Stability Enhancement Using Hyaluronic Acid Gels for Delivery of Human Fetal Progenitor Tenocytes

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Tendon afflictions are very common, and their negative impact is high both at the workplace and in leisure activities. Tendinopathies are increasing in prevalence and can lead to tendon ruptures, where healing is a long process with outcomes that are often disappointing. Human fetal progenitor tenocytes (hFPTs) have been recently tested in vitro as a potential cell source to stimulate tendon regeneration. The aim of the present study was to compare different commercial hyaluronic acid (HA) gels, which could be used to resuspend hFPTs in a formulation that would allow for good delivery of the cells. No medium or growth supplement was used in the formulation in order to make it therapeutically dispensable. These conditions are stringent for cells, but surprisingly, we found that different formulations could allow a good survival for up to 3 days when stored at 4°C (refrigerator stable). The gels must allow a good survival of the cells in parallel with a good stability of the preparation over time and sufficient viscosity to remain in place if deposited on a wounded location. Moreover, the cells must conserve their ability to attach and to proliferate. hFPTs were able to survive and to recover from all of the tested gels, but some products showed some advantages over others in terms of survival and viscosity. Finally, the Ostenil Tendon HA gel fulfilled all of the requirements and presented the best compromise between a good survival and sufficient rheological characteristics to create an interesting cell delivery system.

Key words: Cell therapy; Hyaluronic acid gel; Tendon healing;
Human fetal progenitor tenocytes (hFPTs); Cell stability

INTRODUCTION

Tendon afflictions are very common, and various anatomical areas are affected, including the Achilles tendon, various hand tendons, and the rotator cuff, all of which are among the major tendons exposed to injury. In the US alone, more than 25 million sprains and strains of soft tissues such as tendons and ligaments are reported yearly (23) with an impact that is significant both at the workplace and in leisure activities. Our population is becoming older, and aging is correlated with an increase in chronic degeneration of tendons, presently referred to as tendinopathies (7,21). Elderly people are not the only ones affected, and with the democratization of sport practice, tendinopathies have been increasing in younger individuals during recent decades as well (4,18).

Tendon homeostasis relies on good behavior of tenocytes, which are the cells responsible for the secretion of all extracellular matrix components and the maintenance of a fine balance between synthesis and catabolism of the extracellular matrix (11). In the case of acute loadings, the net balance of collagen levels is negative for up to 18–36 h after exercise and then becomes positive up to 72 h. Thereafter repeated loadings without sufficient rest can lead to an amplification of the collagen breakdown (15). The extracellular matrix becomes more disorganized and weaker, and many changes in the structure are detectable (14). Tendinopathies are not only painful but also make tendons more prone to ruptures (12). In cases of rupture, tendon healing relies on a cascade of events. Tenocytes have an important role as they are

responsible for the formation of new extracellular matrix. Unfortunately, the quality of the new tissue never reaches that of the original tissue prior to injury and scar tissue, and adhesions are frequent, which often leads to decreased mobility and reruptures (10,20,24).

Therefore, tenocytes have an important role both in tendinopathies and in tendon ruptures, and their modulation would be very interesting to improve the outcomes obtained. Human fetal progenitor fibroblasts have shown excellent results in modulating regeneration of tissues such as skin toward a less fibrotic process (2,9). We recently tested human fetal progenitor tenocytes (hFPTs) as a potential cell source for tendon regeneration and showed that it was possible to create cell banks with very high numbers of cells and with respect to good manufacturing practice (GMP), which is a prerequisite for potential clinical use (5). These cells were shown to have stimulating abilities toward adult tenocytes, along with very good genotypic and phenotypic stability, and the banks allow the availability of an off-the-shelf stock of cells that is easily accessible and can be rapidly used. Such results are promising, but to be delivered to the afflicted site, the cells need a support scaffold such as a matrix or a gel. Seeding cells within gels presents the advantage of being easily injectable, and as surgery is not required for their use, they could be particularly useful for treatment of tendinopathies and simple acute injuries. The presence of a medium with serum or other growth factor supplement is not feasible in a preparation destined to be injected into the body. Therefore, we decided to work with formulations containing only hFPTs, phosphate-buffered saline (PBS), and hyaluronic acid (HA) gels. HA is a glycosaminoglycan that is naturally present in numerous tissues, including tendons, and numerous gels based on this molecule are commercially available with a market authorization for injection. The variation of the degree of cross-linking of HA in the gel leads to characteristics that can be very different, notably the viscosity of the gel (22). In this study, we decided to compare a selection of HA gels in search for one formulation that, on one hand, would allow for survival of hFPTs and, on the other hand, would have good rheological characteristics, with the aim of creating a preparation that can be used for therapeutic interventions.

