**Leonardo do Nascimento**

# Ação *in vitro do* Ácido Hialurônico na Viabilidade e Proliferação de Condrócitos Humanos

Caxias do Sul

2019

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Dissertação apresentada à Universidade de Caxias do Sul como pré-requisito para obtenção de Título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Asdrubal Falavigna

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**AÇÃO IN VITRO DO ÁCIDO HIALURÔNICO NO CONTROLE DA OSTEOARTRITE**

*Leonardo do Nascimento*

Dissertação de Mestrado submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul, como parte dos requisitos necessários para a obtenção do título de Mestre em Ciências da Saúde, Linha de Pesquisa: Engenharia e Terapia Celular

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## Dedicatória

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**Sumário**

Esta dissertação de Mestrado Acadêmico Stricto Sensu é apresentada no formato exigido pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul. A mesma é constituída da secção de “Introdução com referências bibliográficas”, a inclusão do artigo original submetido/publicado em periódico Qualis A na classificação da Coordenação de Aperfeiçoamento de Pessoal em Nível Superior (CAPES), as “Considerações Finais e Perspectivas” e Anexos.

# 1 INTRODUÇÃO

A osteoartrite (OA) é uma doença comum, heterogênea e multifatorial que promove uma destruição das cartilagens, a exposição do osso subcondral e a deformidade articular [1,2]. A OA tem uma etiologia e apresentação clínica variada. O diagnóstico é feito através da combinação de dados clínicos, laboratoriais e radiológicos, com uma progressão diferente para cada caso [2].

A perda da função e qualidade da cartilagem articular (CA) é acelerada pelo aumento da idade, sobrepeso e na execução das atividades de repetição [2,3,4]. A incidência de OA é de 35% nos pacientes acima de 50 anos e de 80%, na faixa de idade superior aos 65 anos [5,6]. Estima-se que mais de 67 milhões de pessoas serão acometidas nos Estados Unidos até 2030 [7]. O impacto econômico da OA tem aumentado progressivamente por meio de custos diretos e indiretos em decorrência dos procedimentos cirúrgicos corretivos e questões previdenciárias [8]. A OA também tem avançado no Brasil devido ao aumento da expectativa de vida, práticas esportivas, atividades de repetição e sobrepeso [9].

A CA é um tecido avascular, responsável pela absorção de impacto e atrito, sendo composto principalmente por cartilagem hialina e pela presença de um único tipo de célula, o condrócito [10,11,12]. O condrócito é uma célula mesenquimal responsável pela síntese da matriz extracelular (MEC) e pelo crescimento e manutenção da homeostase através da produção de enzimas, fatores de crescimento e mediadores inflamatórios [13,14,15]. A MEC é composta por 70% de água e macromoléculas do tipo colágenos II, glicoproteínas não colagênicas, hialurano e proteoglicanos (PGs) [16]. O fenótipo dos condrócitos e da MEC variam em quatro diferentes zonas: zona superficial, zona intermediária, zona profunda e zona calcificada [17,18].

A OA altera o equilíbrio da função do condrócito e ativa os fatores pró-inflamatórios e catabólicos [19]. Os condrócitos reduzem sua capacidade metabólica e a concentração de proteoglicanos, formando uma MEC de baixa qualidade incapaz de restaurar a superfície articular original [20,21]. A degeneração progressiva dos condrócitos induz à apoptose [22,23,24].

A OA do joelho é amplamente estudada por estar associada com incapacidade física [25]. A grande maioria dos tratamentos não-cirúrgicos da OA de joelho é ineficaz. Entre as alternativas, existe o reforço muscular, a perda de peso e

a administração intra-articular (IA) de corticóides, plasma rico em plaquetas, células-tronco mesenquimais adultas e ácido hialurônico (AH) [26,27].

O AH é um glicosaminoglicano (GAG), componente da cartilagem e da sinóvia, que atua como lubrificante e amortecedor elástico da articulação [28]. Com o envelhecimento, existe uma redução de 33% a 50% na concentração de AH nas articulações [29,30]. A reposição IA de AH visa restaurar o líquido sinovial, protegendo a cartilagem contra a erosão e reduzindo a inflamação sinovial [30]. Diferentes tipos de viscossuplementação de AH foram aprovados pelo *Food and Drug Administration* (FDA) para uso nos Estados Unidos. Cada fórmula difere pelo peso molecular, tempo de meia vida, concentração, estrutura molecular, frequência, custo e volume de injeção. O Synvisc One ® (Sanofi-Aventis) Hylan G-F 20 possui alto peso molecular (6 milhões de Daltons), com indicação de injeção única IA para grandes articulações, por ter uma grande semelhança ao hialurano. O hilano G-F 20 tem uma elasticidade de 111±13 Pascais e uma viscosidade de 25±2 Pa.s. A elasticidade e a viscosidade do líquido sinovial do joelho de indivíduos dos 18 aos 27 anos de idade são de elasticidade de 117±13 Pascais e uma viscosidade de 45±8 Pa.s. Os hialuranos são degradados e seus produtos de decomposição não são tóxicos. No Brasil, o produto é aprovado para uso comercial pela Anvisa (registro nº 80149670008).

O AH tem sido usado com a finalidade de reparo da cartilagem inflamatória degenerada em pacientes que não toleram os efeitos adversos das terapias farmacológicas ou que não tiveram resposta nos tratamentos não-farmacológicos [31,32]. Para pacientes com OA não-inflamatória, a viscossuplementação é uma alternativa viável. O AH pode fornecer benefícios, pois reduz a dor e melhora função, sendo indicado principalmente nos jovens ou àqueles que apresentam comorbidades que proíbem a artroplastia [33]. A resposta ao AH varia de acordo com a gravidade da OA, idade, sexo, tempo de instalação dos sintomas e regularidade da atividade física [34,35]. Existe uma dificuldade em analisar os dados já publicados sobre AH devido a baixa qualidade do estudo científico, persistindo incertezas da sua eficácia e uma desconexão entre as recomendações das associações de ortopedia e a prática médica [8,36,37,38,39]. O presente estudo visa analisar a resposta *in vitro* do uso de AH em CA de pacientes com OA de joelho. O condrócito submetido a cultivo em AH teria melhor potencial de proliferação e diminuiria a apoptose dos condrócitos [40].

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## 3 ARTIGO

# *In Vitro* Action of Hyaluronic Acid in Viability and Proliferation of Human Chondrocyte

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## Abstract

**Introduction and background:** Degenerative changes of the cartilage represent an important cause of pain and disability in the world population. Several treatment alternatives for remodeling of the cartilage are part of the treatment arsenal. The use of intra-articular hyaluronic acid has shown promising results. Mechanisms of action are not fully comprehended, and studies are needed to evaluate their effectiveness.

**Objectives:** Measure the efficacy of hyaluronic acid to repair osteoarthritis-affected femur-tibia (knee) joint cartilage using *in vitro* models of 2D and 3D chondrocyte culture.

**Design:** *In vitro* study of human cartilage of the knee affected by osteoarthritis that required surgical treatment. The diagnosis of osteoarthritis was confirmed by clinical and radiological examination. Cartilage was obtained from patients undergoing surgical treatment for joint correction of osteoarthritis of the knee. Samples were cultured with hyaluronic acid (intervention group) or vehicle solution (control group). In monolayer or 2D culture, proliferation and cell viability were measured, and nuclear morphometry was analyzed by immunofluorescence. The 3D culture model evaluated the diameter and fusion ability of the spheroids.

