Long term morphological characterization of mesenchymal stromal cells 3D spheroids built with a rapid method based on entry-level equipment

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Received: 29 July 2015 / Accepted: 22 March 2016 / Published online: 29 March 2016
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Abstract Three-dimensional (3D) spheroids of mesenchymal stromal cells (MSC) have been demonstrated to improve a wide range of MSC features, such as multilineage potential, secretion of therapeutic factors, and resistance against hypoxic condition. Accordingly, they represent a promising tool in regenerative medicine for several biological and clinical applications. Many approaches have been proposed to generate MSC spheroids. They usually require specific generation systems, such as rotatory

bioreactors or low-attachment plates, and each approach has its own disadvantages. Furthermore, an over-time analysis of morphological homogeneity and architectural stability of the spheroids generated is rarely provided. In this work we adapted the “pellet culture” method to obtain homogenous spheroids of MSC and maintain them *in vitro* for long term studies. We analysed their outer and inner structure over a 2-month period to provide morphological and architectural information regarding the spheroids generated. Quantitative and qualitative data were obtained using brightfield and confocal microscope imaging coupled to a computational analysis to estimate volume, sphericity, and jaggging degree. In addition, histological evaluation was performed to more thoroughly assess the cellular composition and the internal architecture of the 3D spheroids. The results provided show that MSC spheroids generated with the proposed approach are homogeneous and stable, from both morphological and architectural points of view, for a period of at least 15 days, approximately between day 15 and day 30 after their generation. Accordingly, the approach proposed serves as a rapid, cost-effective, and efficient method to generate and maintain MSC spheroids using common entry-level laboratory equipment only.

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Keywords Mesenchymal stromal/stem cells · 3D multicellular spheroids · Pellet culture method · Morphological analysis · Microscopy · Computational analysis

Introduction

Mesenchymal stromal cells (MSC) are widely used for tissue engineering applications because of their capacity to promote tissue regeneration (Zakrzewski et al. 2014) while modulating immune responses (Prockop and Oh 2012). Currently, there are more than 300 registered clinical trials (www.clinicaltrials.gov) in which MSC are used to treat several different diseases, with applications ranging from bone and cartilage repair to spinal cord, cardiac, and bladder regeneration (Kramer et al. 2012). Three-dimensional (3D) MSC aggregates, typically known as “spheroids”, have been demonstrated to improve several MSC features, such as multilineage potential, secretion of proangiogenic and chemotactic factors, resistance against hypoxic condition (Sart et al. 2014), and, moreover, to enhance stemness (Cesarz and Tamama 2016) and anti-inflammatory properties (Ylöstalo et al. 2012). Cellular spheroids are essentially micro-tissues built in vitro to resemble the native configuration of cells in vivo (Berenzi et al. 2015), with a microenvironment that allows direct cell–cell signalling and cell–matrix interactions, thus better preserving MSC phenotype and innate properties (Bartosh et al. 2010). Spheroids are the preferred format for many bioprinting applications (Mironov et al. 2009). Furthermore, 3D MSC spheroids can easily differentiate into different cell types, for instance chondrocytes (Zhang et al. 2010) and hepatocytes (Zhong et al. 2015). Accordingly, they are a potent configuration for cell therapy and tissue engineering research. In practice, MSC spheroids already represent an extremely promising tool for several applications ranging from regenerative medicine (Piccinini et al. 2014) to anti-inflammatory treatments (Bartosh et al. 2010) and oncologic therapies (Saleh and Genever 2011). The deep characterization of cell behaviour and patterning in 3D structures can further improve MSC features, while refining to clinical applications (Sasai 2013).

