media for a total of 6 days before analyses were performed.

Table 1. Supplements to media

Group	Day 1	Day 4
1	Control, no additives	Control, no additives
2	1 mg/ml LMWHA (15-40 kDa) (Hyaluronan (Low MW), Bio-Techne, USA)	1 mg/ml LMWHA
3	1 mg/ml HMWHA (> 950 kDa) (Hyaluronan (High MW), Bio-Techne, USA)	1 mg/ml HMWHA
4	1 mg/ml LMWHA, 50 ng/ml TNFα (Human TNF-alpha Recombinant Protein, PeproTech®, Thermo Fisher Scientific Inc., USA), 10 ng/ml IL-1β (Human IL-1 beta Recombinant Protein, PeproTech®, Thermo Fisher Scientific Inc., USA)	1 mg/ml LMWHA
5	1 mg/ml HMWHA, 50 ng/ml TNFα, 10 ng/ml IL-1β	1 mg/ml HMWHA
6	Control, 50 ng/ml TNFα, 10 ng/ml IL-1β	Control, no additives

The high concentration of the cytokines used in this study was chosen based on a previous study using eAD-MSCs in order to produce a clear effect on cells (Brandt et al. 2018). Concentration of HA was chosen based on the concentration in healthy equine SF (Matisioudis et al. 2019).

2.5 Viability assay

2.5.1 Experiment

Cell viability was measured with the Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. It is a colorimetric method used to determine metabolic activity and viability. To detect interferences due to assay chemistry, measurements for each of the six treatment media were taken in the absence of cells. To measure cell viability and interference, 20 µl of the assay reagent was added to each well. After two hours of incubation, photometric measurements were made with a plate reader (EnSpire™ 2300 Multilabel Reader, PerkinElmer, USA) at a wavelength of 490 nm.

2.5.2 Data analysis

One-way ANOVA with multiple comparisons test was used to analyse statistical differences among the absorbance means of the experimental groups. Normal distribution of data was tested and Bartlett's test was performed to check for equal standard deviation between groups, ensuring requirements of the data are met for the chosen analysis. Statistics and graphs were done in GraphPad Prism 10. P-values < 0,05 were used as the cut-off to determine statistical significance.

2.6 Phase-contrast microscopy

Images of cells of every experimental group were taken using the Leica DM IL LED Inverted Laboratory Microscope (Leica Microsystems GmbH, Germany) to show general cell morphology and monitor the cells over the course of the experiments. Images were taken on days 4 and 6 using cells cultured in 6 well plates. The camera used for imaging was the Axiocam 208 color (Carl Zeiss AG, Germany).

2.7 Immunocytochemistry and fluorescence microscopy.

Immunocytochemistry was performed to image the cells and visualise the presence of CD44 on the cell surfaces.

ROTI®Histofix with 4 % formaldehyde (Carl Roth GmbH + Co. KG, Germany) was used to fix cells in preparation for immunocytochemistry. The cells were first washed in PBS, then covered with the fixing agent for 10 minutes. The fixed cells were washed in PBS at least three times for 5 minutes each to ensure no fixing agent remained.

The fixed cells were permeabilised with 0,5 % Triton® X-100 (Sigma-Aldrich, USA) in PBS for 5 minutes at room temperature, then washed three times for 5 minutes each in PBS. Cells were blocked for one hour at room temperature with 5 % rat serum in PBS. After blocking, the primary antibody (BD Pharmingen™ APC Rat Anti-Mouse CD44, BD Biosciences, USA) was diluted 1:100 in 5 % rat serum and incubated over night at 4 °C. The cells were again washed at least three times in PBS for 5 minutes each to remove all the excess antibody. The secondary antibody (Invitrogen™ Rabbit anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Thermo Fisher Scientific Inc, USA) was diluted 1:200 in PBS and incubated protected from light for one hour at room temperature, after which the cells were again thoroughly washed with PBS. 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) (DAPI Stain, Cell Signaling Technology, USA) in PBS was used to counterstain for 5 minutes at room temperature protected from light. The cover glasses were mounted onto microscopy slides using mounting medium (Fluoromount for microscopy, SERVA Electrophoresis GmbH, Germany).Fluorescence microscopy and imaging of the cells was done using the Zeiss Axioskop 2 plus microscope (Carl Zeiss AG, Germany) and the Olympus DP72 camera (Olympus Corporation, Japan).