MATERIALS AND METHODS

Cell Culture

hFPTs were grown in a monolayer in polystyrene tissue culture flasks of 75 cm^2 with filter screw caps (Code No. 90076; TPP, Trasadingen, Switzerland) and were placed in cell culture incubators at 37°C in a humidified atmosphere containing 5% CO_2. They were seeded at a density of 3,000 cells/cm^2 at passage 5 from a cryopreserved vial from our cell bank. hFPTs were isolated from the Achilles tendon of a male 14-week-gestation organ donation according to a protocol approved by the State Ethics Committee (University Hospital of Lausanne–CHUV, Ethics Committee Protocol No. 62/07: 14-week-gestation organ donation, registered under the Federal Transplantation Program and its Biobank complying with the laws and regulations). The detailed cell banking procedures were described previously (5,19). The medium was changed every 3 to 4 days and was composed of standard growth medium composed of Dulbecco's modified Eagle's medium (DMEM; Code No. 41966-029; Gibco, Life Technologies Ltd., Paisley, UK) containing 25 mM dextrose, 1 mM sodium pyruvate, and supplemented with 5.97 mM L-glutamine (L-glut: Code No. 25030-024; Gibco) and with 10% fetal bovine serum (FBS; Code No. F7524; Sigma-Aldrich, St. Louis, MO, USA) without antibiotic supplementation. When full confluence was reached, the cells were detached with TrypleE (Code No. 12605-010; Gibco), counted with a hemocytometer, and transferred in desired amounts into new tubes. They were then centrifuged at $250 \times g$, the supernatants were discarded, and they were rinsed twice with PBS (NaCl 6.8 g/L, Na_2HPO_4 1.5 g/L, KH_2PO_4 0.4 g/L; Code No. 100 0 324; Bichsel, Interlaken, Switzerland) to eliminate all residues in the medium. hFPTs were then resuspended with PBS to the desired concentration and were ready to be used at passage 6 for the different experiments.

Comparison of Cell Survival in Commercial HA Gels

hFPT survival was evaluated in seven different commercial gels containing HA in various amounts (Table 1). hFPTs were initially tested with a concentration of 40,000 cells per 100 µl of gel. A cell suspension of 4 million cells/ml in PBS was prepared, and 10 µl was dispatched in 9 wells of a 96-well plate (Code No. 3596; Corning Inc., Corning, NY, USA) (40,000 cells per well). There were as many plates prepared as conditions tested. The cells of each well were then resuspended with 90 µl of a specific gel and homogenized with a displacement pipette (Code No. F148504; Microman, Gilson, Middleton, WI, USA). One plate was evaluated directly after resuspension (0 day). The preservation was then evaluated at room temperature (RT) and at 4°C after 24 h. As the results were better at 4°C (mean survival of 75% for 4°C compared to 66% at RT), other plates were prepared to test survival at the 4°C temperature for multiple time points (1, 2, 3, and 7 days). The cells did not receive any medium or growth supplement during the preparation for the storage conditions. The survival was evaluated with a LIVE/DEAD solution (Code No. L-3224; Molecular Probes, Life Technologies Ltd., Paisley, UK). On the desired day, 100 µl of LIVE/DEAD solution (prepared according to the manufacturer's instructions) was added to the wells and mixed

Table 1. Selection of Commercial Hyaluronic Acid (HA) Gels

Gel	Concentration in HA	Manufacturer
Hyalgan	1%	KELA Pharma, St-Niklaas, Belgium
Ostenil	1%	TRB Chemedica, Haar, Germany
Ostenil Plus	2%	TRB Chemedica, Haar, Germany
Ostenil Tendon	2%	TRB Chemedica, Haar, Germany
Sinovial 0.8%	0.8%	IBSA, Lodi, Italy
Sinovial 1.6%	1.6%	IBSA, Lodi, Italy
Synolis V-A	2%	Anteis, Plan-les-Ouates, Switzerland

with the displacing pipette. After 30 min of incubation at RT and with protection from light, images were taken with an inverted microscope equipped for fluorescence (IX81; Olympus, Tokyo, Japan) and a digital camera (iXon; Andor Technology Ltd., Belfast, UK). Green and red channels were recorded on a stack of 11 layers covering a total depth of 600 μm for each gel (60-μm space between slices) in the lower third portion. The final image was obtained within ImageJ (National Institutes of Health, Bethesda, MD, USA) through a standard deviation z-projection to obtain one layer composed of two color channels and converted to 16 bits. The final images were then analyzed with Cell Profiler (Broad Institute, Cambridge, MA, USA) to obtain the number of live and dead cells and a ratio representing the cell survival; calculations were done for each image. The identification of primary objects (live and dead cells) within the two color layers was done for a diameter of 10 to 120 pixels with automatic threshold strategy and distinction of clumped objects based on the shape with dividing lines based on intensity. Each identification was controlled manually to check the accuracy of the method. The image processing and analysis are summarized in Figure 1. As there were less visible cells in some conditions, on day 7 the bottoms of the wells were also analyzed for sedimentation, and an image was taken for each gel on a single layer with both colors.

Rheology on HA Gels

The dynamic viscosity of each gel was measured with a Rheometer System CVOR 150 CE/WIN (Bohlin Instruments GmbH, Pforzheim, Germany) within a plate–plate system at RT. The diameter of the rotating plate was 10 mm, the distance between the plates was 800 μm, and it was possible to place 80 μl of gel in between. A shear rate of 1 Hz was applied for 20 s of precharge, and the dynamic viscosity was then recorded at the same shear rate for 20 s and measured every 2 s. The measurements were then repeated twice with fresh gel. Finally, for each gel, the points were plotted as the mean of the three replicates. The dynamic viscosity was obtained by calculating the mean of the 11 points obtained from the three replicates, and the standard deviation was calculated on the basis of the same points.

Comparison of Cell Survival as a Function of Viscosity

The survival results after resuspension and after 3 days at 4°C are summarized in Table 2. The results are presented such that more than 85% of live cells are represented by "+++," from 60 to 85% "++," and for less than 60% "+." The degrees of viscosity are indicated in Table 2 as follows: the gels with a viscosity higher than 50 Pas are represented by "+++," from 10 to 50 Pas "++," and for less than 10 Pas "+." In parallel, a linear correlation was determined by plotting the survival results after 1 day of storage at 4°C as a function of the viscosity of the gels in Excel software (Microsoft, Redmond, WA, USA). The root mean squared error was used for the determination of the regression equation and the coefficient of determination (R^2).