**Results**: Hyaluronic acid improved cell viability and proliferation potential when compared to the control group, and no change in nuclear morphology characteristics was observed. In the 3D evaluation, there was an improvement in the cellular feedback mechanism, increasing the survival and maintenance of the chondrospheres.

**Conclusion:** 2D and 3D culture with hyaluronic acid demonstrates improved chondrocyte viability and proliferation when compared to the control group.


**Keywords:** hyaluronic acid, osteoarthritis, viscossuplementation, cartilage, joint disease, chondrosphere

## 1 Introduction

Osteoarthritis (OA) is a common, heterogeneous, multifactorial disease that promotes cartilage destruction, subchondral bone exposure, and joint deformity [1,2]. The incidence of OA is 35% in patients over 50 years and 80% in the age group over 65 years [3,4]. It is estimated that more than 67 million people will be affected in the United States by 2030 [5]. OA has also advanced in Brazil due to increased life expectancy, sports practices, repetitive activities and overweight [6].

The articular cartilage (AC) is an avascular tissue, responsible for the absorption of impact and friction, being composed mainly by hyaline cartilage and the chondrocyte [7,8,9]. The chondrocyte is a mesenchymal cell responsible for synthesis of extracellular matrix (ECM) and the growth and maintenance of homeostasis through the production of enzymes, growth factors and inflammatory mediators [10,11,12].

OA alters the balance of chondrocyte function and activates pro-inflammatory and catabolic factors [13]. Chondrocytes reduce their metabolic capacity and proteoglycan concentration, forming a low quality ECM unable to restore the original articular surface [14,15]. OA of the femur-tibial (knee) joint is widely studied because it is associated with physical disability [16]. The vast majority of non-surgical treatments of knee OA are ineffective. Among the alternatives, there is muscle strengthening, weight loss and intra-articular (IA) administration of corticoids, platelet rich plasma, adult mesenchymal stem cells and hyaluronic acid (HA) [17,18].

HA is a glycosaminoglycan (GAG), component of cartilage and synovium, which acts as a lubricant and elastic schock absorver of the joint [19]. HA has been used in patients who do not tolerate the adverse effects of pharmacological therapies or who had no response in non-pharmacological treatments [20,21]. It is hard to analyze the already published data on HA due to the low quality of the scientific study, a disconnection between the recommendations of the orthopedic associations and the medical practice [22,23,24,25,26]. This study intends to analyze the *in vitro* response of the use of HA in AC of patients with knee OA.

## 2 Method

This is an *in vitro* experimental study. The study was approved by the Ethics Committee of the University of Caxias do Sul (protocol number 2.503.102).

## 2.1 Patient Eligibility Criteria

We selected symptomatic patients with knee cartilage wear and refractory to the conservative treatment of analgesic and anti-inflammatory medication, postural care, motor physical therapy and muscular strengthening. The patients were referred for radiological investigation of simple X-rays in anteroposterior and supported profile. The clinical-radiological concordance was fundamental for the surgical indication.

The choice of surgical treatment was made by the patient after a conversation with the doctor and his/her relatives, being cleared their doubts as to the result of the treatment and to the postoperative care. Patients were informed of the need to send fragments of the knee cartilage to the laboratory if they accepted to participate in the study.

Inclusion criteria were as follows:

- Patients with degenerative knee cartilage pathology refractory to conservative treatment.
- Radiological evidence of involvement of the lateral or medial aspect of the femorotibial compartment, which may include the presence of one or more Kellgren-Lawrence grade 3 or 4 osteophytes (Table 1) [27].
- Radiological evidence of joint impairment according to Ahlback's classification (Table 2), grade 3 or 4 [28].
- Patient agreed to participate in the study signing a free and informed consent document.

The exclusion criteria were:

- Presence of prior surgery, trauma, tumor or infection in the joint.
- Diagnosis of rheumatological, metabolic or autoimmune diseases.
- Continuous use of anti-arthrosis medication or corticosteroids.
- Presence of genu varum or genu valgum.

## 2.2 Collection of Biological Material and Preparation for Transport

Human knee AC were collected from 3 patients submitted to total knee arthroplasty with OA (age range 47-68 years, 3 women). Demographic data are presented in Table 3.

The collection was performed in a surgical center, with trained staff, under the supervision of the researcher. In aseptic conditions, cartilage samples were collected from the tibial support area of the knee. The material was washed twice in physiological solution and placed in a sterile tube containing 7mL of 1X DPBS (Dulbecco's Saline Phosphate Buffer, code number BR30013-05, LGC Biotechnology, SP, Brazil) plus 1% P / S Penicillin 10.000U / mL-Streptococcus 10.000µg / mL, code number BR30110-01, LGC Biotechnology, SP, Brazil). The tubes were transported to the Laboratory of Cell Therapy of the University of Caxias do Sul, being maintained at an average temperature of 37ºC.

**2.3 Isolation and Culture of Cells**

The isolation of the chondrocytes of the knee cartilages followed the methodologies published in the literature with modifications [29-34]. The material was mechanically dissociated into petri dish and reconditioned in its container, maintaining a maximum capacity of 10mL by adding the biological material and DPBS1X ((Dulbecco's Buffer Saline Phosphate, code number BR30013-05, LGC Biotechnology, SP, Brazil). The material was maintained for 30 minutes in a humidified oven at 37°C with 5% $CO_2$. After that time, the tissue was enzymatically dissociated with the aid of 0.2 mg / ml Pronase (Nuclease-free, isolated from *Streptomyces griseus*, Cat. No. P5147-1G, Sigma-AldrichÂ®, Missouri, USA) for 1 hour, followed by 1mg/ml Collagenase type I (from *Clostridium histolyticum*, Cat. No. C0130-1G, Sigma-AldrichÂ®, Missouri, USA) for 2 The cell suspension was centrifuged (1000rpm for 10min) and the pellet resuspended in 1ml DMEM (High Glucose Plus (DMEM)) was added to the suspension, Dulbecco's Modified Eagle's L-Glutamine, Code BR11056-05, LGC Biotechnology, SP, Brazil) supplemented with 10% FBS (Fetal Bovine Serum, filtered, Sterile, Inactivated, Code 10-bio500-l, LGC Biotechnology, SP , Brazil) and 1% P/S (Penicillin 10.000U / mL-Streptococcus 10.000µg/mL, code number BR30110-01, LGC Biotechnology, SP, Brazil), which were divided into two 25cm² bottles containing medium DMEM (DMEM-High Glucose Plus - Dulbecco's Modified Eagle's Medium with L-Analyl and L-Glutamine, Code BR11056-05, LGC Biotechnology, SP, Brazil) supplemented with 10% FBS (Fetal Bovine Serum, Filtrate, Sterile, Inactivated, 10% bio-500-l, LGC Biotechnology, SP,

Brazil) and 1% P / S (Penicillin 10.000U / mL-Streptococcus 10.000µg/mL solution, code number BR30110-01, LGC Biotechnology, SP, Brazil). The cells were packed in a humidified oven at 37°C with 5% CO2.