2.7.1 Image analysis

Images were analysed qualitatively to confirm the presence of CD44 expression in cells of different experimental groups. No further data analysis was performed.

2.8 Flow cytometry

2.8.1 Sample preparation and measurement

Six days after treatment, the cells were harvested for flow cytometry, to quantify the CD44 protein expression. The medium was aspirated from each well and the cells washed with 1 ml of PBS. The PBS was removed and 1 ml of accutase solution (Invitrogen™ Accutase - Enzyme Cell Detachment Medium, Thermo Fisher Scientific Inc., USA) was added per well and put in the incubator for 5 to 10 minutes until all cells were detached from the well surface. The contents of the wells of the same treatment were transferred to one 2 ml Eppendorf tube and 1 ml of medium was added to each tube. The tubes were centrifuged at 440 g for 5 minutes and the supernatant was discarded. The cells were counted and divided into two parts containing more than 10^6 cells. One part was used as unstained control. The unstained cells were washed in PBS, centrifuged at 440 g for 5 minutes and resuspended in 100 µl of PBS.

The remaining cells were resuspended in 50 µl of a 1:1000 dilution of BD Horizon™ Fixable Viability Stain 620 (BD Biosciences, USA) to perform live/dead staining. The stain was incubated in the dark for 15 minutes at room temperature. After incubation, 100 µl of PBS (+3% FBS) was added to each tube. The tubes were then centrifuged again. The cells were washed by removing the supernatant, adding 300 µl of PBS and centrifuging, which was done twice. Pellets were resuspended in 50 µl of blocking solution (5 % rat serum) and incubated for 15 minutes at 4°C. According to the protocol used by Hagen et al., the antibody (BD Pharmingen™ APC Rat Anti-Mouse CD44, BD Biosciences, USA) was diluted 1:100 (Hagen et al. 2020). After blocking, 50 µl of the antibody dilution was added to each tube, and again incubated for 15 minutes at 4°C. After incubation, the cells were again washed twice as described earlier. The stained cells were resuspended in 100 µl of PBS. Flow cytometry was measured using the CytoFLEX S Flow Cytometer (Beckman Coulter, Inc., USA).

The flow cytometry experiment was performed three times. Two replicates of the experiment were successful. A third attempt was unsuccessful, most likely because the protocol was modified to include fixation of the cells using a 2 % formaldehyde solution (ROTI®Histofix with 4 % formaldehyde diluted with PBS), which caused the cells to be lost during washing steps for unknown reasons.

2.8.2 Data analysis

FlowJo™ v10 was used to analyse and visualise flow cytometry data. The gating strategy is depicted in Figure 2. Also, gating for live cells using the BD Horizon™ Fixable Viability Stain 620 was used in CD44 stained cells (not depicted as the vast majority of the cells were alive in each measurement). Data were analysed descriptively and visually. No further statistical analyses were performed due to constant expression of CD44 in the cells among all groups.