Evaluation of Cell Survival and Recovery With Increased Cell Concentration and Packaging in Syringes

The survival of hFPTs was then evaluated with improved formulations at a density of 500,000 cells per 100 μl of gel and with packaging within syringes. The same gels as above were used, except for Hyalgan, which was not available for this experiment (due to supply problem). The cells were resuspended at a concentration of 50 million cells/ml in PBS, and 80 μl was dispatched in wells of a 48-well plate. The cells of each well were resuspended with 720 μl of a specific gel and homogenized with a displacement pipette to reach the final concentration. The cell suspension was then simply aspirated with the syringe (without needle) to reach 500 μl, the air bubbles were eliminated, a cap was placed on the syringe, and the final preparation with 2.5 million cells in a total volume of 500 μl was kept at 4°C for 72 h.

The survival was evaluated with a LIVE/DEAD assay following a similar protocol as in the previous assay. For each gel, 100 μl of preparation was extruded through a 22-gauge needle in a well of a 96-well plate, and 100 μl of the LIVE/DEAD solution was added. The only difference was a recording on only three layers representing a depth of 120 μm instead of 600 μm, as the concentration was higher than previously accomplished. The
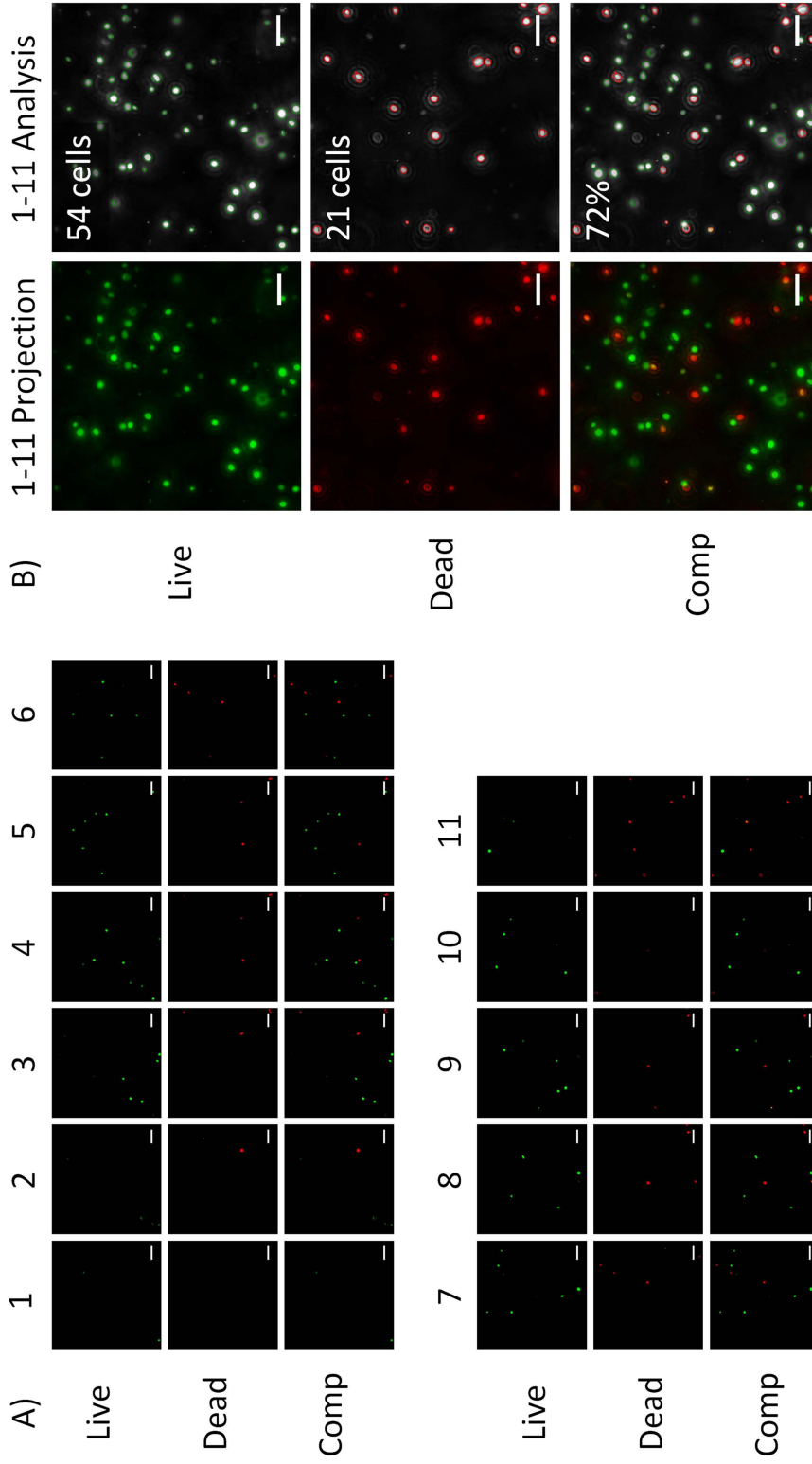
Figure 1. Image processing and analysis. (A) For the different gels, a stack of pictures was taken with multiple layers (either 11 or 3 layers depending on the concentration, always with 60-μm interspace between 2 layers) and two color channels (green for live cells and red for dead cells). A composite image with the two channels was created by merging the two color channels (comp). (B) For each channel, the multiple slices were merged through a standard deviation z-projection within ImageJ software to obtain one projection representing the cells present in the whole portion analyzed (600 μm in the present case with 11 layers, 120 μm for 3 layers). The live and the dead cells were detected (circled in green or red) and counted automatically within Cell Profiler. The two color channels were fused on the same image, and the ratio of live cells was indicated. Scale bars: 100 μm.