The cells received 2mL of DMEM (DMEM-High Dulbecco's Modified Eagle's Medium with L-Analyl and L-Glutamine, Code BR11056-05, LGC Biotechnology, SP, Brazil) supplemented with 10% FBS , 10% urea, code number BR30110-01, LGC Biotecnologia, SP, Brazil) every 3 days until completing 14 days, when the first complete exchange of media was made, since there was cell adhesion verification. The culture was established around 26 days, with 25cm² containers containing 80% of cellular confluence. About 106 cells were used for flow cytometry analyzes.

### 2.4 Analysis and characterization of isolated cells (chondrocytes) by Flow Cytometry

The cell population established in the culture was determined by flow cytometry (FACScalibur, Becton Dickison Immunocytometry Systems, San Jose, USA) with the following antibodies: anti-CD105, anti-CD73, anti-CD90, anti-CD45, anti-CD34, anti-CD11b, anti-CD79a, anti-CD14, anti-CD117 and anti-HLA-DR [35,36].

### 2.5 General cell culture protocols

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/mL streptomycin at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5 % $CO_2$ in air. For in vitro assays, three independent experiments were performed in triplicate for each patient sample.

### 2.6 Cell viability assay

The number of viable cells with metabolically active mitochondria were determined based on the mitochondrial reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) according to the method described by Nicoletti et al., 2014 [37]. The cells were treated for 24 h and 48 h at different concentrations of SynvicsOne® range from 10 µM to 500 µM. The cell

viability was calculated using the equation: Cell viability (%) = ($Abs_s/Abs_{control}$)100; where $Abs_s$ is the absorbance of cells treated with different SynvicsOne® concentrations and $Abs_{control}$ is the absorbance of control cells (incubated with SynvicsOne® vehicle only). The experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation. $P<0.05$ was indicative of statistical significance versus control group.

### 2.7 Cell counting

To assess the proliferation grade, the human chondrocytes cells were seeded at 15-20×$10^3$ cells per well in 24-well plates for 24 h. Then, the cells were treated for 24 h and 48 h with SynvicsOne® (10 to 500 µM). After this period of incubation, the medium was collected and 200 µl of trypsin/EDTA solution was added to detach the cells, which were counted in a hemocytometer. The cell number of the control group (non-treated cells) was considered 100%. The experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation. $P<0.05$ was indicative of statistical significance versus control group.

### 2.8 Nuclear morphology and morphometric analysis

The 4',6'-diamino-2-fenil-indol (DAPI) staining was carried out to establish the nuclear morphology of the human chondrocytes cells. Briefly, the cells were seeded in 24 well plates and incubated with different SynvicsOne® concentrations for 24 h to 72 h. After incubation, the cells were washed three times in 1% PBS, and fixed with 4% formaldehyde at room temperature, for 15 min. The fixed cells were then washed with 1% PBS, permeabilized with 0.1% Triton X-100 in 1% PBS and stained with a 300 nM DAPI solution (Santa Cruz, CA) at room temperature, for 10 min. The nuclear morphology of the cells was examined under a fluorescent microscope (Carl Zeiss MicroImaging GmbH, Germany). DAPI staining clearly delineates de nuclear morphology that allows the quantification of the nuclear roundeness and solidity measurements by Image J Software. Data from control cells (untreated) are used to set the parameters of the normal population. The morphometric parameters were

calculated considered 100 events for each SynvicsOne® concentration, three times in triplicate. The experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation. $P<0.05$ was indicative of statistical significance.

### 2.9 Preparation of spheroids for 3D *in vitro* culture

In the last decades the culture in 3D in spheroids has shown promising results for evaluation of several tissues [38]. Human chondrocytes from 3 passages were detached from plastic and resuspended in complete growth medium. For cell seeding to spheroids formation, a suspension of $2 \times 10^6$ cells was prepared in 190ul of DMEM supplemented with 50 µg/ml ascorbic acid (Sigma-Aldrich®, Missouri, EUA), 1.25 µg/ml human albumin, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich®, Missouri, EUA) and Insulin-Transferrin-Selenium, ITS 1X (Lonza). Cell density was chosen according to recommendations of the silicone mold manufacturer MicroTissues 3D Petri Dish micromolds (Sigma-Aldrich®, Missouri, EUA). The cell suspension was carefully placed into each 81-well nonadhesive agarose micromold, and the seedings chambers were placed in 6-well culture plates and covered with complete growth media for 1 hour. Spheroid microplates were then incubated at 37°C in a humidified atmosphere with 5% $CO_2$ for 3 days with change of medium post-24h.

### 2.10 Estimation of spheroids diameter and fusion ability

After 3 days, the spheroids formed were exposed to different concentrations of SynvicsOne® (100 µM and 500 µM) and the average diameters (µM) were evaluated for 1 day, 3 days and 7 days in culture using an inverted microscope (Axio Vert A1, Carl Zeiss, Germany). The diameters were colected from 10 spheroids per sample and analysed by Carl Zeiss software (MicroImaging GmbH, Germany). In parallel, the spheroids ability to fusion was examined. Spheroids in culture (control and SynvicsOne® 500 µM) were placed in close proximity in wells pre-coated with agarose, where spheroids surfaces are touching each other. Bright-field images spheroids doublets were obtained at points 12 h, 24 h and 72 h using light inverted microscopy (Axio Vert A1, Carl Zeiss, Germany).

## 3 Results

All samples collected resulted in viable cultures for experimentation. Chondrocytes removed from the cartilages damaged by OA presented potential for differentiation and proliferation (Figure 1).

The phenotypic characterization showed that the cell surface antigens did not show an expression pattern for adult mesenchymal stem cells. In addition, the HLA-DR antigen showed high expression characteristic for differentiated cells. This data infers that the isolated and cultured population actually has a great possibility of being chondrocytes originating from fragments of knee cartilage (Table 4).

### 3.1 Isolation and Culture of the Chondrocyte

Isolated chondrocyte samples were found to be viable in 2D cultures in both the control and HA groups. There was a higher chondrocyte proliferation in cultures that received 100μM and 500μM HA concentrations. This increase remained in cultures evaluated at 24hs and became more evident in cultures with 48hs when compared to the control (Figure 2). The isolated cells had maintenance of viability up to 72 hours and greater proliferative capacity in HA cultures when compared to the control group, suggesting that this medium favors more favorable conditions of chondrocyte conservation and proliferation (Figure 3).

### 3.2 Morphometric analysis of the chondrocytes

DAPI staining provides nuclear morphological features (area, roundness and solidity) and might be related to several mechanisms that affect cell survival processes. The human chondrocytes exposed to different concentrations of SynvicsOne® up to 72hs, not presented morphological signs of nuclear irregularities by morphometric analysis. All cells analyzed presented a regular round shape and a well-defined nuclear surface, without signs of nuclear fragmentation neither pro-apoptotic effects (Figure 4).

### 3.3 3D Viability and Proliferation

Chondroesphere assesment demonstrated the physiological feedback mechanism of unidentified condrocytes in conventional culture media. The maintenance of cellular viability was observed in all the analyzed groups, with results superior to the spheroid diameter of the group submitted to HA when compared to the control group. In the group submitted to HA, concentrations of 500μM, were more effective in maintaining the diameter of the after 7 days of analysis, followed by the concentration of 100μM and finally the control group (Figure 5).

### 3.4 Influence of Viscosity on 3D culture

The evaluation of the free spheroids in agarose covered wells showed the intrinsic capacity of chondrospheres grouping in the attempt of rearrangement and reestablishment of the new articular tissue. The increased viscosity in the medium with HA, even at higher concentrations did not alter or delay clustering of the spheres (Figure 6).

