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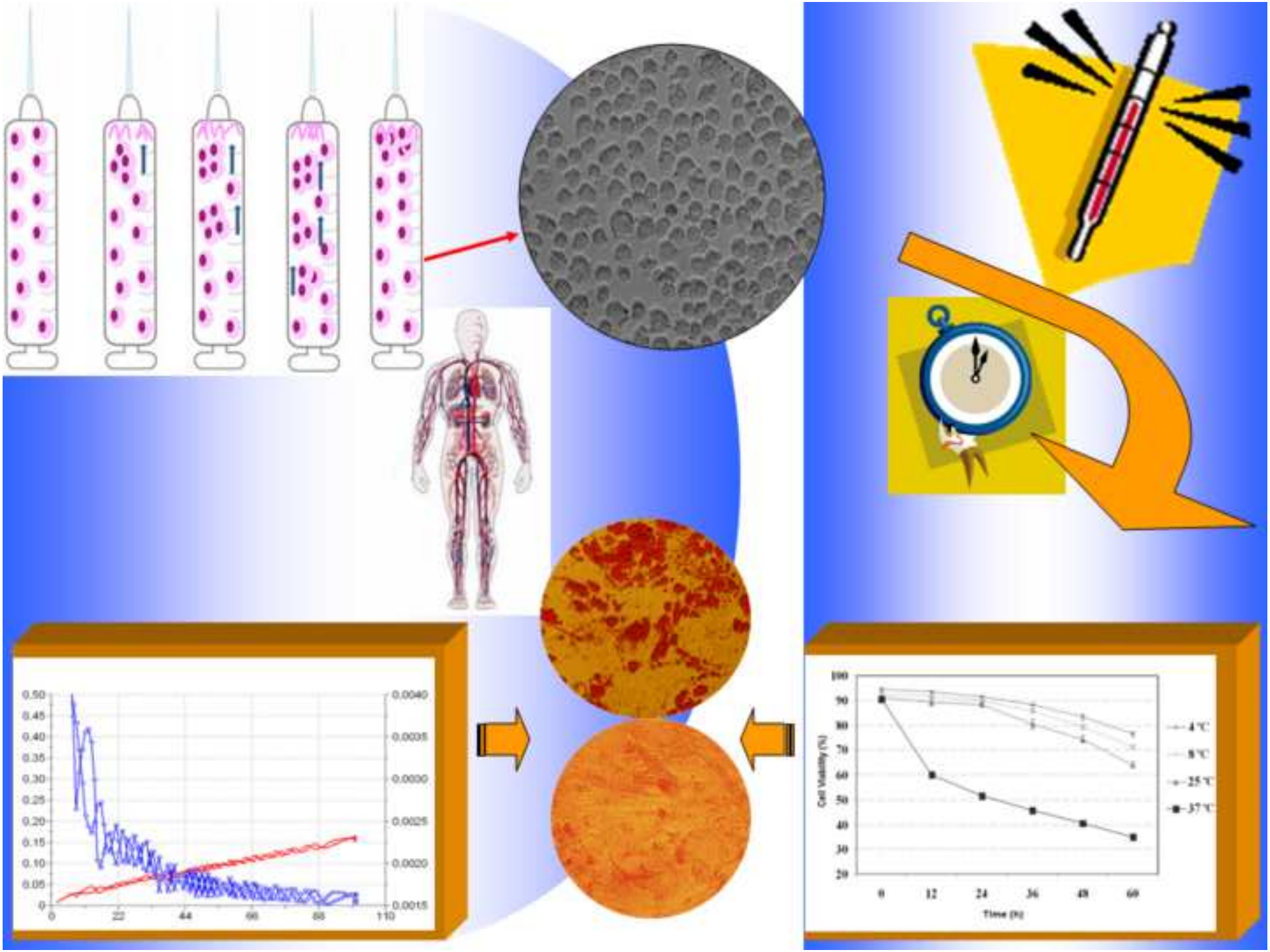
*Graphical Abstract



Title

Study of the stability of packaging and storage conditions of human mesenchymal stem cell for intra-arterial clinical application in patient with critical limb ischemia.