Table 2. Summary of the Cell Survival and of the Rheology Results for the Seven Commercial HA Gels

Gel	Ostenil	Ostenil Plus	Ostenil Tendon	Sinovial 0.8%	Sinovial 1.6%	Synolis V-A	Hyalgan
Cell survival (resuspension)	+++	+	++	+++	++	+	+++
Cell survival (conservation 3 days, 4°C)	+++	+	++	++	++	+	+++
Viscosity	++	+++	+++	+	++	+++	+

+, <60% live cells (survival), >10 Pas (viscosity).
++, 60–85% live cells (survival), 10–50 Pas (viscosity).
+++, More than 85% live cells (survival), >50 Pas (viscosity).

images were also taken in the lower third portion and with the same interspace of 60 μm between slices, as in the previous experiment.

In parallel, a recovery assay was performed in monolayer culture. For each gel, 100 μl was diluted to 35 ml of standard growth medium, and hFPTs were then dispatched in 12 wells of two six-well plates (evaluation of four time points in triplicate for each gel) with 2 ml per well to obtain a seeding of approximately 3,000 cells/cm² at passage 6 (each solution was counted to know the actual number of seeded cells). The plates were incubated at 37°C and the cells were counted in three wells for each gel at days 4, 7, 11, and 14. The cells were rinsed with PBS, detached with TrypleE, and transferred to a 15-ml tube. The TrypleE was inactivated by the addition of an equal volume of medium. The cells were centrifuged at $250 \times g$, the supernatant was discarded, and the cells were resuspended in an adapted volume to be counted with a hemocytometer. On the same days, the medium was replenished in the remaining wells. The population doublings (PDs) were determined at each time point (equation 1), and the results were plotted as PDs as a function of time (PDs allow to avoid biases due to slight variations in seeding densities).

$$PD = \frac{\ln\left(\dfrac{\text{final cell number}}{\text{initial cell number}}\right)}{\ln(2)} \quad (1)$$

The results from hFPTs grown in six wells at passage 6 directly from monolayer culture and which had never had contact with any gel were used as the control.

RESULTS

Comparison of Cell Survival in Commercial HA Gels

Figure 2 presents the cell survival of hFPTs within the different gels for the different conditions tested.

Directly after cell resuspension within the gel, the survival was determined to be excellent with Hyalgan, Sinovial 0.8%, and Ostenil with 94%, 93%, and 88% live cells, respectively. There was a weaker proportion of live cells within Sinovial 1.6% and Ostenil Tendon with 75%

and 74% live cells, respectively. Finally, Synolis V-A and Ostenil Plus presented a relatively weak survival directly after resuspension with just as many cells alive as dead (respectively 57% and 50% live cells).

After 24 h of storage, the cell survival was better for every gel stored at 4°C compared to RT, except for Ostenil Plus. Taking the results of all gels together, the mean survival was 66% (ranging from 40% to 85%) at RT and 75% (ranging from 54% to 91%) at 4°C. Hyalgan presented only a limited number of visible cells after 24 h at RT, but no decrease in cell number was seen with the storage at 4°C.

After 48 h at 4°C, the survival rates followed the same trend as for 24-h storage at 4°C. There was again a decreased number of resuspended cells for Hyalgan.

After 72 h at 4°C, the same trend was seen except for a drop in survival for Ostenil Plus and Sinovial 0.8%. There was a decreased number of resuspended cells both for Hyalgan and Sinovial 0.8%.

After 1 week at 4°C, the survival dropped for the other gels, namely, Hyalgan, Ostenil, Ostenil Tendon, Sinovial 1.6%, and Synolis V-A. The observation of the bottom of the wells (Fig. 3) showed that many more sedimented cells stayed at the bottom of the wells with Hyalgan and Sinovial 0.8% than with the other gels. There was also sedimentation with Ostenil, but to a lesser extent.

Rheology

After 20 s of precharge, the recording of the dynamic viscosity during 20 s was seen to be very stable and reproducible between the triplicates for each gel. Hyalgan and Sinovial 0.8% presented very low degrees of viscosity, which were at 1.1 and 3.2 Pas, respectively. Ostenil and Sinovial 1.6% were slightly more viscous with viscosities of 11.3 and 12.2 Pas, respectively. Ostenil Plus and Ostenil Tendon had intermediate viscosities of 64.5 and 60.4 Pas, respectively. Finally, Synolis V-A was the most viscous with 122.0 Pas (Fig. 4).