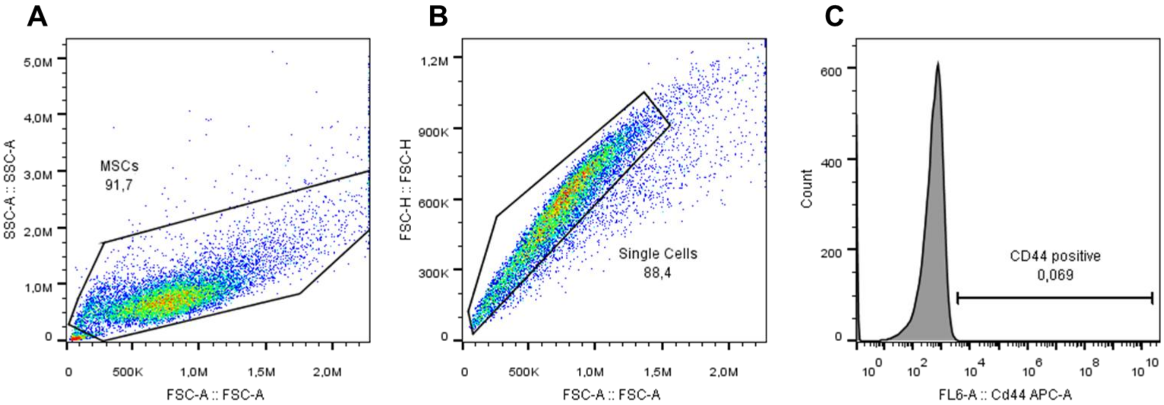

Figure 2. Gating strategy: Unstained cells of group 2 are used to depict the gating strategy. Forward scatter (FSC) and side scatter (SSC) gating was used to gate for MSCs (A). FSC area and FSC height were used to gate for single cells (B). The gate for CD44 positive cells is shown in a histogram comparing APC fluorescence signal (CD44) and cell count (C).

3 Results

3.1 Viability assay

To determine the effects of various conditionings (Table 1), cell metabolic activity and viability was analysed by Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay and compared between groups.

In experiment 1, viability of cytokine induced cells cultured in the presence of LMWHA (4) and HMWHA (5) was significantly higher compared to the control (1) and cytokine induced cells control (6). Absorbance in wells containing cells cultured with HA without cytokine induction (2, 3) was not statistically different compared to the control (1). Viability of cytokine induced cells did not differ significantly from the control (1) (Figure 3).

In experiment 2, viability of cytokine induced cells cultured in the presence of HMWHA (5), however not of cytokine induced cells cultured with LMWHA (4), was significantly higher compared to the control (1). No statistically significant difference in viability was seen between cytokine induced cells cultured with HA (4, 5) compared to cytokine induced cells not cultured with HA (6). Absorbance in wells with cells treated with LMWHA (2) did not differ significantly from the control (1). Cells cultured in the presence of HMWHA (3) had a significantly lower viability compared to the control (1). Absorbance in wells with cytokine induced cells (6) was significantly higher compared to the control (1) (Figure 4).

In experiment 3, viability of the cytokine induced cells cultured in the presence of LMWHA (4) had a significantly higher viability compared to the control (1), as well compared to cytokine induced cells cultured without HA (6). Viability of cytokine induced cells cultured with HMWHA (5) was significantly higher compared to the control (1) but not compared to cytokine induced cells not treated with HA (6). Viability of cytokine induced cells cultured without HA (6) was significantly higher compared to the control (1) (Figure 5).

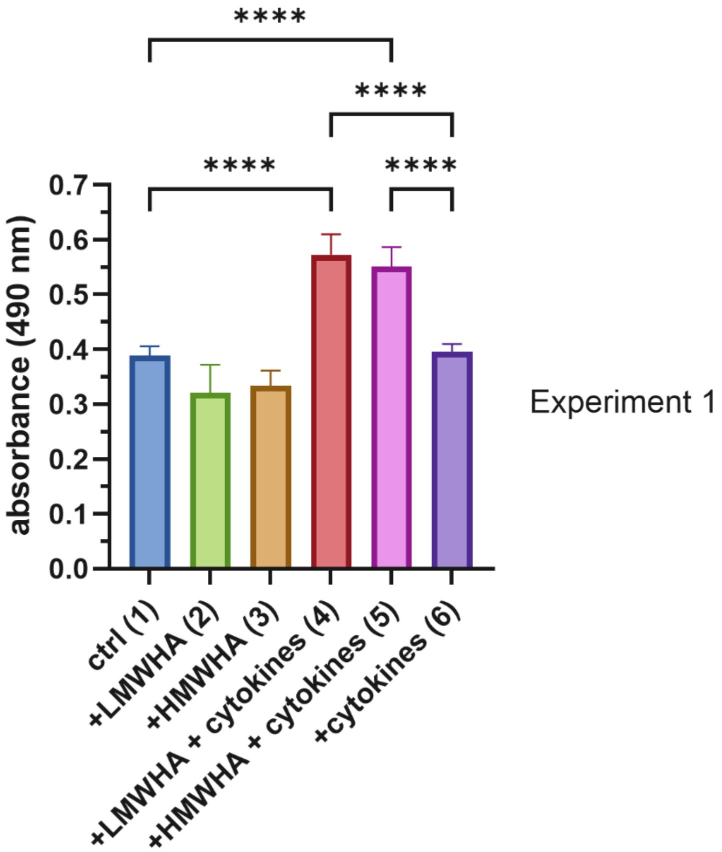

Figure 3. MSC viability after six days of treatment, experiment 1: Cell viability measured via Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay. Absorbance measured at a wavelength of 490 nm. Data is shown as mean ± standard deviation (n = 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

