Evaluation of different commercial hyaluronic acids as a vehicle for injection of human adipose-derived mesenchymal stem cells

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Abstract

Objective: The main purpose of this study is to evaluate, in vitro, the cytotoxicity of different commercial brands of hyaluronic acids to be used as a vehicle for injection of human adipose-derived mesenchymal stem cells (AD-MSCs).

Methods: AD-MSCs were divided into seven groups: one control group where AD-MSCs were cultivated with phosphate-buffered saline (PBS) and six other groups where AD-MSCs were cultivated with different commercial brands of hyaluronic acid. AD-MSC viability analysis was performed after 4, 24, and 48 h in contact with each treatment, using the trypan staining method on a Countess automated cell counter (Thermo Fisher Scientific).

Results: The results clearly demonstrated a significant difference in cell viability when AD-MSCs were exposed to different hyaluronic acids when compared with the control group.

Conclusion: These data suggest that hyaluronic acid can be used as a vehicle for injection of human AD-MSCs, but caution is needed to choose the best product, aiming at its future therapeutic application.

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Avaliação de diferentes ácidos hialurônicos comerciais como veículo de injecção para células mesenquimais humanas derivadas do tecido adiposo

RESUMO

Objetivo: Avaliar in vitro, de forma direta, a citotoxicidade de ácidos hialurônicos como veículo de injecção para linhagens de células-tronco mesenquimais (CTMs) obtidas de tecido adiposo humano.

Métodos: As CTMs foram divididas em sete grupos, os quais foram expostos ao ácido hialurônico de seis marcas comerciais, além do contato com tampão fosfato-salino PBS (grupo controle). Após quatro, 24 e 48 horas, foi feita a análise da viabilidade celular através do contador Countess pelo método de coloração com Trypan Blue (Thermo Fisher Scientific).

Resultados: Os resultados demonstraram uma diferença significativa na viabilidade celular quando essas linhagens de CTMs foram expostas aos diferentes ácidos hialurônicos em comparação com o grupo controle.

Conclusão: Os dados sugerem que o ácido hialurônico pode ser usado como veículo de injecção para CTMs, porém é necessária cautela na escolha do melhor produto para aplicação terapêutica futura.

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Introduction

Articular cartilage lesions are among the most frequent musculoskeletal pathologies, and the most difficult to treat. This is due to the fact that the chondral tissue is subject to constant stimuli; moreover, it has a low potential for repair due to its vascular and lymphatic circulation deficiency.¹,²

Among the currently available surgical treatment options, one that presents encouraging results is the autologous chondrocyte implant described in 1994 by Brittberg et al., in which, after collection of healthy cartilage, cell expansion and culture are performed and, at a second time, the chondrocytes are implanted in the chondral lesion.³,⁴ Due to the limitations for this procedure, such as unavailability and degeneration of donor cartilage, problems with in vitro chondrocyte expansion, and its high cost, viable optional forms have been sought to treat this important joint lesion.⁵,⁶ Thus, mesenchymal stem cells (MSCs) have been identified as a good treatment option. Among the various sources of production, adipose-tissue derived MSCs (AD-MSCs) are highlighted, as they present potentiality for regeneration and differentiation in cartilaginous tissue, are available in large amounts in the body and can be obtained with easy and non-invasive techniques.⁷-⁹ Hialuronic acid is considered an excellent vehicle for MSCs in tissue repair, due to its viscosity and physicochemical properties, and it has gained prominence in tissue bioengineering⁹,¹⁰ (Table 1). Nonetheless, no data assessing the potential of MSCs when exposed to different hyaluronate molecular weights, brands, and viscosities are available.

This study is aimed at evaluating in vitro, in a direct way, the cytotoxicity of different brands of hyaluronic acid on MSC lineages obtained from human adipose tissue.

Material and methods

Ethical considerations

All human tissue samples for MSC isolation were obtained after the informed consent form was signed by the donor or guardian, in agreement with the Research in Human Beings Ethics Committee of the University Hospital and Institute of Biosciences of the University of São Paulo (protocol no. 040/2005).

Collection, isolation, and expansion of MSCs

AD-MSCs were isolated using methods previously described by the present group.¹¹-¹⁴ Adipose tissue was collected in cesarean sections from the abdominal subcutaneous region of the patient. After collection, the samples were stored in a sterile flask and transported to the laboratory in thermal boxes with temperature control between 4 ºC and 24 ºC. All samples were processed within 48 h.

Briefly, to isolate the AD-MSCs, the adipose tissue samples were washed in phosphate-buffered saline (PBS) 1x, pH 7.4, with 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies), and then dissociated with 0.075% GMP (Serva) collagenase at 37 ºC for 30 min. Then, the infranatant was centrifuged at 300 × g for 5 min, and the cell pellet formed was plated on culture bottles, following a ratio of 1000–3500 cells per cm².