Comparison of Cell Survival as a Function of Viscosity

Table 2 summarizes the survival of hFPTs directly after resuspension in their respective gels and after 3 days of storage at 4°C alongside their degree of viscosity.
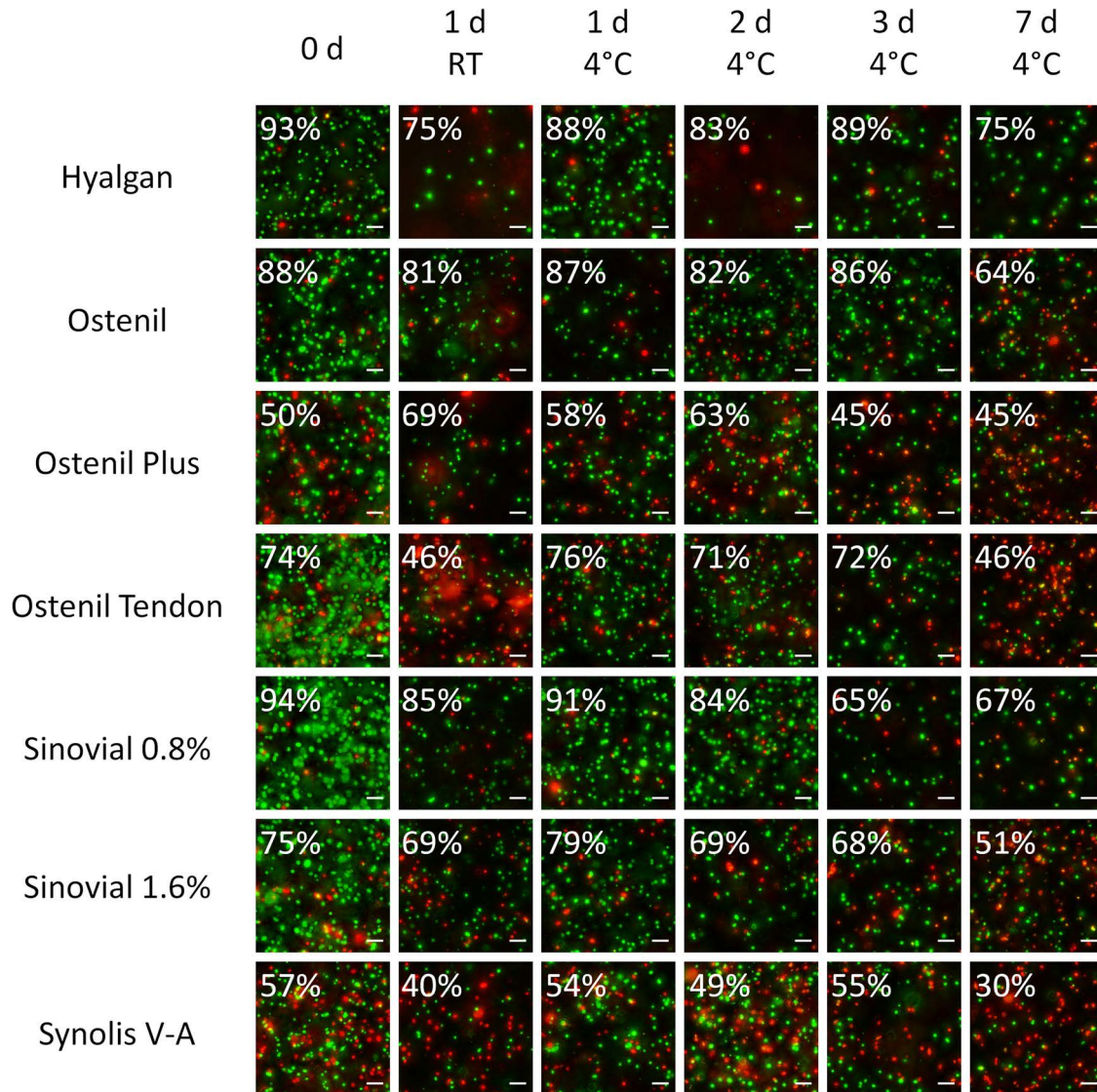
Figure 2. Cell survival of hFPTs within different commercial HA gels. The images present the survival of hFPTs within HA gel for different conditions with various time points (0 to 7 days) and temperatures (RT or 4°C) of storage. The live cells appear in green (metabolically active), while the dead cells appear in red (membrane ruptured). Each image was obtained by projection of 11 slices covering a depth of 600 μm into one final image. The percentage indicates the ratio of live cells among the total number of cells for each image. Scale bars: 100 μm.
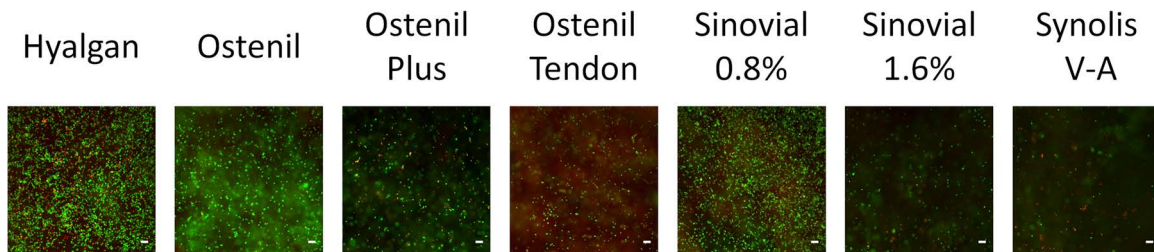

Figure 3. Sedimentation of hFPTs at the bottom of wells after 7 days at 4°C. After 7 days of storage, the presence of sedimented cells at the bottom of the wells was more pronounced in Hyalgan and Sinovial 0.8% and to a more limited degree in Ostenil. Each image was obtained as one single layer with both color channels. The other gels were not affected. Scale bars: 100 μm.
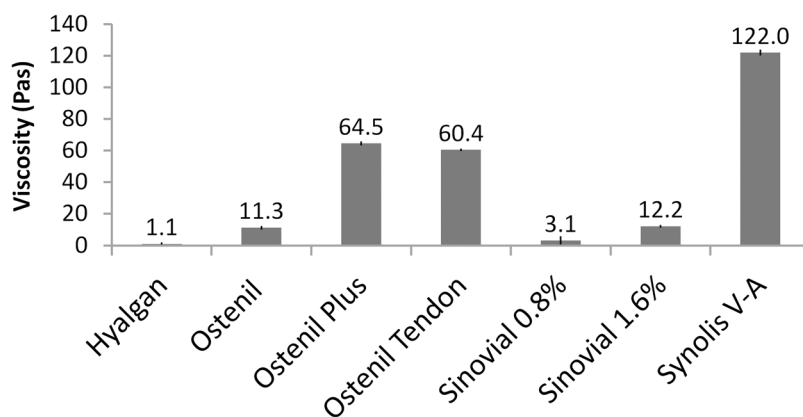
Figure 4. Dynamic viscosity of HA gels. The dynamic viscosity of the different commercial HA gels represents the mean of the 11 measures made in 20 s (based on triplicates) and with the standard deviations shown as error bars.