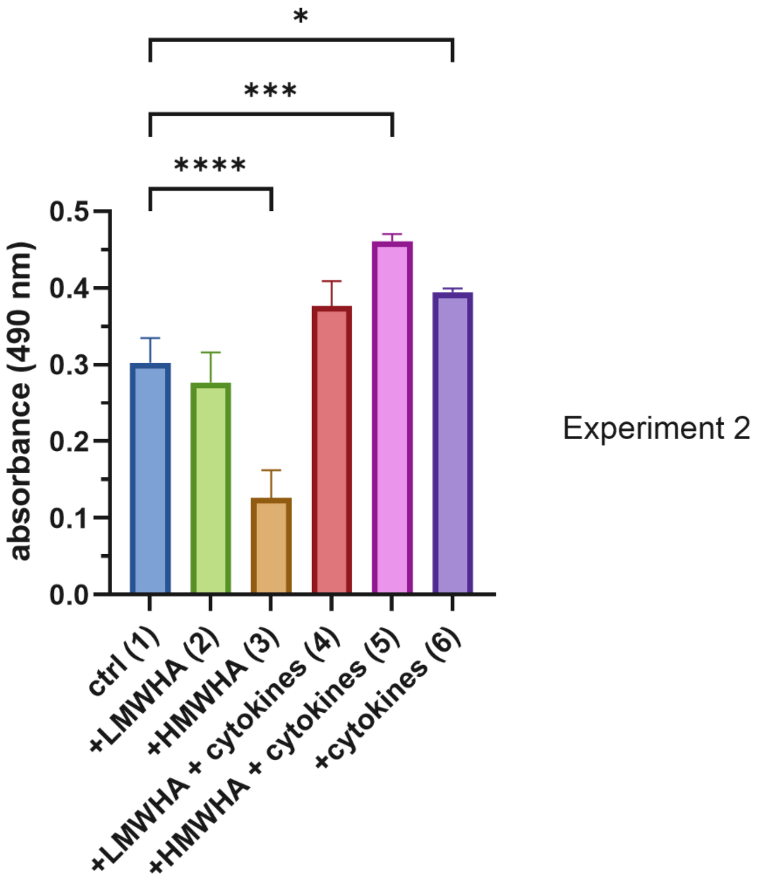

Figure 4. MSC viability after six days of treatment, experiment 2: Cell viability measured via Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay. Absorbance measured at a wavelenght of 490 nm. Data is shown as mean ± standard deviation (n = 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