Several approaches have been proposed in the literature to generate spheroids composed of cancer cell lines (Lin and Chang 2008), and most can be applied when constructing human MSC spheroids (Sart et al. 2014). These approaches can be coarsely divided into two categories: (a) Dynamic approaches, using spinner flasks (Teixeira et al. 2014) and rotating vessel bioreactors (Wang et al. 2014); (b) Static approaches, based on non-adhesive surfaces

(Hildebrandt et al. 2011), gels (Cosson and Lutolf 2014) or hanging-drop plates (Bartosh et al. 2010). Three major aspects must be considered when evaluating the advantages and disadvantages of each approach: efficiency, cost, and size of the produced spheroids (Mehta et al. 2012; Alimperti et al. 2014). In particular, Iwai et al. (2016) recently discussed the disadvantages of the methods today available to produce spheroids, meanwhile proposing their new generation approach, relying on a temperature-modulated charged surface deposition built on purpose. On one hand, the dynamic approaches generally provide high spheroid generation efficiency, but require expensive specialized equipment. On the other hand, static approaches are cost-effective, but characterized by poor generation efficiency and heterogeneity in terms of size and shape (Hildebrandt et al. 2011), and we recently proved that the morphological homogeneity of the spheroids used for in vitro therapeutic screening is fundamental for the biological relevance of data obtained (Zanoni et al. 2016). However, the lack of over-time analysis that assesses spheroid morphological homogeneity and biological stability hampers the selection of an approach suitable for a given study or application. For instance, the MSC spheroids used by Suenaga et al. (2015) for bone regeneration purposes in an animal model are implanted 1 day after generation. The regeneration analysis carried out after 2 months could yield different results whether the spheroids are used in their morphological and architectural stability range.

Among the different spheroid generation systems, the “pellet culture” method (Johnstone et al. 1998) provides a cost-effective and extremely rapid method to generate 3D MSC spheroids. This generation method does not necessitate specialized equipment: it only requires sterile polypropylene conical tubes and a benchtop centrifuge, available in most of biological laboratories. The spheroids obtained are characterized by cell–cell interactions analogous to those that occur in condensation during embryonic development (Furukawa et al. 2008). The generation efficiency is really high, i.e. one spheroid per tube, and only 5 min of centrifugation and a few days of incubation are needed to generate the spheroids (König et al. 2011). Thanks to the short time required for generation, the spheroids obtained are characterized by the absence of a necrotic core, whereas it is present in the spheroids generated with most of the

other systems (Wartenberg and Acker 1995), this causing an overall response heterogeneity and low proliferation rate of the composing cells (Occhetta et al. 2015). Furthermore, the pellet culture method is the most known generation method that allows tuning the size of the obtained spheroid by simply controlling the number of cells seeded in the tubes (Rossi et al. 2005), although there is not a validated protocol to obtain the spheroids, and typically the researchers adopt their own modified version (Zhang et al. 2010) of the original method outlined by Johnstone et al. Nonetheless, the pellet culture method is the perfect solution for researchers that want rapidly producing large, spherical-shaped MSC spheroids, of a desired size, without high costs and needs of specialized equipment.

In the literature there are several studies presenting biological analyses performed (e.g. DNA quantification, reverse transcription and real-time PCR, ELISA assay) to assess the differentiation ability of MSC spheroids generated with the pellet culture method (Ruedel et al. 2013; Im et al. 2011). For instance, Hildebrandt et al. (2011), by using the von Kossa staining proved the osteogenic differentiation of MSC spheroids after 35 days of cultivation. Similarly, Ong et al. (2006) investigated the differentiation ability of human MSC spheroids into hepatocytes, and after for 4 weeks of differentiation they implanted the spheroids into the liver of hepatectomized rats to assess the engraftment potential of these transdifferentiated cells. Longer term studies were performed by Mauck et al. (2006) that analysed the chondrogenic differentiation ability of bovine MSC over a 10-week period. Of course, the cited works also comment the morphological appearance of spheroids to try find out some correlation with biological features. While most of the considerations are usually expressed in one paragraph, or little more, it is worth citing the work of Hildebrandt et al., which dedicated almost a whole Section to discuss the correlation between the diameter-normalized metabolic long term activities (3 weeks; Im et al. 2011) of MSC and osteogenic induced spheroids (Hildebrandt et al. 2011). In spite that visual appearance of spheroids is guessed as being holder of useful information to be correlated with biological parameters of different spheroid populations, the literature lacks any methodological approach analysing long term morphological and architectural homogeneity and stability of the spheroids generated with the pellet culture method.