Figure 5 presents hFPT survival after 24 h at 4°C as a function of the viscosity of the gel in which they were resuspended. The data follow a visible trend, and the coefficient of determination of the linear regression line is 0.84. The slope of the regression line equals −0.003, meaning that 0.3% of survival is lost for each supplementary unit of Pas.

Cell Survival and Recovery After 72 h at 4°C
With Higher Cell Concentration and
With Packaging in Syringes

After 72 h of storage at 4°C, hFPT survival at higher concentration in syringes ranged from 81% for Ostenil Plus to 94% for Sinovial 0.8% as seen with the LIVE/DEAD experiment (Fig. 6A). Because of the high concentration, the number of cells for determining survival was higher than in the overall gel screening experiment (mean of 287 cells per final image for the higher concentration instead of 128 cells for the lower concentration), despite a

recording on a depth five times lower. The recovery assay (Fig. 6B) highlighted the fact that the cells extruded from all syringes were able to attach and proliferate in monolayer culture, and 90% to 100% confluency was reached after 7 days for Ostenil, Ostenil Plus, Ostenil Tendon, and Sinovial 0.8% and after 10 days for Sinovial 1.6% and Synolis V-A. The proliferation with confluency reached after 7 days was really close to what was found for cells from the same passage that were grown in monolayer culture and that had never been in contact with any gel (Fig. 6B, control).

DISCUSSION

hFPTs present very interesting characteristics in vitro and could be an optimal cell source for regeneration of tendons (5). Nevertheless, a scaffold is required to deliver the cells to the wounded site, and the use of an HA gel could be feasible if it allows hFPTs to survive when seeded inside and retains flexibility for manipulation. Furthermore, with
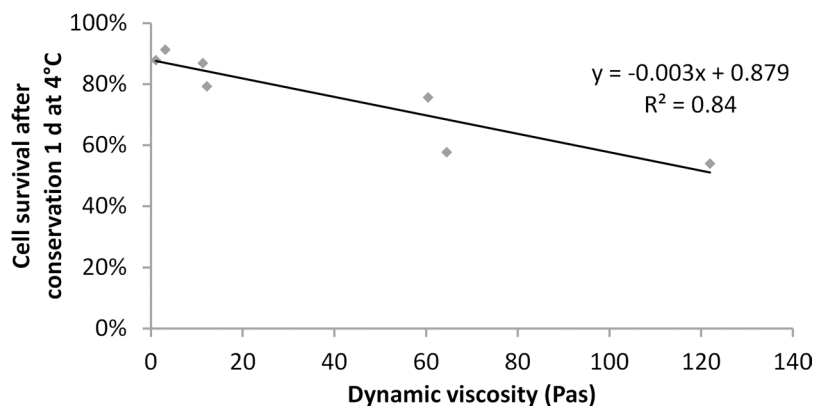

Figure 5. Linear correlation between dynamic viscosity and hFPT survival after 1 day at 4°C. The linear correlation between the dynamic viscosity of the gel and the cell survival after 1 day at 4°C was very high with a coefficient of determination (R^2) of 0.84. With a slope of −0.003, for each supplementary unit of Pas in one gel, 0.3% of survival was lost for hFPTs.
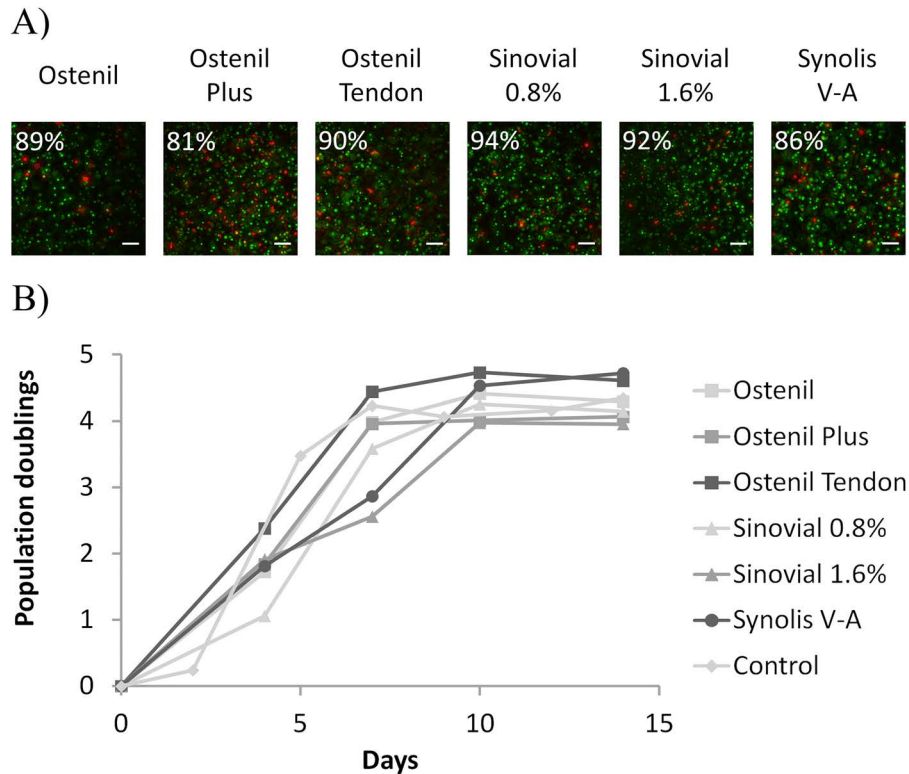
A)