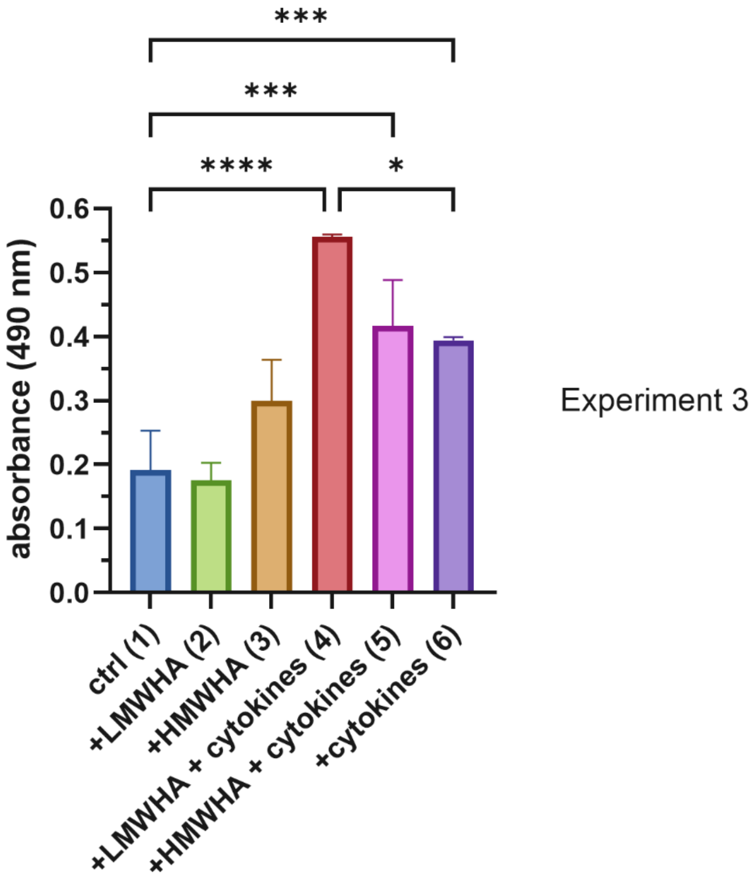

Figure 5. MSC viability after six days of treatment, experiment 3: Cell viability measured via Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay. Absorbance measured at a wave-lenght of 490 nm. Data is shown as mean ± standard deviation (n = 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.2 Phase-contrast microscopy

The eAD-MSCs had a fibroblast-like, elongated appearance with multiple projections (multipolar). Cells grew attached to the culture plate. No obvious differences in cell morphology were seen between experimental groups. After seven days of total culture time, cells were organised into a dense network covering the bottom of the plate. (Figure 6)

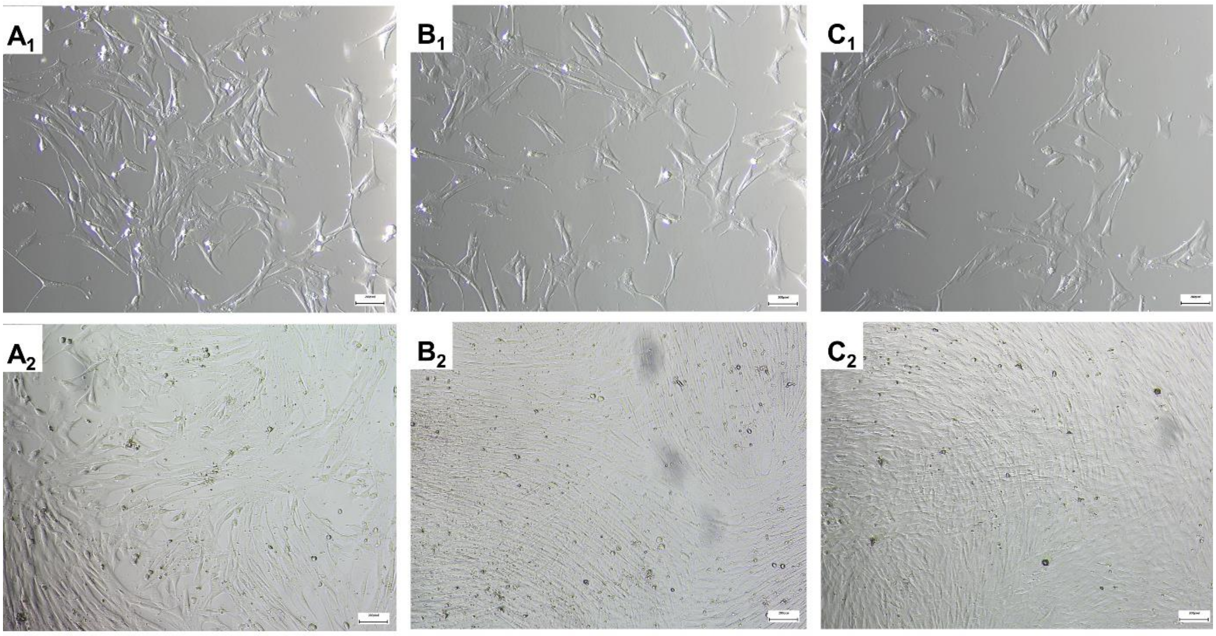

Figure 6. Phase-contrast microscopy images of eAD-MSCs: Images of cells of group 1 (A), group 4 (B) and group 5 (C) were taken on day 4 (A_1, B_1, C_1) and day 6 (A_2, B_2, C_2) of treatment. Images were taken at 10 x magnification. Images depicted were chosen representatively for eAD-MSC morphology seen in all groups. Scale bar = 200 pixel.