## 4 Discussion

The treatment of AC affected by OA in several stages remains a major challenge in the engineering of tissues and biomaterials [39]. In this study, we isolated cartilage chondrocytes from patients with advanced degrees of knee OA. Chondrocyte characterization was confirmed by flow cytometry, demonstrating compatibility with literature data [35,40,41.42].

The most investigated cell sources for AC repair are chondrocytes, adult mesenchymal stem cells (MTCs) and more recently the use of two or more cell populations in a synergistic *in vitro* co-culture environment [43-48]. The identification of new techniques and protocols with better chondrocyte culture conditions has demonstrated greater phenotype maintenance and cellular restoration in AC [49,50]. We performed protocol of culture of 3D chondrocytes isolated from human AC with advanced degree of OA. The spheroid culture model has become a powerful tool for chondrocyte survival research, and its potential has been explored for the repair of lesions in patients with OA [51,52]. We observed a physiological response of the

chondrosphere, maintaining the mechanism of feedback and proliferation of *in vitro* cartilage. In addition to cohesion, adhesion and compaction, the spheroids portray self-organizing characteristics of the cartilaginous tissue, maintaining the cell type [53]. The initial chondrosphere dimension between 350 and 400 μM corresponds to the physiological limits required for nutrition and diffusion of oxygen in the chondroesphere [54]. Maintenance of the chondroesphere diameter (~ 400μM) after the seventh day of culture in the HA group contrasts with the control group, which presented a physiological decrease in its diameter. The decrease in the physiological diameter of the chondrosphere in 7 days suggests loss of feedback with cell compaction and alteration of its phenotype [55]. This data supports the hypothesis that HA improves the feedback mechanism, increasing the viability and capacity of tissue regeneration.

The HA and other biomaterials can potentiate processes that promote the formation of AC providing an effective means for proliferation and spheroid organization for OA [53,55]. Even with increasing viscosity in the culture with HA, there was no influence of chondrosphere fusion. This data suggests that this characteristic does not change the physiological organization of AC. HA seems to be not enough adhesive to inhibit the spread and cellular dedifferentiation of the chondrogenic phenotype and allow the transport of diffusive nutrients to spheroids [53].

HA did not alter the chondrocyte count, demonstrating no cytotoxic effect [56]. In 3D cultures, greater chondrocyte viability was identified in the groups that received higher concentrations of HA (500μM) when compared to the usual *in vivo* dose (between 50 and 100 μM) and to the control group. This data interfered directly with the effectiveness of the culture. *In vitro* and animal studies have reported that the efficacy of the dose and administration of HA will be better known with a better understanding of its concentrations in clinical practice [57,58].

The positive chondrocyte response in cell culture makes AC with OA a possible cell donor, especially in culture protocols associated with HA. This suggests that chondrocytes previously damaged by OA grown in HA can be used for implants in areas with greater need for functional correction. This data is confirmed by the literature when hydrogels formed from alginate, fibrin, HA, gelatin and polyethylene glycol (PEG) have demonstrated properties that can be manipulated to influence cell growth, differentiation and chondrocyte behavior [59,60].

Implantation of isolated chondrospheres in cartilaginous defects, has been reported [61,62]. However, the combination of spheroids and biomaterials has not been extensively explored in tissue engineering [63]. In our study, HA demonstrated to be a viable medium for the chondrocyte, potentiating the culture in relation to the control group. HA influences directly chondrosphere maintenance and may increase the clinical response of implants in patients with OA or isolated joint lesions.

As a limitation of our research, we have no means to evaluate the supernatant environment, with differentiation of markers and tissue necrosis. The evaluation of the resistance potential of the chondrosphere load in the different groups will also give us more data for future research.

A future perspective of the group would be to standardize the methodology, and evaluate as physiological variables, such as charge and supernatant of the media. The *in vitro* study of different types of cartilage with various loads, such as a cartilage of the human hand and intervertebral disc, is being started.

## 5 Conclusion

Chondrocyte cultures in 2D and 3D human cartilage with OA showed superior results when exposed to HA. Higher HA concentrations have been shown to be more effective in increasing chondrocyte viability and proliferation.

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## Conflicts of interest

The authors declare no conflict of interest.

## Author Contributions Statement

L.N. and A.F. designed the experiments, analyzed the data and wrote the manuscript; M.F. and N.F.N. contributed in *in vitro* experiments and histological analysis; D.M. contributed with cell characterization by flow cytometry. L.N. and A.F. participated of the final approval of the version to be submitted.

**Table 1. Kellgren and Lawrence system for classification of osteoarthritis of knee [27]**

| Grade | Description |
|---|---|
| 1 | Doubtful joint space narrowing (JSN) and possible osteophytic lipping |
| 2 | Definite osteophytes and possible JSN on anteroposterior weight-bearing radiograph |
| 3 | Multiple osteophytes, definite JSN, sclerosis, possible bony deformity |
| 4 | Large osteophytes, marked JSN, severe sclerosis and definite bony deformity |

**Table 2. Ahlbäck classification of osteoarthritis of the knee joint [28]**

| Grade | Description |
|---|---|
| 1 | Joint space narrowing (less than 3 mm) |
| 2 | Joint space obliteration |
| 3 | Minor bone attrition (0-5 mm) |
| 4 | Moderate bone attrition (5-10 mm) |
| 5 | Severe bone attrition (more than 10 mm) |

**Table 3. Demographic Data**

| | |
|---|---|
| Patients, *n* | 3 |
| Gender, female, *n* | 3 |
| Age, mean, years | 59.3 ± 8.8 (47 - 68) |
| Duration of symptoms, months | 23 ± 8.2 (12 - 32) |
| Kellgren-Lawrence, 3/4, *n* | 0/3 |

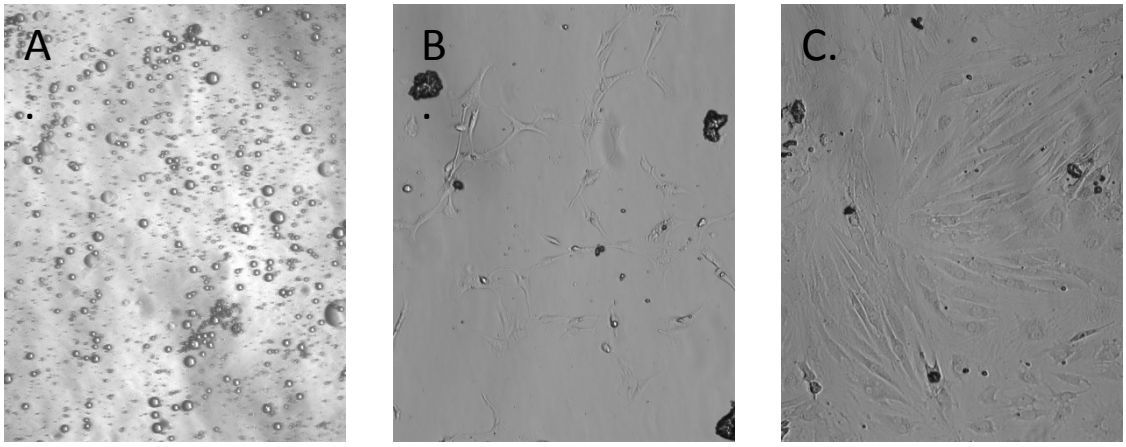
Data at the time of collection of articular knee cartilage.