For in vitro expansion of the AD-MSCs, the animal serum-free MSC culture system StemPro MSC SFM (Thermo Fisher Scientific) was used according to the supplier’s recommendations (proliferation medium). Each lineage was maintained at 37 ºC in a humidified atmosphere with 5% CO₂ until reaching at least 1 × 10⁶ cells to be cryopreserved in liquid nitrogen at −196 ºC with the StemPro MSC SFM kit. To achieve the
number of cells desirable for the experiments, they were thawed in a 37 °C water bath and plated in culture bottles with the abovementioned proliferation medium.

**Immunophenotyping**

In order to confirm the mesenchymal origin of the obtained AD-MSC lineages, cell membrane protein expression analysis was performed by flow cytometry, using the methods previously described by the present group. To that end, the adherent cells obtained in stage five were incubated with the following conjugated antibodies: CD29-Pe-Cy5, CD31-Pe, CD34-PerCP, CD45-FITC, CD73-Pe, CD90-R-Pe, CD105-Pe, HLA-ABC-FITC, HLA-DR-R-Pe (Becton Dickinson), according to the manufacturer’s recommendations. As a negative control, cells were incubated with PBS instead of the primary antibody. A total of 10,000 events were acquired with the Guava EasyCyte flow cytometer (Guava Technologies) and analyzed with Guava ExpressPro software (Guava Technologies).

**Differentiation test**

hASC and hUCUT were submitted to differentiation protocols to evaluate their MSC properties. Thus, the adherent cells obtained in stage 5 were submitted to adipogenic, chondrogenic, and osteogenic in vitro inductions with the StemPro differentiation kit (Life Technologies) according to the manufacturer’s recommendations. Non-induced control samples were maintained in proliferation medium throughout the assay.

**Adipogenesis**

Adipogenic differentiation was confirmed on day 21 by staining with Oil Red O (Sigma). For this, the cells were set with 4% paraformaldehyde for 30 min, washed with distilled water, and stained with a working solution of 0.16% Oil Red O for 20 min.

**Chondrogenesis**

To confirm chondrogenic differentiation, after 21 days of induction the cells were stained with 1% toluidine blue for 1 min, washed with distilled water, and then treated with a wash of 70%, 95%, and 100% ethyl alcohol solution, respectively.

**Osteogenesis**

Osteogenic differentiation was assessed by the Von Kossa stain. Briefly, the cells were stained with 1% silver nitrate (Sigma) for 45 min under ultraviolet light, stained with 3% sodium thiosulfate (Sigma) for 5 min, and then contrasted with van Gieson. At the end of this step, the cells were carefully washed with ethyl alcohol and allowed to dry completely.

**Experimental groups**

In step eight, MSCs were divided into seven groups, which were exposed to hyaluronic acid from six commercial brands and phosphate-saline buffer – PBS (control group):

Group 1: AD-MSC in contact with Fermathron® (Hyaltech),
Group 2 AD-MSC in contact with Orthovisc® (J&J),
Group 3 AD-MSC in contact with Synvisc® (Sanoﬁ),
Group 4 AD-MSC in contact with Synovium® (LCA Pharmaceutical),
Group 5 AD-MSC in contact with Suprayal® (Zodiac),
Group 6 AD-MSC in contact with Osteonil® (TRB Chemedica),
Group 7 AD-MSC in contact with PBS (Thermo Fisher Scientiﬁc).

In the preparation process, the cells were detached from the culture bottle with TrypLE reagent (Thermo Scientiﬁc) and centrifuged at 300 × g for 5 min, counted in the Countess apparatus (Thermo Fisher), and resuspended in hyaluronic acid or PBS at the concentration of. After 4, 24, and 48 h of incubation at room temperature and atmosphere, cell viability analysis was performed through the Countess counter by the Trypan Blue staining method (Thermo Fisher Scientiﬁc), according to the supplier’s recommendations. Two independent experiments were performed, each with three different AD-MSC strains (AT9, AT11, and AT13). For each time interval, a single viability reading was made, and the same experimental conditions were followed.

**Statistical analysis**

The numerical data obtained were presented as a mean ± standard deviation. Statistical analysis was performed using Student’s t-test (p < 0.05).