B)

Figure 6. Cell survival (A) and recovery (B) of hFPTs at a concentration of 500,000 cells per 100 μl in syringes after 72 h of storage at 4°C. (A) The live cells appear in green, while the dead cells appear in red. Each image was obtained by projection of three slices covering a depth of 120 μm into one final image. The percentage indicates the ratio of live cells among the total number of cells for each image. Scale bars: 100 μm. (B) The recovery of the cells extruded from the syringe is shown for 2 weeks of monolayer culture. The points represent the cumulative population doublings up to confluency. The results obtained for hFPTs that have never been in contact with gel and grown at the same passage were added for comparison (control).

regard to clinical application in patients, it is necessary to determine the stability of the cells within the gel. It is advantageous if the formulation can be stored for some time, as the delivery to operating sites can take time and delays in surgery schedules can occur.

First, it is important to anticipate regulatory requirements and to be in accordance with the laws and recommendations of the relevant government bodies and institutions. The HA gels tested all received a market authorization with the possibility to be injected. The use of animal-derived products such as FBS has been challenged lately, and such products were omitted in order to retain the potential of being injected in a clinical setting; hFPTs were rinsed and resuspended only with PBS before being mixed with an HA gel, without any medium or any other growth supplements. This introduces more difficulties for maintaining living cells, but it is compulsory for the potential use of hFPTs for therapeutic intervention in the future.

The different experiments were conducted in an incremental process, which means the techniques were adapted experiment after experiment. For example, once primary results were obtained, we changed the concentrations to be closer to what could be used in clinics. This explains why the results appear as a single replicate for an overview of multiple gels and why no further statistical information is added. However, each well was resuspended individually, and the trend for a specific gel remained the same throughout the time points with the same gels consistently presenting good survival and always the other gels consistently presenting lower survival. By increasing the cell concentration, we obtained better survival, but the trend was the same among the different gels. Thus, our discussion and conclusion are based on several experimental evaluations for each and every gel. After 24 h of storage, either at RT or at 4°C, the results showed that storage by refrigeration was better in terms of cell survival, and this observation could be seen for all of the gels except Ostenil Plus. Tendons are known to have a relatively slow metabolism (11,17), but tenocytes still require nutrients for their survival. By diminishing the temperature, the metabolic activity of the cells is also diminished and so

are their requirements. The absence of nutrients may then have less impact under such conditions, and this could explain a better survival at refrigerated temperatures up to a certain limit. Between day 2 and day 3, there was a drop in survival of more than 15% for Ostenil Plus and Sinovial 0.8%. The other gels underwent a similar drop for cell survival between day 3 and day 7. It is a major advantage to have a cellular preparation that can be stored for some time before its clinical application. It was the case in this study with live cells representing still 65% to even 89% in five gels after 3 days of conservation when refrigerated. This storage duration allows flexibility and corresponds, for example, to what is found in our hospital for other therapies, such as platelet-rich plasma (PRP) injections (1).

The suspension of hFPTs within Ostenil Plus and Synolis V-A led to negative results in terms of cell survival. This yield was found to already be decreased just after homogenization of the cells within gels and without any storage phase. These two gels presented the weakest proportion of live cells for each time point as expected. This effect was not translated into one single initial preparation, as the cells were resuspended for each time point in their individual wells. These gels were the most viscous of the selection, and this could probably explain the low overall survival even with the delicate use of displacement pipettes. With increased viscosity, the shear stress applied on the cell surface during resuspension and homogenization is increased and probably leads to more membrane ruptures. Indeed, there was a strong linear correlation between cell survival after 24 h at 4°C and viscosity as seen by the coefficient of determination of 0.84, and gels with a high viscosity should be carefully selected for resuspension of hFPTs. The negative survival of cells within Ostenil Plus can nevertheless not only be explained by the viscosity of the gel, as the survival was much better within Ostenil Tendon, which had a very similar viscosity. This implies that viscosity is probably not the only factor favoring cell death and indicates that these effects may also be due to the gel composition.

Ostenil, Sinovial 0.8%, and Hyalgan showed the best cell survival rates throughout the time points studied. These three gels presented the lowest viscosity, and it is probable that only a limited number of cells were damaged during resuspension. The survival rates remained high for Hyalgan and Ostenil up to 3 days at 4°C, which shows a good tolerance of hFPTs for these two gels. With Sinovial 0.8%, there was a greater decrease in cell survival after 3 days of storage at 4°C, which potentially indicates a weaker tolerance in the long term. Despite excellent survival results, some other characteristics of these three gels, such as the fact that Hyalgan frequently presented less visible cells within the observed layers than in the

other gels and that Sinovial 0.8% also did after 3 days, should be explored. The observation of the bottom of the wells after 1 week of storage at 4°C highlighted the presence of many more sedimented cells in these two gels, which demonstrates that a dynamic viscosity of less than 5 Pas is insufficient to maintain the cells in suspension in the long term. Ostenil was subject to a lower sedimentation, and the other gels did not seem to be affected. This indicates that the preparation with a gel of low viscosity could become nonhomogenous over time, and as the final preparations are destined to be packaged in syringes, it would not be easy to resuspend the cells prior to injection. Thus, such gels could be appropriate for preparations that would be used rapidly, but not for preparations that would be stored. Moreover, even if the poor viscosity would not cause any problems for intratendinous injection, it could be responsible for leakage in the case of peritendinous application, for example, at the surface of a wounded site. Among these three gels, Ostenil seems the least affected by sedimentation and could be a more desirable candidate.