**Table 4. Phenotypic characterization in cells isolated from knee cartilage**

| Phenotype | Cell phenotype isolated from knee cartilage (%) | International society for Cellular Therapy position statement [35] | Cellular Therapy position statement [40,41] | International society for Cellular Therapy position statement [35,42] |
|---|---|---|---|---|
| CD11b | 23.01% | Negative | - | - |
| CD14 | 91.50% | Negative | - | Negative |
| CD19 | - | Negative | - | - |
| CD34 | 22.07% | Negative | - | Negative |
| CD45 | 18.50% | Negative | - | Negative |
| CD73 | 49.60% | Positive | - | Positive |
| CD90 | 99.70% | Positive | - | Positive |
| CD105 | 80.94% | Positive | - | Negative |
| CD117 | 90.50% | - | Positive | - |
| CD79a | 11.60% | - | - | Negative |
| HLA-DR | 98.60% | Negative | - | Negative |

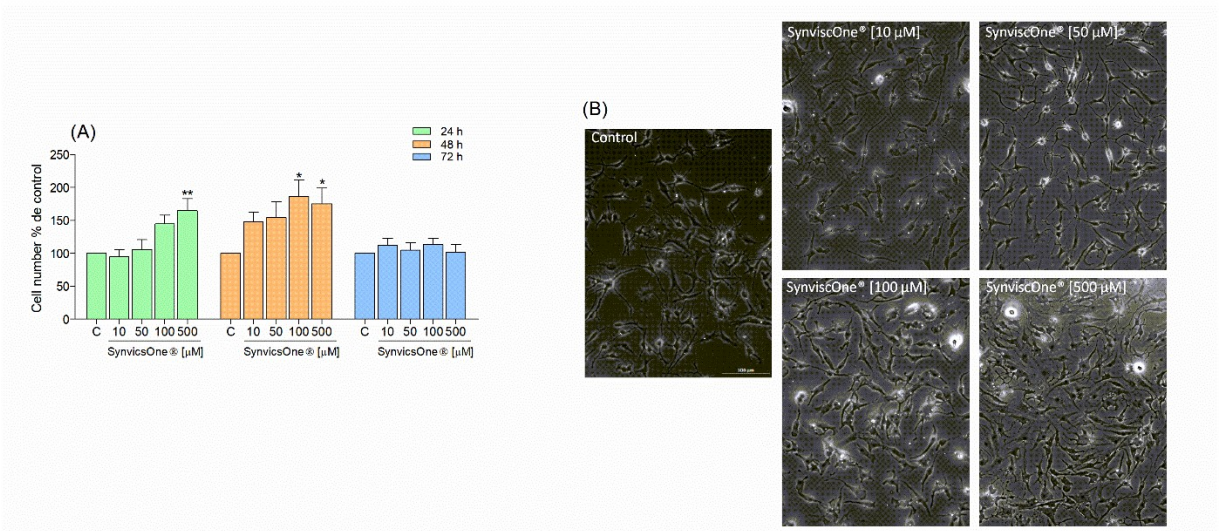
Results of cell surface antigens used in the phenotypic characterization of isolated knee cartilage cells compared to literature data [35,40,41,42].

**Figure 1. Isolation and culture of chondrocytes**



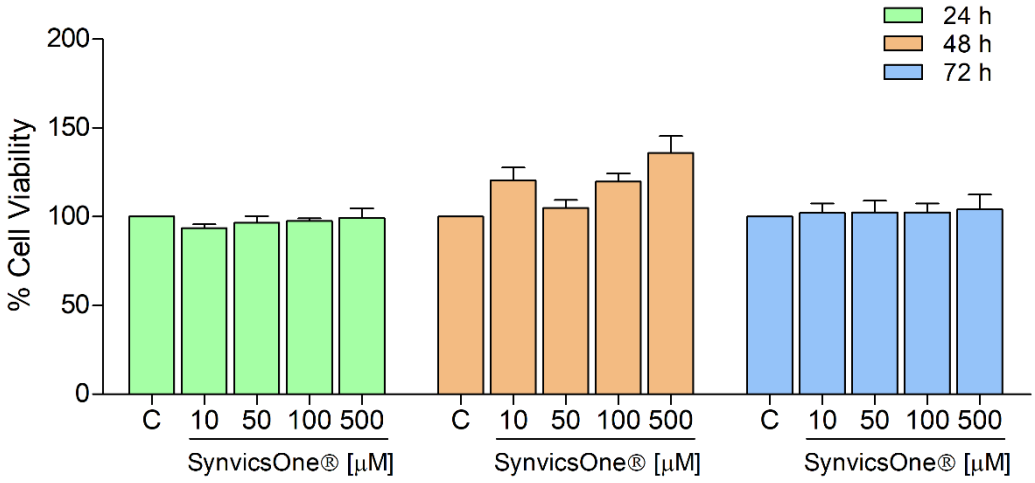

A. Isolation of Knee Chondrocytes, B. Chondrocyte culture onset (14 days) and C. Established culture with 80% confluency (26 days). Objective 10x.