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**Table 1 – Physical and chemical properties of different hyaluronic acids.**

| Product       | Molecule        | Concentration | Molecular weight (MDa) | Elasticity (Pa 2.5 Hz) | Viscosity (Pa 2.5 Hz) | Source                  | Crosslinked molecule |
|---------------|-----------------|---------------|------------------------|------------------------|-----------------------|-------------------------|----------------------|
| Suprayal®     | Sodium hyaluronate | 1%            | 0.75                   | Viscoelasticity 1.2    | Bacterial fermentation | No                      |                      |
| Fermathron®   | Sodium hyaluronate | 1%            | >1                     | Viscoelasticity 0.84   | Bacterial fermentation | No                      |                      |
| Synvisc®      | Hylan G-F 20    | 0.8%          | 6.0                    | 111                    | 25                    | Avian                   | Yes                  |
| Orthovisc®    | Sodium hyaluronate | 1.5%          | 1–2.9                  | 60                     | 46                    | Bacterial fermentation | No                  |
| Synovium®     | Sodium hyaluronate | 2.5%          | >2.8                   | 160                    | 159                   | Bacterial fermentation | No                  |

Characteristics of normal synovial fluid: elasticity = 117 Pa 2.5 Hz; molecular weight = 3–4 MD; viscosity = 45 Pa 2.5 Hz.
Results

Establishment and selection of cell lineages

Three AD-MSC lineages were obtained: AT8, AT11, and AT13. They were followed in vitro, and the morphology of each of them was evaluated during the stages. All lineages presented morphology similar to MSCs (Fig. 1) and excellent proliferation rate in the stages evaluated, in an animal serum-free medium. Therefore, they are excellent candidates for cell expansion protocols for future therapeutic applications.

Immunophenotyping

To confirm the mesenchymal origin of the adherent cell lineages obtained (AT8, AT11, and AT13), the authors used flow cytometry to evaluate the expression of a panel of cell membrane proteins. Samples were positive for CD29 and CD90 adherent cell markers; negative for the CD31 endothelial marker; negative for the CD34 and CD45 hematopoietic markers; positive for the CD73 and CD105 mesenchymal markers; positive for HLA-ABC, and negative for HLA-DR (Fig. 2). This pattern is in accordance with the immunophenotyping panel set forth by the International Society of Cell Therapy.15

Differentiation test

The plasticity of adipose cells obtained from adipose tissue (lineages AT8, AT11, and AT13) was evaluated after 21 days of culture in a medium that induces specialized cell types of mesodermal origin. The osteogenic (Fig. 3A), adipogenic (Fig. 3B), and chondrogenic (Fig. 3C) inductions were confirmed by the presence of calcium deposits, lipid vacuoles, and extracellular matrix rich in mucopolysaccharides, respectively. Together, these results indicate the mesenchymal origin of isolated adherent cells and the potential for multipotent in vitro differentiation.15

Hyaluronic acid contact test

The results found in the study indicated a difference in cell viability according to the hyaluronic acid used to culture the MSCs in comparison with the control group, as shown in Table 2.

In the first 4 h, only the cells in contact with Orthovisc® presented a significantly reduced viability in relation to the control group. The same effect was observed in the cells in contact with Orthovisc® for 24 h and 48 h (p < 0.05). However, after 24 h, all treatments, except for the cells in contact with Supracyal®, had significantly reduced viability when compared with the PBS control group. Finally, after 48 h, both cells in contact with Orthovisc® and Synvisc® had significantly reduced viability when compared with the PBS control group.

Thus, Supracyal® was the most suitable hyaluronic acid for use as a vehicle for AD-MSC transport in cell therapy trials, maintaining the viability at levels very close to that of the cells treated with the PBS control solution.

Regarding viscosity and cell manipulation, the hyaluronic acids Fermatron® and Supracyal® presented the best performance. Synovium® acid presented very high viscosity, which hindered cell manipulation. In turn, Orthovisc® and Synvisc® presented intermediate viscosity, but also hindered cell manipulation.

Discussion

Regenerative medicine has been viewed with great interest by orthopedists, as it has become an attractive option for the treatment of chronic conditions, such as joint cartilage lesions and arthritis. This is due to the fact that the treatments available to date are insufficient for complete lesion healing, which often results in decreased mobility and loss of patient autonomy. This tissue regeneration therapy uses cell therapy as a strategy, in which stem cells are used for lesion treatment and repair. Thus, due to their potential differentiation capacity in bone and cartilaginous tissue, MSCs are very promising and can be isolated from several sites in the human organism, such as bone marrow, fat, periosteum, and synovial membrane, among others.16,17

Adipose tissue is considered a good candidate as a source for cell therapy because it can be accessed by relatively simple and non-invasive techniques and has a high concentration of MSCs, approximately 10–100 times more MSCs per volume than bone marrow.18