3.3 Immunocytochemistry and fluorescence microscopy

Staining of CD44, as well as counterstaining of the nuclei with DAPI, was successful in all experimental groups. Visually, no differences of CD44 expression or localisation could be discerned between groups. CD44 seems to be abundantly expressed on the surface of eAD-MSCs of all experimental groups (Figure 7).

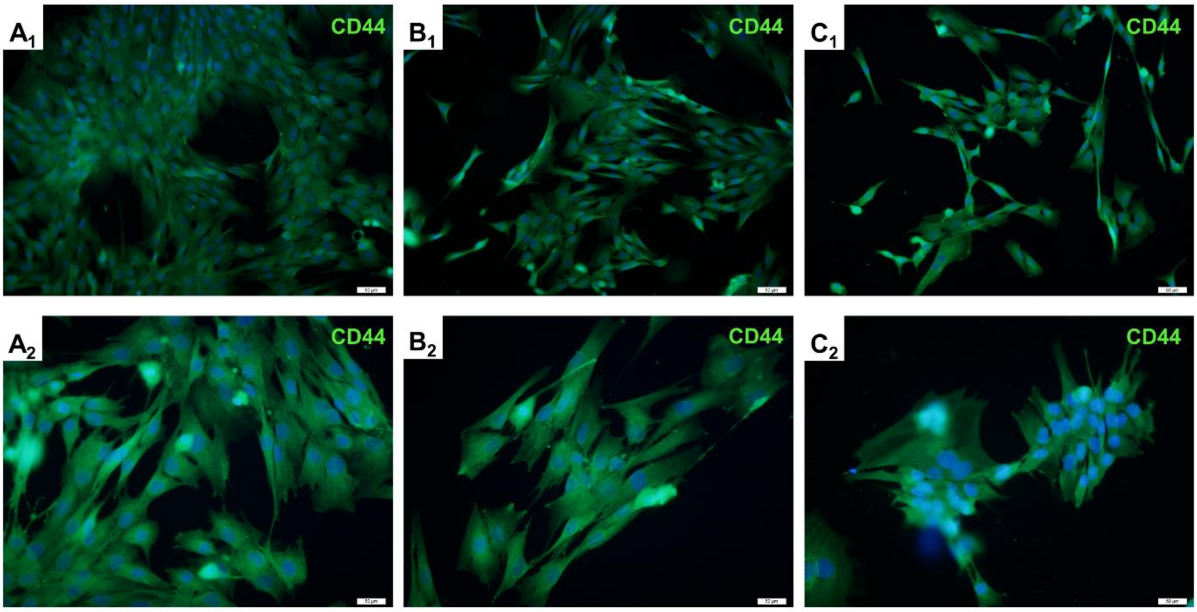

Figure 7. Fluorescence microscopy images: Selection of quality images representing results seen in all groups. Cells in group 1 (A), group 4 (B) and group 5 (C) were imaged at 20x magnification (A_1, B_1, C_1) and 40x maginifcation (A_2, B_2, C_2). Green fluorescence signal corresponds to CD44, blue signal corresponds to nuclei stained with DAPI. Scale bar = 50 µm.

3.4 Flow cytometry

Practically no difference in CD44 positive cells could be seen between cells cultured with LMWHA and HMWHA and between cells cultured with HA and the control. Cytokine induction also made no difference regarding fraction of CD44 postive cells and HA treatment did not modify this. Regardless of culture environment, CD44 was found to be ubiquitously expressed in eAD-MSCs. CD44 expression levels seem similar between experimental groups (Figure 8). The successful replicate of the experiment also showed very similar results.