In this study we used the pellet culture method in order to define a standardized procedure for the generation of morpho-architecturally homogenous and large MSC spheroids. Accordingly, we started by setting up the procedure for generation and long term maintenance of the MSC spheroids in vitro. Subsequently, we investigated the over-time morphological homogeneity and architectural stability of the spheroids, in order to provide statistical data on their size, shape, and long term maintenance efficiency. In practice, by using freely available software only we monitored the population of the spheroids for a 2-month period. Quantitative and qualitative data obtained through brightfield and confocal imaging were analysed using computational analysis to estimate volume, sphericity, and jagging degree. In addition, histological evaluation was performed to deeper assess the cellular composition and the internal architecture of the spheroid. The data obtained provide the users with important information about the method efficiency and the over-time morpho-architectural homogeneity and stability of the MSC spheroids generated with the proposed approach, this helping them in better planning experiments based on MSC 3D models.

In conclusion, we investigated a spheroid generation and maintenance approach that is a quick, efficient, and cost-effective method to obtain stable and homogeneous human MSC spheroids of a desired size; this method could increase successful outcomes of laboratories engaged in the development of MSC applications taking advantage of 3D constructs, without necessitating the purchase of specialized equipment.

Materials and methods

MSC isolation and culture

MSC were obtained from the bone marrow of patients that underwent elective surgery at Rizzoli Orthopaedic Institute (Bologna, Italy). The local Ethical Committee approved the study and patients signed informed consent before the donation. MSC were isolated using gradient separation (Ficoll-Paque PREMIUM, density 1.073 g/mL; GE Healthcare, Uppsala, Sweden) and plastic adherence, as previously described (Pierini et al. 2013). Briefly, after the gradient separation step,

viable mononucleated cells (seeding density: 400,000 cells/cm²) were seeded in complete medium composed of α -modified minimum essential medium (α -MEM; BioWhittaker, Lonza, Verviers, Belgium) supplemented with 20 % lot-selected fetal bovine serum (FBS; Lonza, Basel, Switzerland), 1 % GlutaMAXTM (Gibco, Life Technologies, Paisley, UK). After 48 h of culture, the medium was changed to remove non-adherent cells. When cells reached 70–80 % confluence, they were detached by trypsinization (TripLeTM Select; Life Technologies) for 5 min at 37 °C, counted and expanded at a seeding density of 2000 cells/cm².

Spheroids production procedure

The method used to generate MSC spheroids is schematically represented in Fig. 1. This is an adaptation of the pellet culture method proposed by Johnstone et al. (1998) to quickly obtain MSC spheroids, and it can also be used for generating spheroids from some cancer cell lines and co-cultures of cancer cells and MSC (Fig. 2). Expanded MSC were trypsinized, counted, and 2.5×10^5 cells (such as

in Zhang et al. (2010) and Muraglia et al. (2003)) were placed in a 1.5 mL polypropylene conical tube with a screw cap (Primo Euroclone, Milan, Italy) and suspended in 0.5 mL Dulbecco's Modified Essential Medium–High Glucose (DMEM-HG; Euroclone, Milan, Italy) supplemented with 10 % FBS (Gibco). The aliquots were spun in a bench top centrifuge at 500 g for 5 min. The tubes were then incubated in humidified atmosphere at 37 °C with 5 % CO₂, with loosened caps to ensure adequate gas exchange. After 72 h, the cell pellets became compact spherical aggregates, and could be detached from the tube's bottom, and handled without losing their integrity. The spheroids were maintained inside the tube used for generation and the culture medium was changed twice a week. It is worth noting that by using polypropylene conical tubes the MSC spheroids can be easily cultured for long periods (i.e. ≥ 14 days (Friedrich et al. 2009)), keeping surface integrity and shape memory. On the contrary, if maintained in a standard plastic multi-well plate, such as, a 96-well (Vinci et al. 2012) or a 384-well plate (Tung et al. 2011), the MSC spheroids quickly adhere to the bottom of the plate, losing three-dimensionality (Fig. 3).