**Figure 2. Qualitative and Quantitative Cell Proliferation**



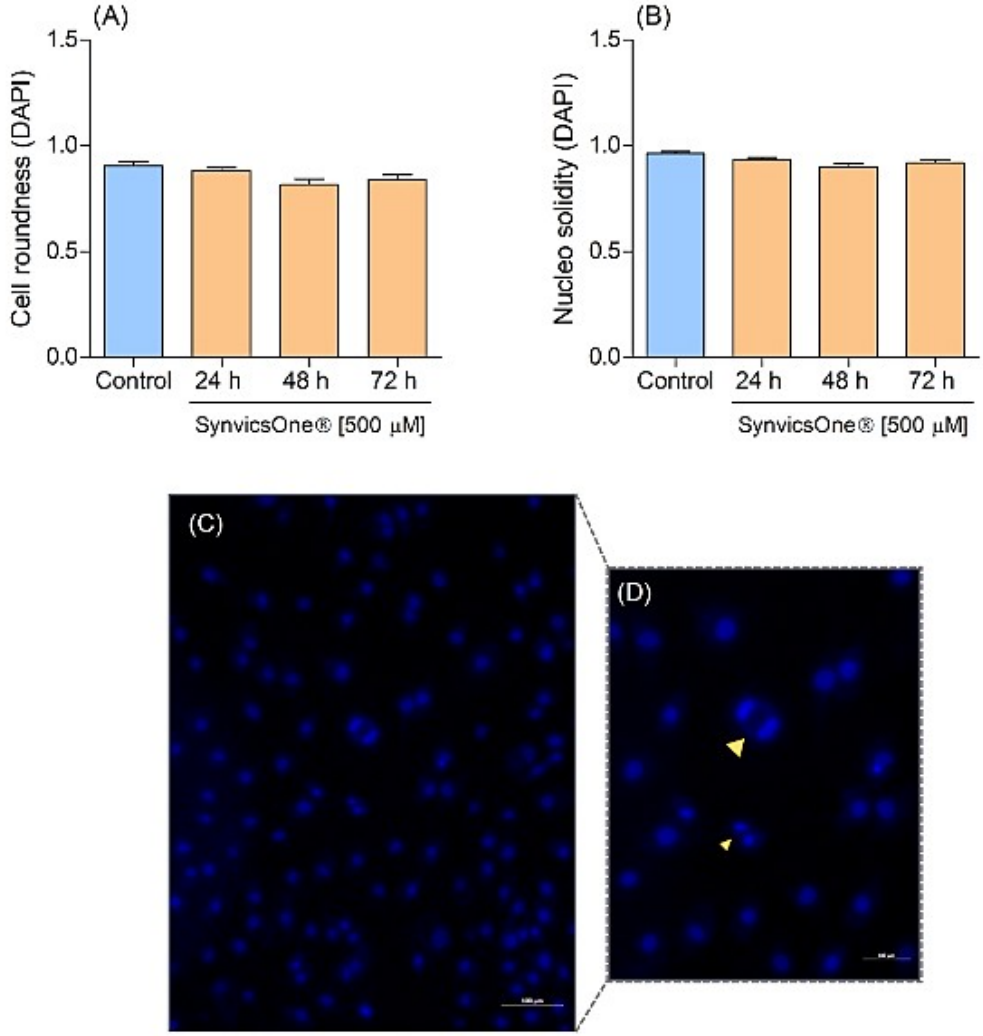

(A) Effect of incubation with SynviscOne® (10-500 µM) on human chondrocytes cell proliferation after 24, 48 and 72 h (n=3). Each column represents the mean ± SD. *p<0.05 *versus* control. (B) Representative images of human chondrocytes exposed to different concentrations of SynviscOne® (10-500 µM) 48 h post incubation (magnification 20x).

**Figure 3. MTT - Quantitative**



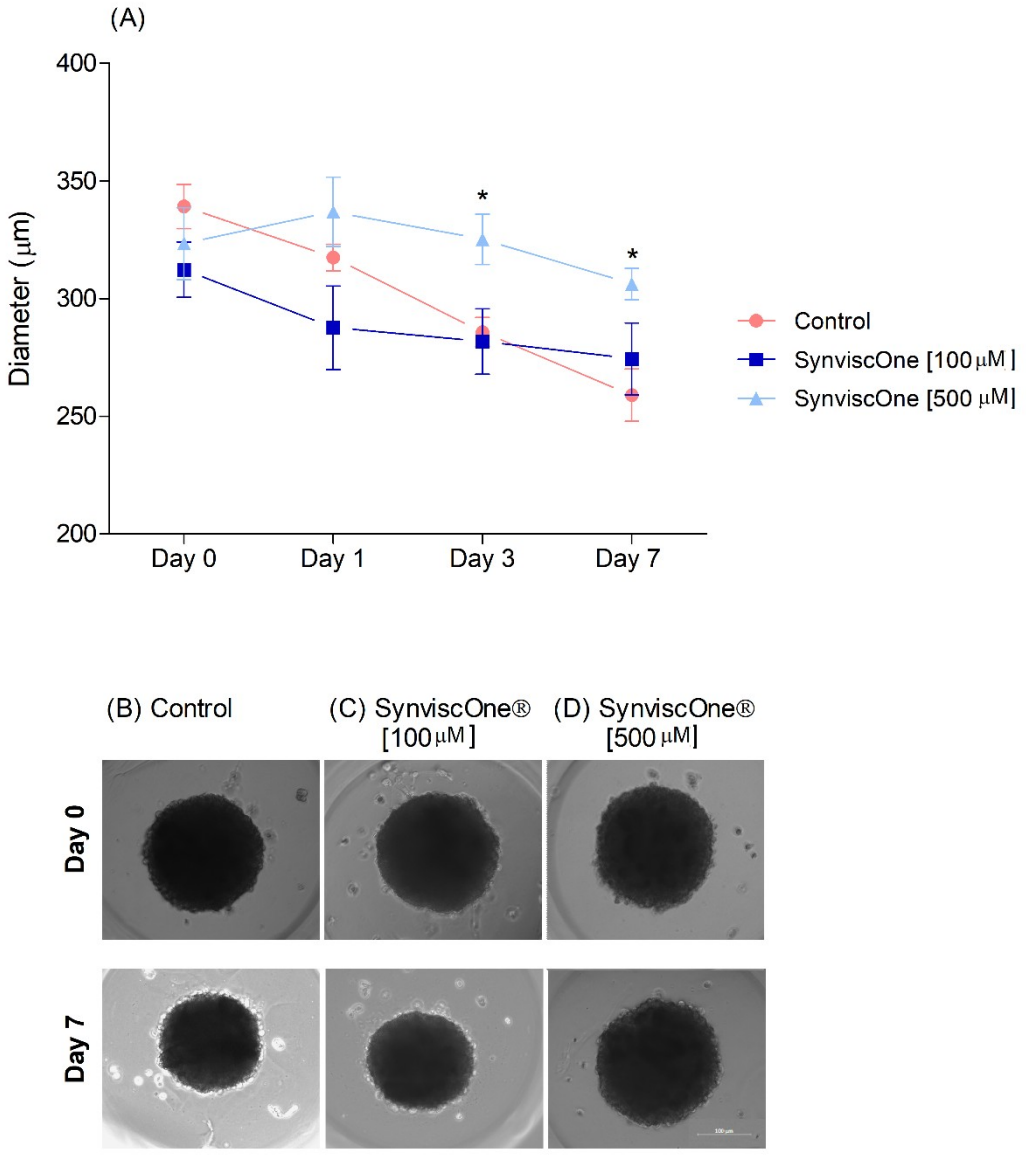

Effect of incubation with SynviscOne® (10-500 µM) on human chondrocytes cell viability after 24, 48 and 72 h (n=3). Each column represents the mean ± SD. *p<0.05 *versus* control.

**Figure 4. DAPI – Qualitative and Quantitative**



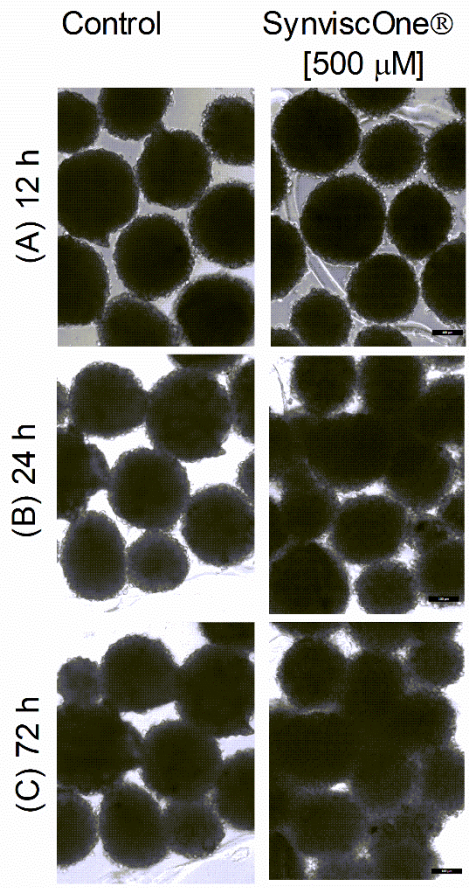

(A) Cell roundness and (B) nucleo solidity as a parameter of morphometric nuclear analysis of human chondrocyes after 24, 48 and 72 h of incubation with SynviscOne® (500 µM). Each column represents the mean ± SD. *p<0.05 versus control. (C) Representative images of chondrocyte cells depicted a regular round shape and a well-defined nuclear surface and (D) mitotic figures (yellow arrow) in late anaphase with the appearance of two discs migrating toward opposite poles of the cell on 72 h of SynviscOne® (500 µM) (magnification 20x).