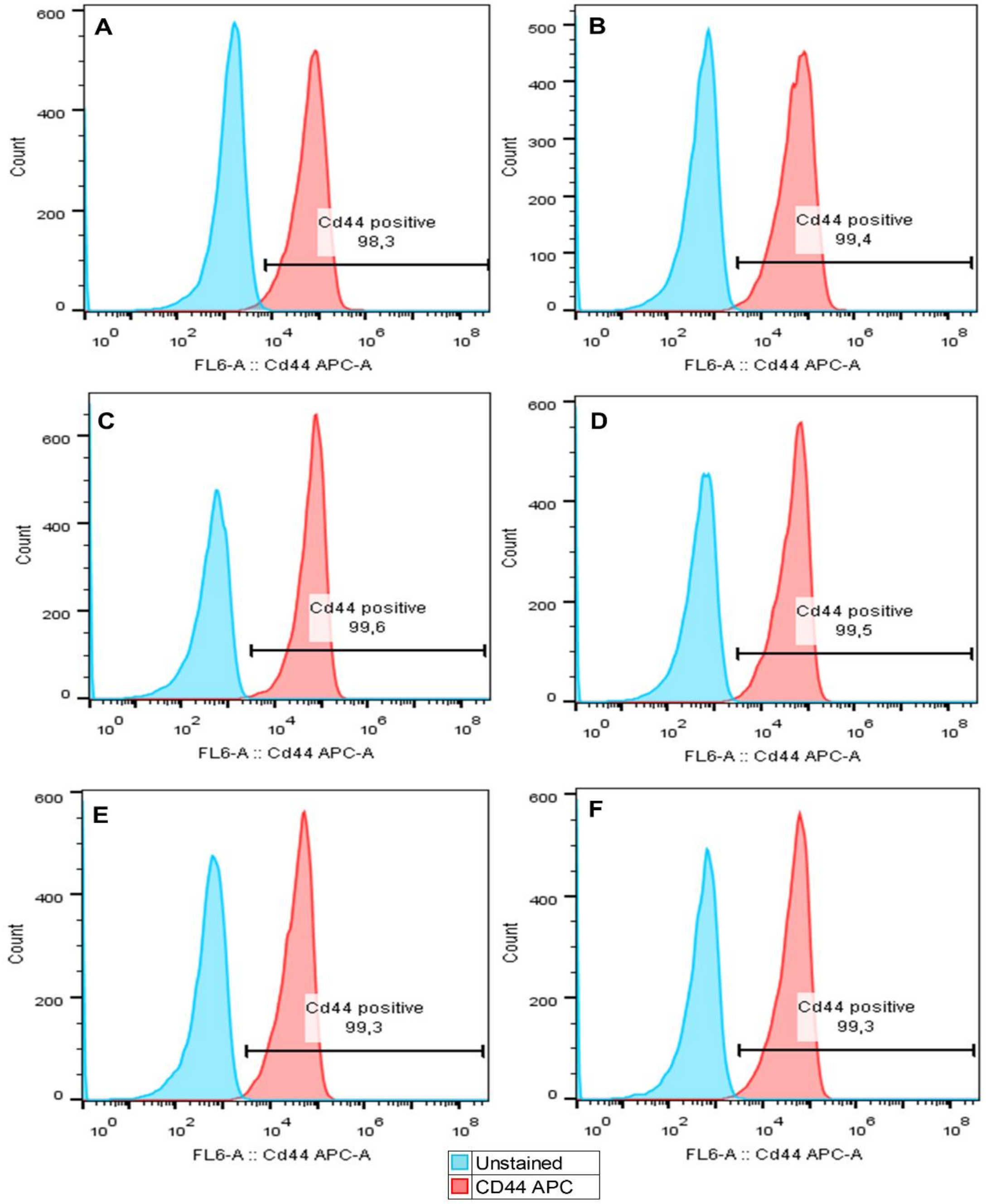

Figure 8. CD44 expression flow cytometry results: APC fluorescence signal (x-axis) is plotted against cell count (y-axis) for all experimental groups: group 1 (A), group 2 (B), group 3 (C), group 4 (D), group 5 (E), group 6 (F). Signals of unstained cells (blue) and labelled cells (red) are combined in each graph. Amount of CD44 positive cells is given in percent for each experimental group.