Fig. 1 Schematic diagram of the procedure followed to generate MSC spheroids. Tubes containing single-cell suspension aliquots were centrifuged at 500 g for 5 min. A spheroid

was generated for each tube. To support cell aggregation, all tubes were then incubated for 72 h. After that, the spheroids were solid and ready to be analysed. (Color figure online)

Fig. 2 The pellet culture method can also be used to generate 3D co-cultures between MSC and cells of several cancer cell lines. From left to right: spheroids of PC3 (ATCC#CRL-1435),

U2OS (ATCC#HTB-96), U87MG (ATCC#HTB-14) cells, mixed with MSC in a 1:1 ratio. The reported images were acquired 1 week after the spheroids' generation

Fig. 3 Brightfield images of three representative MSC spheroids (named as SPa, SPb, and SPc) generated according to the pellet culture method and maintained in low attachment flat-bottom 96-well plates (Non-Tissue Culture Treated Pate, Becton–Dickinson, Franklin Lakes, NJ, USA). After 4 days all the spheroids adhered to the bottom of the plate, thus losing their three-dimensionality

Computational analysis

A set of 25 MSC spheroids was monitored for statistical analysis. To examine changes in shape and morphology, a brightfield image was acquired for each spheroid twice a week, after transferring for the imaging time the spheroids to a plastic flat-bottom 24-well plate. Images were acquired with an inverted Nikon Eclipse TE2000-U microscope (Nikon, Amsterdam, Netherlands), equipped with a Nikon DS-Vi1-U3 CCD colour digital camera (1/1.18" CCD vision sensor, square pixels of 4.40 μm side, 1600×1200 pixel resolution, RGB 8-bit grey level) and a Nikon Plan Fluor 4×/0.13NA objective. First of all, the acquired images were flat-field corrected for inhomogeneous illumination intensity (Smith et al. 2015). Then, all the images were segmented and analysed by using *AnaSP*, the open-source software described in Piccinini (2015), freely available at: <http://sourceforge.net/p/anasp>. Volume and sphericity were considered to evaluate morphological stability and homogeneity over time. Furthermore, we also analysed the indentation of the spheroid's border, defining a new feature named *jagging degree*. Starting from the binary masks obtained by using *AnaSP*, the volume (V) of each

spheroid was computed by using *ReViSP* (Piccinini et al. 2015) (freely available at: <http://sourceforge.net/p/revisp>), a software specifically designed to accurately estimate the volume of spheroids and to render an image of their 3D surface (Fig. 4a). The “roundness” of the spheroids (Ahammer et al. 1999), technically called sphericity (S), was estimated according to the formula proposed by Kelm et al. (2003), and reported in Eq. 1:

$$S = \frac{\sqrt{4A\pi}}{P}, \quad (1)$$

A and P are the area and the diameter of the spheroid analysed, respectively. S ranges from 0 to 1, where $S = 1$ indicates that the cross-section of the spheroid is a perfect circle. Finally, the jagging degree (J) was computed according to Eq. 2:

$$J = 1 - \frac{\hat{P}}{P}, \quad (2)$$

$\hat{P}$ is the perimeter of the convex hull of the spheroid and the ratio $\hat{P}/P$ represents the convexity. J ranges from 0 to 1 and $J = 0$ indicates that the spheroid border is perfectly smooth, free of jagged fringes.

Fig. 4 **a** Starting from the 2D images acquired by imaging in brightfield each spheroid, volume, and 3D mesh were automatically obtained by using *ReViSP*. **b** Representative images of MSC spheroids visually defining the concepts of sphericity and jagginess degree. (Color figure online)

Figure 4b shows some representative spheroids, useful to better visualize the meaning of sphericity and jagginess degree.

Viability analysis

Cell viability was assessed by using the LIVE/DEAD[®] viability/cytotoxicity Kit L-3224 (Life Technologies, Monza, Italy) according to manufacturer's protocol. After 10 min staining in a humidified atmosphere at 37 °C with 5 % CO₂, the spheroids were directly imaged with a Nikon Eclipse Ti microscope equipped with an A1R confocal laser scanner (Nikon), temperature and CO₂ controllers (Okolab, Ottaviano, Naples, Italy) and a Nikon Plan Fluor 10×/0.30NA Ph1 DLL objective lens. z-stacks of images were acquired every 3 μm and 3D rendering was performed with NIS elements software using the *Alpha-blending* algorithm.