**Figure 5. Diameter of the spheroid**




(A) Determination of the spheroid diameter as a result of their maturation process when exposed to SynviscOne® (100 - 500 µM) up to 7 days. Data are represented as mean ± SD. *p<0.05 versus control. Representative images of (B) control spheroids and spheroids exposed to (C) SynviscOne® (100 µM) or (D) SynviscOne® (500 µM).

**Figure 6. Spheroids fusion**




Spheroid retained their fusion capability when exposed to SynviscOne®. Representative images of control spheroids and spheroids exposed to SynviscOne® (500 μM) in close contact after (A) 12 h, (B) 24 h and (C) 72 h. Note an expressive fusion in C (magnification 20x).

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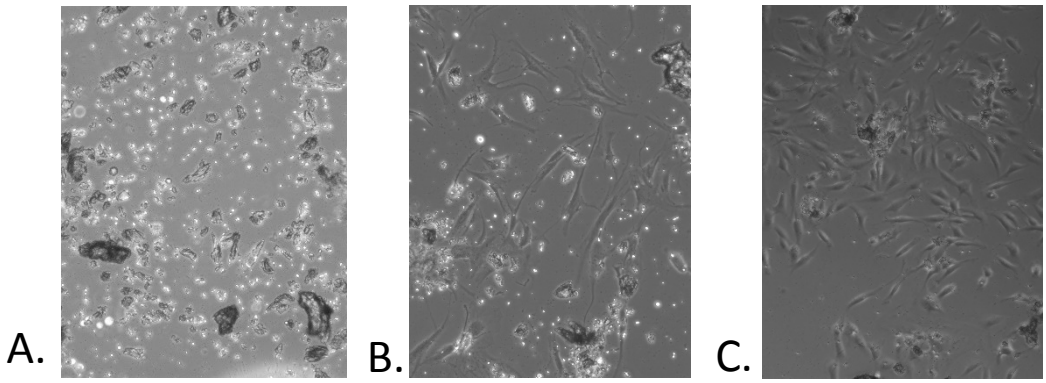
## 4. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

A padronização da metodologia, e a avaliação de variáveis fisiológicas como carga e sobrenadante do meio, possibilitará novos projetos de pesquisa visando a confecção de biomateriais para reposição de cartilagem articular. Tipos distintos de cartilagem submetidos a diferentes cargas, cartilagem da mão e disco intervertebral, serão também avaliadas e analisadas as respostas com o estudo da cartilagem do joelho.

A caracterização e a cultura de condrócitos da cartilagem provenientes de pacientes com osteoartrite em região carpo-metacárpica permanece um desafio. No período do mestrado, houve a possibilidade de caracterizar e cultivar as células da cartilagem da mão. Este protocolo desenvolvido para caracterização e cultivo das células da mão é pioneiro na literatura, tendo sua continuidade como projeto de doutorado. Duas metodologias foram aplicadas para a verificação do melhor protocolo a ser estabelecido para o isolamento, o protocolo padrão utilizado com cartilagem do joelho (Figura 1) e um novo protocolo com testagem de diferentes tempos de ação enzimática (Figura 2).

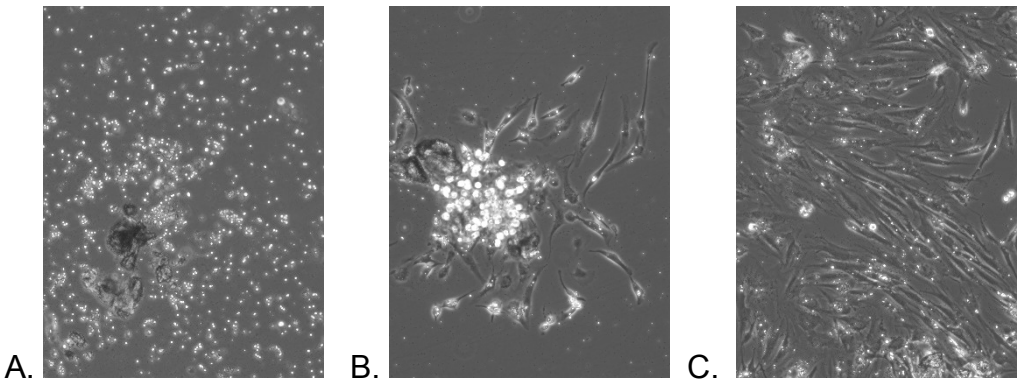
Em torno de $10^6$ células foram utilizadas para as análises de citometria de fluxo. A fenotipagem mostrou elevado perfil de expressão para HLA-DR, o que nos mostra que o material celular avaliado pode ser considerado como células diferenciadas (Tabela 1).

**Figura 1 - Método 1 de Isolamento de condrócitos da cartilagem da mão.**




A) Isolamento celular, B) Iinício da aderência e cultivo celular próximo aos debris teciduais e C) Cultura com confluência próxima de 80%. Objetiva de 10x.

**Figura 2 - Método 2 de Isolamento de condrócitos da cartilagem da mão**.




A) Isolamento celular, B) Início da aderência e cultivo celular próximo aos debris teciduais e C) Cultura com confluência próxima de 60%. Objetiva de 10x.

**Tabela 1. Caracterização fenotípica condrócitos de mão.**

| Fenótipo | Fenótipo das Células isoladas da cartilagem de mão Metodologia 1 (%) | Fenótipo das Células isoladas da cartilagem de mão Metodologia 2 (%) | Posicionamento da sociedade internacional para terapia celular (1) | Posicionamento de Terapia Celular (2,3) | Posicionamento da sociedade internacional para terapia celular (1,4) |
|---|---|---|---|---|---|
| CD11b | 5.99% | 23.20% | Negative | - | - |
| CD14 | 93.60% | 73.40% | Negative | - | Negative |
| CD19 | - | - | Negative | - | - |
| CD34 | 10.90% | 25.60% | Negative | - | Negative |
| CD45 | 14.70% | 17.10% | Negative | - | Negative |
| CD73 | 49.60% | 66.40% | Positive | - | Positive |
| CD90 | 99.20% | 78.50% | Positive | - | Positive |
| CD105 | 91.20% | 71.20% | Positive | - | Negative |
| CD117 | 24.60% | 24.70% | - | Positive | - |
| CD79a | 7.90% | 25.60% | - | - | Negative |
| HLA-DR | 47.44 | 38.10% | Negative | - | Negative |

Caracterização fenotípica nas células isoladas da cartilagem de mão utilizando antígenos de superfície celular com a metodologia 1 e 2 e comparados com dados da literatura [1,2,3,4].