However, isolated cell therapy is not yet capable of repairing large defects. In this sense, tissue engineering is seen as a promising strategy, as it uses scaffolds as biomaterials to mimic the extracellular matrix architecture of the specific tissue and has a great influence on cell growth and differentiation.17 This fact has already been observed by Hiadaka et al.,19 He et al.,20 and Marra et al.21 in 2007 and 2008, when successful results were observed in the use of scaffolds with progenitor cells to replace bone, cutaneous, and adipose tissue, respectively.
Fig. 2 – Immunophenotyping of adherent cells obtained from adipose tissue. Gray histograms represent the population of non-antibody labeled cells (negative control). Black histograms represent the population of cells labeled with the respective antibodies described in each line. The graphs show the number of cells versus fluorescence intensity.
Fig. 3 – The potential for differentiation of adherent cells isolated from adipose tissue. The first column represents the negative control lineages (not induced) and the second column, the lineages treated with induction medium after 21 days of culture. (A) Osteogenic induction. Differentiation was confirmed by the presence of calcium deposits (dark brown) through von Kossa staining. 50 x magnification. (B) Adipogenesis induction. The differentiation was confirmed by the presence of droplets of cytoplasmic lipids (red) by Oil Red O staining. 200 x magnification. (C) Chondrogenic induction. The differentiation was confirmed by toluidine blue staining, which allows visualizing the phenomenon of metachromasia, characteristic of extracellular matrix rich in mucopolysaccharides. 50 x magnification.

Table 2 – Viability of adipose tissue-derived human mesenchymal stem cells in contact with different commercial hyaluronic acids. Mean and standard deviation expressed as a percentage of viability for each treatment evaluated.

| Treatment    | 4h Mean | 4h SD  | 24h Mean | 24h SD  | 48h Mean | 48h SD  |
|--------------|---------|--------|----------|---------|----------|---------|
| Fermathom®   | 81.6    | 10.535 | 74.8     | 4.021   | 76.7     | 13.812  |
| Orthovisc®   | 67.4    | 10.433 | 66.5     | 5.792   | 59.6     | 13.124  |
| Synvisc®     | 61.7    | 7.506  | 67.8     | 12.631  | 58.2     | 14.332  |
| Synovium®    | 72.8    | 14.943 | 72.5     | 7.401   | 62.8     | 18.073  |
| Suprhyal®    | 84.1    | 6.809  | 81.2     | 9.915   | 81.6     | 9.717   |
| Osteonil®    | 71.4    | 14.3   | 59.1     | 16.282  | 71.7     | 11.218  |
| PBS          | 90.0    | 3.464  | 83.5     | 2.933   | 77.4     | 12.347  |

*p < 0.05.

Hyaluronic acid is commonly used in tissue engineering as a biomaterial in hydrogel structures. Therefore, understanding its functions is fundamental for creating more efficient structures.10

One of the explanations for the superior results of the PBS is due to its isotonic nature, which makes this the most commonly used reagent in biological research. Phosphate salts are non-toxic to living cells and have a high buffering capacity. pH maintenance is important for cell viability, as well as in the process of recovering and revitalizing cells. In turn, hyaluronic acid is one of the main components of the extracellular matrix and significantly contributes to cell proliferation and migration.

Based on the present results, it was observed that the cell viability of the AD-MSCs is strongly associated with the type of hyaluronic acid used for culture. Hyaluronic acid is one of the main components of the extracellular matrix and significantly contributes to cell proliferation and migration.

Furthermore, it is believed that the molecular weight may be responsible for the differences observed. Among the brands researched, Suprhyal® presented the highest amount of viable stem cells at the end of the analyzed period and the lowest molecular weight (0.75 MDa) among the evaluated groups. This can be explained by the evidence that high molecular weight hyaluronic acid acts as an inhibitor of angiogenesis and cell proliferation, in addition to having an anti-inflammatory and immunosuppressive effect.10

Therefore, it can be suggested that cell viability may be directly related to the concentration and molecular weight of the hyaluronic acid used and that this may be a determining factor in the success of the surgical treatment of chondral lesions.

However, it is important to note that the abovementioned results regard the influence of hyaluronic acid on stem cells in the context of tissue engineering and not their use to provide viscosupplementation in the conservative treatment of osteoarthritis; for the latter purpose, there is evidence of the
clinical benefit and the probable superiority of high molecular weight hyaluronic acid.\textsuperscript{22–24}

**Conclusion**

The data suggest that hyaluronic acid can be used as an injection vehicle for MSCs, but caution is needed in the choice of the best product according to the viability and cytotoxic effect of the different brands of hyaluronic acids. Thereby, the therapeutic application will be performed more safely.

**Conflicts of interest**

The authors declare no conflicts of interest.

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