Histological analysis and immunostainings

To carry out analysis of the internal architecture, spheroids were prepared for cryosectioning and then stained using hematoxylin and eosin (HE). The 3D MSC aggregates were washed in PBS 1×, transferred in cryomolds, embedded in O.C.T. TM Compound (Tissue-Tek, Sakura, Leiden, Netherlands) and placed in dry ice (−70 °C) in a Styrofoam container to allow rapid freezing. Cryosections of 5 μm thickness were mounted onto glass slides and stained with HE according to standard procedures. For immunostaining, cryosections were fixed in 4 % paraformaldehyde

for 10 min at RT, washed three times in PBS 1×, permeabilized for 10 min in PBT (PBS 1× + 0.1 % Triton×100), then incubated in blocking solution (PBS 1× + 5 % BSA) for 30 min. Primary antibodies diluted in blocking solution were added and incubated overnight at 4 °C. After three washes in PBS 1×, the secondary antibody diluted in PBS 1× was added and incubated for 45 min at RT. The primary antibodies used were: mouse anti-Col1a (NFI/20) [1:500] sc-80760 and rabbit anti-Fibronectin (H-300) [1:100] sc-9068 (Santa Cruz Biotechnology, Dallas, TX, USA). The secondary antibody used was anti-mouse IgG-Cy3 [1:100] C2181 (Sigma Aldrich, St. Louis, MO, USA) and anti-rabbit FITC [1:50] F0205 (Dako, Milano, Italy). Nuclei were stained with Hoechst 33,342 (5 μg/mL) (Life Technologies) for 10 min at RT after secondary antibody step. Coverslips were mounted after washes in PBS 1× in Fluoromount-G solution (Southern Biotech, Birmingham, AL, USA) and imaged by confocal microscopy.

Statistical analysis

MATLAB (© The MathWorks, Inc., Natick, MA, USA) was used for statistical of computed morphological parameters. To assess morphological homogeneity of the MSC spheroids, meant as the variability of the features between the different spheroids at each time point i , mean ($\bar{X}_i$) and standard deviation (s_i) were computed. Then, to study the temporal stability of the different features, we analysed the temporal distribution of their mean values $\bar{X}_i$, by computing their

temporal mean ($\bar{X}$) and standard deviation (s). All the values resulting within the bounds $\bar{X} \pm 2s$ were considered stable over-time.

Results and discussion

In the following paragraphs we present and discuss the key results of our over-time analysis of MSC spheroids, generated and maintained according to the proposed method, with particular emphasis on their morphological and architectural homogeneity and stability over-time.

Efficiency of spheroid generation and maintenance approach

Efficiency is defined as the ratio among the number of spheroids actually and theoretically available. It is a crucial parameter of the systems and the methods used to generate spheroids. In general, the efficiency is particularly low for static systems. For example, hanging drop plates showed generation efficiency lower than 50 % (Hildebrandt et al. 2011), meaning that a high number of wells fail to produce a spheroid. Accordingly, an extra-amount of plates/wells has to be used to ensure the desired number of spheroids, thus affecting the planning and the cost of the approach.

In order to evaluate the efficiency of the proposed approach, we prepared 25 conical tubes and monitored the number of spheroids available by periodically imaging them over a period of 2 months. Figure 5 reports the number of spheroids available over time. To evaluate generation efficiency we counted the spheroids available 72 h after centrifugation (day 0). All tubes contained one spheroid each (efficiency

Fig. 5 Efficiency of the proposed method, measured as a function of the reduction of the number of spheroids available over time

100 % at day 0, i.e. 25 spheroids), thus proving the high efficiency of the method at the generation time. During the maintenance in culture, the number of the spheroids available decreases to 19 after 1 month (efficiency 76 %), and to 17 at the end of the analysis (after 2 months: efficiency 68 %). In general, the decrease in the spheroid number can be ascribed to loss of viability, loss of compactness or disaggregation, or to unintentional discarding during manual medium change; this likely occurred at day 25.