## 4.1 Referências das Perspectivas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# 5 ANEXOS

## 5.1 Parecer do CEP

## UNIVERSIDADE DE CAXIAS DO SUL - RS

### PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** Ácido Hialurônico no Controle da Osteoartrite: Avaliação in vitro em Modelos Distintos de Cartilagem

**Pesquisador:** Asdrubal Falavigna

**Área Temática:**

**Versão:** 1

**CAAE:** 82869517.1.0000.5341

**Instituição Proponente:** Fundação Universidade de Caxias do Sul - FUCS/RS

**Patrocinador Principal:** Financiamento Próprio

**DADOS DO PARECER**

**Número do Parecer:** 2.503.162

**Apresentação do Projeto:**

Trata-se de pesquisa intitulada - Ácido Hialurônico no Controle da Osteoartrite: Avaliação in vitro em Modelos Distintos de Cartilagem. O presente estudo compreende experimentos in vitro com a proposta de estudar a ação do ácido hialurônico em modelos de cartilagem articular com osteoartrite das articulações fêmuro-tibial, carpo-metacárpicas e disco intervertebral degenerado. Pacientes com indicação clínica e radiológica para as patologias citadas nos três grupos serão submetidos a cirurgia corretiva para dano articular. O paciente que participar do estudo terá a cartilagem encaminhada ao laboratório, ao invés da mesma ser descartada. O estudo consistirá de três grupos de pacientes selecionados para coleta de material articular. O Grupo 1 apresentando artrose femuro-tibial (G1FT); o grupo 2 apresentando artrose carpo-metacárpica do polegar (G2CM); e o grupo 3 apresentando doença degenerativa discal (G3DD).

Cada grupo será formado por cinco pacientes. Foi calculado um tamanho amostral de 5 pacientes por grupo, com um poder de 80%, e uma probabilidade de erro alfa de 5%, através de avaliação de estudos já existentes na literatura que possuem avaliação de 3 a 8 pacientes em sua totalidade.O material coletado no transoperatório das cirurgias referidas será encaminhado ao laboratório de Terapia Celular da Universidade de Caxias do Sul, onde será processado pela dissociação mecânica e enzimática dos tecidos e analisado por equipe treinada para ser utilizado em cultivo celular. As células cultivadas a partir das amostras de cartilagem articular com osteoartrite, serão incubadas com diferentes concentrações de ácido hialurônico, a fim de verificar o crescimento das células de cartilagem retiradas de sítios com ou sem carga. A expressão de mecanorreceptores e os testes de viabilidade celular serão avaliados na ausência ou presença(pré-tratamento de 24, 48 e 72 horas) do ácido hialurônico incubados em DMEM e 10% SFB. Nesse sentido, após o período da infusão no cultivo dessas células, as mesmas serão verificadas quanto a regeneração utilizando marcadores imunocitoquímicos e moleculares, previamente definidos e avaliados nas células sem a presença da matriz.

**Objetivo da Pesquisa:**

Objetivo Primário: Avaliar a ação in vitro do uso do ácido hialurônico no reparo de cartilagem articular que sofreu ou não influência de carga em três tipos distintos de cartilagem humana – mão, joelho e disco intervertebral. Objetivo Secundário: - Analisar in vitro a proliferação e a viabilidade das células de cartilagem que sofreram efeito de carga – disco intervertebral e joelho.- Analisar in vitro a proliferação e a viabilidade de células de cartilagem que não sofreram carga – articulação carpo metacárpica do polegar.- Avaliar a resposta regenerativa da cartilagem exposta ao ácido hialurônico.- Avaliar a expressão dos mecanorreceptores em cartilagens com ou sem carga.

**Avaliação dos Riscos e Benefícios:**

Riscos:Segundo os Itens II.22 e IV.3.b, da Resolução CNS nº 466 de 2012 toda pesquisa apresenta riscos nas dimensões física, psíquica, moral,intelectual,social, cultural ou espiritual do ser humano. Além disso, a participação na pesquisa, pode acarretar riscos ligados à manutenção do sigilo e confidencialidade durante a coleta e uso dos dados. Apesar de estes riscos existirem em qualquer pesquisa, os pesquisadores responsáveis pelo estudo manterão o máximo de sigilo e confidencialidade possível quanto as informações do paciente. A pesquisa será realizada somente com o material da cartilagem ou do disco intervertebral descartado na cirurgia. Portanto, os riscos que podem vir a existir do processo cirúrgico estão relacionados somente ao tratamento da doença e não a pesquisa proposta.Benefícios: Através deste estudo poderemos ter benefícios para futuros pacientes que sofrem com comprometimento das articulações: (1) melhor entendimento da doença, de seus possíveis fatores etiológicos e dos mecanismos associados; (2) melhor conhecimento dos fatores degenerativos e suas implicações no ambiente celular; e (3)contribuir com os aspectos terapêuticos que possam melhorar as abordagens da doença.

**Os benefícios obtidos na presente pesquisa auxiliarão futuros pacientes a terem menos sofrimento físico, psicológico e social. Comentários e Considerações sobre a Pesquisa:**

Projeto aparentemente ético, posto que cumpre os preceitos éticos de proteção ao participante do estudo.

**Considerações sobre os Termos de apresentação obrigatória:**

FR assinada pelo prof. Dagoberto Godoy; TCLE adequado; Aquiescência do Hospital Saúde assinado pelo seu diretor técnico.

**Conclusões ou Pendências e Lista de Inadequações:**

Não há pendências.

**Considerações Finais a critério do CEP:**

Diante do exposto, o Comitê de Ética em Pesquisa da Universidade de Caxias do Sul, de acordo com as atribuições definidas nas Resoluções CNS 466/12 e CNS 510/16, aprova o projeto para dar início à pesquisa.

É dever do CEP acompanhar o desenvolvimento da pesquisa, por meio de relatórios parciais e final. Solicitamos que os relatórios contemplem o andamento da pesquisa, as modificações de protocolo, cancelamento, encerramento, publicações decorrentes da pesquisa e outras informações pertinentes. Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e as suas justificativas.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento | Arquivo | Postagem | Autor | Situação |
|---|---|---|---|---|
| Informações Básicas do Projeto | PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1058058.pdf | 23/12/2017 20:18:20 | | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | Termo_Saude_PDF.pdf | 23/12/2017 20:17:20 | Asdrubal Falavigna | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | TCLE_HA_cartilagem.docx | 23/12/2017 20:17:06 | Asdrubal Falavigna | Aceito |

| | | | | |
|---|---|---|---|---|
| Projeto Detalhado / Brochura Investigador | Projeto_PB.docx | 23/12/2017 20:16:55 | Asdrubal Falavigna | Aceito |
| Folha de Rosto | Folha_de_Rosto_PB.pdf | 23/12/2017 20:16:41 | Asdrubal Falavigna | Aceito |

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Não

CAXIAS DO SUL, 20 de Fevereiro de 2018

**Assinado por:**
**Luciane**
**Andreia Bizzi**
**(Coordenador)**

### 5.1 Confirmação de submissão

CC: "Natália Fontana Nicoletti" nfnicoletti@ucs.br, "Daniel Marinowic" daniel.marinowic@pucrs.br, "Manuela Peletti-Figueiró" manu.peletti@gmail.com, "Asdrubal Falavigna" asdrubalfalavigna@icloud.com

Dear Dr. do Nascimento:

**Thank you for the submission of your manuscript  In Vitro Action of Hyaluronic Acid in Osteoarthritis Control to Osteoarthritis and Cartilage.**

**You have now successfully completed the on-line submission process. This acknowledgement and any queries are for the corresponding author.** This e-mail has also been copied to each author on the paper. Please bear in mind that all queries regarding the paper should be made through the corresponding author.

The manuscript is undergoing peer review. You will be notified by one of the Editors when the review is complete.

Kind regards,

Editorial Office
Osteoarthritis and Cartilage

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