Ostenil Tendon and Sinovial 1.6% presented intermediate survival results, which were very close to each other. Sinovial 1.6%, even with a viscosity similar to standard Ostenil, presented a lower survival compared to this gel. It is likely that the negative impact on the cells is thus not due to shear stress during resuspension, but simply to a weaker tolerance of the cells toward this gel, and thus it would not be an ideal candidate. Despite a much higher viscosity, Ostenil Tendon presented a similar survival to Sinovial 1.6%. The survival in Ostenil Tendon was also much better than for Ostenil Plus, which shared a similar viscosity. It is probable that some cells died during the resuspension due to shear stress, but Ostenil Tendon did not seem to negatively impact the cells and seemed well tolerated. This gel presents an ideal compromise between a high rate of cell survival and an adapted viscosity for long-term storage and for delivery in zones where leakage would be a problem.

Tendinopathies are already treated with different types of injections; notably PRP (at a volume of 500 μl per site) is frequently used in our hospital. Many cell types have been used for tendon treatment, but the optimal number of cells required remains to be found (3). Based on our experience, we estimate that 2.5 million cells per treatment would be adequate. Concentrations ranging from 1 to 10 million cells are frequently found with stem cells in animal (6,8,16) and in human (13) models through in vivo experimentation. For these reasons, we prepared syringes with 2.5 million cells in 500 μl of gel in the second part of the study to mimic what could be used in clinics for the treatment of tendons. When aspirating the cell/gel formulations with a syringe, we used only the

syringe without a cannula or needle to allow the best protection of the cell preparation. However, when extruding the cells, we used a needle of 22 gauge to be similar to what could be used in the clinic. We did preliminary testing of needles from 20 to 30 gauge with very good results up to 23 gauge and could see a drop in survival with 30 gauge, with the highest impact on more viscous gel preparations. With other cell therapies for tendinopathies using PRP, we generally used 20-gauge needles for direct injection into the tendon (1).

Hyalgan was not available for this experiment (due to supply problems), but hFPTs were tested with all of the other gels, and the results were very encouraging. After 72 h at 4°C, the survival rates presented the same trend to what was found in wells with a lower concentration. Synolis V-A and Ostenil Plus presented the lowest survival, Sinovial 0.8% had the highest survival rates, and the other gels were in the middle. Furthermore, the survival for each gel after 72 h of storage was much higher compared to the previous results with lower cell concentrations, and the differences between gels were lower. It is probable that a higher cell concentration leads to a better preservation during the preparation. Use of larger volumes of the preparation also allowed for easier manipulation and resuspension of cells.

The LIVE/DEAD assay showed that many cells maintained the integrity of their membrane and that they were still metabolically active, but it is important to note that they did not lose their capacity to migrate, attach, and proliferate. The recovery assay highlighted this fact. After 3 days of storage at 4°C, the cells extruded from all the gels were able to attach to culture material and to proliferate. The cells from Ostenil, Ostenil Plus, Ostenil Tendon, and Sinovial 0.8% reached 90% to 100% confluency after 7 days and presented a proliferation profile very close to what was found with cells at the same passage, which had always been grown in monolayer culture and that had not been in contact with any gel. There was a slight delay for the cells from Sinovial 1.6% and Synolis V-A, but they were still able to reach 90% to 100% confluency after 10 days. These results are excellent and do not suggest further criteria for a discriminative choice between the different gels.

On the basis of the global results, Ostenil Tendon HA gel seems the best candidate for further development and for the formulation of one preparation that could be used therapeutically. Indeed, this gel presents a very good compromise between cell survival and sufficient viscosity. Moreover, the recovery of cells after 3 days of storage at 4°C is excellent. With the off-the-shelf availability of hFPTs, it would be possible to prepare cell suspensions in this gel on demand from the clinic. The final cell preparations could be prepared under GMP along with the associated simple logistics to deliver them to the patient. Importantly, they can be stored at 4°C at least up to 72 h, allowing good flexibility for clinical usage.

CONCLUSION

With the view of potentially using hFPT preparations clinically, the use of medium, serum, or any other growth supplement was avoided in the preparations with HA gels. With such conditions, the preservation of the cell suspensions in gels was improved by a refrigerated temperature, and it was possible to store multiple preparations up to 3 days.

hFPTs had low survival in Synolis V-A and Ostenil Plus, even without storage, and these gels are thus not adapted for a formulation containing hFPTs. Hyalgan, Sinovial 0.8%, and Ostenil allowed a high degree of hFPT survival and could be good candidates for the preparation of hFPT suspensions. Because of their low viscosity, they are not adapted for storage and would be more suitable for intratendinous injection than peritendinous injection into a defect, where the risk of leakage would be higher. Ostenil Tendon and Sinovial 1.6% presented intermediate results in terms of cell survival, but as Ostenil Tendon viscosity is approximately five times higher than Sinovial 1.6%, it is a more valuable choice for a formulation that could also be used on the surface of a wound, where a higher viscosity should allow a better maintenance of the cellular preparation. It therefore presents the best compromise between cell survival and adapted characteristics for conservation and delivery. Moreover, it is possible to package hFPT suspensions in syringes with 2.5 million cells in 500 µl of gel, which represents an adapted formulation for a clinical use. With such a formulation, it is now possible to imagine an in vivo delivery of hFPTs to further evaluate and characterize this cell source.

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