Morphological features assessment

Beginning with the brightfield images acquired at the different time points, the computational analysis was carried out using only open source software tools freely available. *ReViSP* was used to estimate the volume of each spheroid (also in case of non-perfect sphericity, Fig. 4a), and *AnaSP* to compute sphericity and jaggging degree. Figure 6a shows the morphology changes of three representative MSC spheroids over-time. It is worth noting that by using polypropylene conical tubes, the MSC spheroids maintain their surface integrity and shape memory for a long time period. Figures 6b, c, and d show $\bar{X}_i$ and s_i of volume, sphericity and jaggging degree, respectively. The region of stability of each feature is delineated by dashed lines and it is defined as the interval within the boundaries $\bar{X} \pm 2s$. Table 1 reports the s_i values obtained.

The volume of the MSC spheroids shows a decreasing trend (Fig. 6b) as already reported in previous studies (Cerwinka et al. 2012). Nevertheless, the loss of spheroid volume is much more limited than the one observed by Hildebrandt et al. (2011). The volume decreased from an initial average value of about 0.4 mm³ (with equivalent diameter of ~900 μm) to nearly 0.1 mm³ (equivalent diameter ~600 μm) at day 21 and was constant to the end of the analysis (i.e., 2 months). Just 15 days from spheroid generation, all the values remain bounded within $\bar{X} \pm 2s$, thus demonstrating good stability of the volume after an initial settling time. Similarly, s_i decreased down to 0.02 mm³ after about 15 days from generation (Table 1), showing a general regularization of the spheroids' population over time and a high degree of homogeneity.

As far as the sphericity is concerned, it was always greater than 0.8 (Fig. 6c) indicating that in general the MSC spheroids obtained with the proposed method

Fig. 6 **a** Brightfield images of the temporal evolution of three representative MSC spheroids (named SP01, SP02, and SP03) acquired at different time points. **b–d** Morphological features trend. To analyse homogeneity and stability of MSC spheroids, we computed at different time points: **b** volume, **c** sphericity, and **d** jaggging degree of a set of 25 spheroids

Table 1 Standard deviation (s) for the different morphological features measured at different days from generation

	Day												
	0	4	7	11	14	18	21	25	28	32	39	46	54
Volume s [mm ³]	0.08	0.04	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02	0.01	0.01
Sphericity s	0.01	0.02	0.02	0.03	0.03	0.01	0.04	0.03	0.03	0.02	0.04	0.03	0.04
Jaggging degree s	0.01	0.01	0.01	0.02	0.02	0.01	0.03	0.03	0.02	0.02	0.03	0.02	0.04

can be considered closed to a perfect sphere ever since generation (i.e. no “spheroidization time” is needed (Zanoni et al. 2016)). In very few cases, the shape was substantially irregular (spheroid with a protuberance, as reported in the example of Fig. 4a). However, the low s_i values (≤ 0.05), obtained for the different time point of analysis (Table 1), confirmed the high homogeneity of the population.

Similarly, the jaggging degree values were always lower than 0.1 (Fig. 6d), proving that MSC spheroids are in general characterized by a very compact border almost free of jags. This suggests a strong integrity of the spheroids, likely indicating a high viability of the

cells composing the outer surface. Again, the s_i values obtained (the worst s_i was 0.04) underlay a high homogeneity of the spheroids during the time in culture.

Despite the expected reduction in volume in the first 15 days after generation, the values computed for sphericity and jaggging degree showed good homogeneity of the spheroid at each time point evaluated and a high stability over time. In conclusion, considering all the morphological features evaluated, we can assume that the spheroids are homogenous and stable for a time interval of at least 1 month, between days 15 and day 45.

Cell viability and internal architecture evaluation

To determine if the observed decrease in the spheroid number (“Efficiency of spheroid generation and maintenance approach” section) can be accounted by loss of MSC viability, cellular vitality was assessed at different time points, using Live and Dead assay and confocal imaging on the whole 3D aggregate. As shown in Fig. 7a, b, for the first month of observation the external shell is composed of viable cells (Calcein positive cells, green in Fig. 7) and a lower percentage of dead cells (EthD-1 positive cells, red in Fig. 7). However, after 2 months in culture the spheroid surface appears less homogenous and compact, as depicted by wider unstained areas (Fig. 7c).