4 Discussion

In this study, one-time cytokine treatment with TNFα (50 ng/ml) and IL-1β (10 ng/ml) was used to induce inflammatory conditions in eAD-MSCs. Simultaneous conditioning of the cells with HA of two different molecular weights, that exceeded the length of cytokine induction, was used to observe the ability of HA to modify the effects of cytokine induction in eAD-MSCs. Effect of HA on non-induced cells was also explored. Cell viability and CD44 expression was determined. Cell morphology was examined using phase contrast microscopy.

Initially, it was hypothesised that cytokine induction would decrease cell viability compared to non-induced cells, based on studies reporting IL-1β (Jiang et al. 2020) and potentially TNFα (Peng and Ying 2014) causing a decrease in cell viability. In this study however, two of three viability assay showed a significant increase of viability in cytokine induced cells (cultured without HA) compared to the non-induced control. These findings are also in accordance with the findings of previous studies in which short-term treatment with TNFα improved the function of dental pulp cells (Ueda et al. 2014) and combined treatment with TNFα and IL-1β increased the proliferation of eAD-MSCs (Brandt et al. 2018).

No clear effects of HMWHA (> 950 kDa) or LMWHA (15-40 kDa) on viability were seen in non-induced cells compared to the control. Contrary to what was hypothesised, LMWHA had no effect on cell viability, and HMWHA caused reduced viability of cells compared to control in one of three experiments. A previous study exploring the effects of molecular weight of HA on the proliferation of dental pulp stem cells similarly found no significant effects (Schmidt et al. 2023).

More interesting findings regarding metabolic activity and viability of MSCs were found in cytokine induced groups. Especially cytokine-induced cells treated with LMWHA showed a significant increase in viability compared to cytokine-induced cells cultured without HA and the control in two of the three experiments. HMWHA treatment of cytokine-induced cells was found to have no, or a slight positive effect on viability compared to cytokine-induced cells not treated with HA or the control. Cytokine induction of eAD-MSCs with TNFα and IL-1β was shown to significantly increase viability *in vitro* compared to the control, and especially LMWHA could enhance this effect. This is in accordance to what was hypothesised. Previous studies have found LMWHA and HA fragments to increase proliferation of endothelial cells and enhance angiogenesis (Lu et al. 2023, Slevin et al. 2007, West et al. 1985). Results of this study point to LMWHA having a positive effect on metabolic activity, viability and proliferation on MSCs in an inflammatory environment. More studies are needed to solidify this finding and to uncover the underlying mechanisms.

CD44 was ubiquitously expressed on eAD-MSCs in this study. The fraction of CD44 positive cell did not differ greatly between experimental groups. Additionally, expression levels of CD44 seem similar between groups. A previous study reported HA to cause a significant increase in CD44 expression in MSCs (Corradetti et al. 2017). However, Moreno et al. explored the effect of HA at a concentration of 1 mg/ml adipose derived MSC culture and found no effect on CD44 expression (Moreno et al. 2015).

This study is not without limitations. To achieve clearer, more consistent results for the viability assay, more repetitions of the experiment and a higher number of replicates per experimental group could be necessary. A source of error that could have contributed to the varying results between experiments is improper mixing of HA with media, due to its high viscosity. This could have resulted in differing concentrations of HA in the culture media, impacting cell viability or changing background absorbance of the media. Imprecise pipetting could also account for some of the variability.

Another limitation of the experimental set-up may be the use of only two inflammatory cytokines, IL-1β and TNFα, to model onset of inflammation. Furthermore, the chosen analyses for this project provided only limited information about the cultured MSCs, namely viability, morphology and CD44 expression. A limiting factor is also the use of only two molecular weights of HA and the high price of the HA.

Taken together, this study brough forward novel findings on how conditioning with HA of different molecular weights affects cell viability during short-term inflammatory conditions and confirmed some of the previous findings about the influence of HA on cells during standard culture conditions. The finding of the impact of LMWHA on cell viability during the onset of inflammatory conditions and not in standard conditions is interesting and worth further exploring. A broader spectrum of analyses to explore different properties of MSCs in the chosen culture conditions will be necessary in future research. Furthermore, cultivation of MSCs with healthy and OA SF may help explore the effects of the joint environment on MSCs. Co-culture with immune cells or chondrocytes, as well as exploration of the secretome of MSCs in various culture conditions might also be of interest.

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