Surprisingly, the computational analysis indicated that the spheroids maintain a high degree of sphericity for 2 months of observation (Fig. 5d). Therefore, to better characterize both outer and inner architecture, cryosections were stained with HE to visualize cellular nuclei and cytoplasm, and with antibodies recognizing

specific Extracellular Matrix (ECM) components: Fibronectin (green in Fig. 7d–f) and Collagen IA (red in Fig. 7d–f).

Based on the HE staining pattern, spheroids appear homogeneous and compact up to a month after generation (Fig. 7d, e). After 2 months, they maintained a rough spherical shape, but lost cellular homogeneity, and large internal unstained regions were clearly visible. Those regions can likely be ascribed to necrotic areas since cells undergoing necrosis display nuclei and cellular membrane disintegration, leading to a loss of structural integrity and cell–cell contacts. The Live and Dead staining pattern and the presence of unstained areas were visible on the external shell of spheroids; these could be therefore attributed to the necrotic regions that develop during time in culture. The increasing degree of jagging of the spheroid’s border, measured by the computational analysis, could be therefore explained by the time-related compactness decrease and by the global loss of architectural integrity of MSC aggregates. In contrast

Fig. 7 **a–c** Cell viability analysis. Representative 3D rendering of confocal z-stacks images of spheroids acquired at different time points, showing the spatial distribution of live (green) and dead (red) cells highlighted by Calcein AM and Ethidium Homodimer 1 dyes, respectively. **d–f** Internal architecture

analysis. Representative images of 5 μm cryosections stained with HE and with antibodies against Collagen IA (red in smaller right panels) and Fibronectin (green in smaller right panels). Merge panels show Hoechst nuclear staining overlapped with Collagen IA and Fibronectin. (Color figure online)

to tumour spheroids where necrotic regions are commonly found in the core region (Ma et al. 2012) due to hypoxia or depletion of substrates or accumulation of metabolic waste products, MSC spheroids' necrotic regions appear randomly distributed on the whole structure. This result may be suggestive of dissimilar mechanisms adopted by MSC aggregates and tumour spheroids for substrate and gas exchanges through the multicellular structure.

To further comprehend the extracellular matrix organization, we analysed the distribution of Fibronectin and Collagen, whose interaction is necessary for ECM architecture and functionality (Sevilla et al. 2013). As shown in Fig. 7d–f, Fibronectin and Collagen localization in the cell–cell contact regions is clearly visible at the beginning of the analysis (asterisks in Fig. 7d–f). After 1 month in culture, Fibronectin fibrils are observed on the surface of the spheroids while Collagen is localized below the fibronectin layer (arrowhead in Fig. 7d–f). After 2 months, as expected from the appearance of necrotic areas by histological analysis (described above), the Fibronectin/Collagen interaction is misplaced, due to cellular membrane disintegration: consequently, spheroids lose ECM mediated shape.

The data acquired regarding the qualitative analysis of viability and the internal cellular architecture corroborate the observation that MSC spheroids are biologically stable spherical aggregates for a time interval of at least 1 month.

Conclusions

To the best of our knowledge, this is the first long term analysis of human MSC spheroids (maintained in culture for 2 months) providing morphological data, such as volume, sphericity and a new feature, the jaggging degree (a possible indicator of surface flaking), and information about outer cellular viability and inner structure composition.

The following are the two major outcomes of this work: (a) the proposed method can be used efficiently to rapidly obtain uniform MSC spheroids with a high sphericity and a “smooth” surface, by using standard equipment available in every biological laboratory; (b) the spheroids obtained have homogeneous and stable morphology and architecture for at least 15 days after 2 weeks from generation.

Other authors have proven that the pellet culture MSC spheroids can keep some of their salient biological features (e.g. the differentiation ability) for a long time. Accordingly, together with our morpho-architectural observations, the proposed pellet culture method can be efficiently used as a reference for laboratories interested in easily obtaining stable homogeneous populations of MSC spheroids without high costs and, moreover, without need for specialized equipment.

Acknowledgments The authors would like to thank Dr. Davide Donati and his staff of the Third Orthopedics and Traumatology Clinic (IOR, Bologna), for providing the cells used in this work and Ms. Charlotte Story (University of North Carolina at Chapel Hill, USA) for editorial assistance and English revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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