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Abstract

Critical limb ischemia (CLI) is associated with significant morbidity and mortality. In this study, we developed and characterized an intra-arterial cell suspension containing human mesenchymal stem cells (hMSCs) for the treatment of CLI. Equally, the stability of cells was studied in order to evaluate the optimal conditions of storage that guarantee their viability from cell processing to the administration phase. The effects of various factors, including excipients, storage temperature and time were evaluated to analyze the survival of hMSCs in the finished medicinal product. The viability of hMSCs in different packaging media was studied for 60 h at 4 ºC. The best medium maintaining hMSCs viability was then selected to test storage conditions (4, 8, 25 and 37 ºC; 60 h). The results showed that at 4 ºC the viability was maintained above 80% for 48 h, at 8 ºC decreased slightly, whereas at room temperature and 37 ºC decreased drastically. Its biocompatibility was assessed by cell morphology and cell viability assays. During stability study, the stored cells did not show any change in their phenotypic or genotypic characteristics and physicochemical properties remained unchanged, the ability to differentiate into adipocytes and osteocytes and sterility requirements were also unaltered. Finally, our paper proposes a packing media composed of albumin 20%, glucose 5% and Ringer's lactate at a concentration of 1×10^6 cells/mL, which must be stored at 4 ºC as suitable for maintaining cells viability (> 80%) and without altering their characteristics for more than 48 h.

Keywords: critical limb ischemia, adipose mesenchymal stem cell, packaging, storage, injectable, intra-arterial.

1. INTRODUCTION

The recent advances of biomedical research and biotechnologies have opened new promising therapeutic strategies, and human mesenchymal stem cells (hMSCs) are attracting increasing interest for possible application in cell therapies for the treatment of several human diseases [1,2]. hMSCs offer considerable therapeutic potential through the development of different cell therapy medical products (CTMP) for clinical use [3], due to their regenerative and immunoregulatory capacities [4,5], which have made them one of the most promising candidates for cell therapy success including regenerative and immune therapies where present conventional treatments are inadequate.

Critical limb ischemia (CLI) is one of the diseases most studied in the field of cell therapy, in particular in diabetic patients, CLI of the leg develops earlier and more intensely, avoiding revascularization [6, 7] CLI is a syndrome manifested by ischemic rest pain, non-healing ulcers, tissue loss and gangrene. The incidence of CLI is estimated to be approximately 500 to 1000 patients per million and year [8]. Patients with CLI are at high risk of amputation, increased in diabetic patients, which leads to a low quality of life, and severe morbidity and mortality, resulting in a significant social and economic impact [9].

Currently the latest advances in this pathology have led to the development of new drugs with stem cells as an alternative to surgical and pharmacological treatment. hMSCs transplantation is one of the most studied therapeutic alternatives in preclinical and clinical stages, to be due to paracrine, immunomodulatory, and differentiation effects [8,10,11].

hMSCs encompass a broad range of anchorage dependent fibroblast-like cells which can be obtained from bone marrow aspirates, skeletal muscle connective tissue, human

trabecular bones, adipose tissue, periosteum, fetal blood and liver, and umbilical cord blood [12]. These cells are characterized by being able to adhere to plastic. They can proliferate *in vitro* and exhibit multilineage differentiation capacity being capable to give rise to diverse cells like osteoblasts, chondrocytes, adipocytes, myocytes, tenocytes and vascular cells and to express several common cell surface antigens [13].

Adipose tissue- derived stem cells have emerged as a new and promising type of stem cells because adipose tissue is an abundant source of stem cells, it lacks donor limitation and it is possible to obtain by a minimally invasive method [14].

Conducting cellular therapeutics is a complex undertaking, and both safety and efficacy measures shall be considered in the establishment of the manufacturing process of a finished medicinal product for cell therapy [15] among all steps involved, research phase, translational phase for scaling-up the protocol for the clinical requirements, establishment of standard operation procedures (SOP), validation runs, regulatory registration, storage and transportation [16]. The use of hMSCs for clinical application requires a high number of cells, which entails the *in vitro* expansion in a certified laboratory under good manufacturing practice (GMP) conditions [17]. After culturing and having obtained the necessary number of cells, hMSCs must be loaded in a suitable dosage form for their administration. hMSCs can be formulated into liquid and semisolid dosage forms. Current methods of cell delivery involve the use of injections and microencapsulation. The use of several biomaterials for microencapsulation results in an impenetrable membrane to cells, and requires strong mechanical disturbances such as pressurized nozzles, emulsification, or stirring during droplet generation leading to cell degradation [18]. A cell suspension can be parentally administered directly in the damaged organ/tissue whilst offering medical devices use possibility. Attempts of CLI

treatment with intra-arterial hMSCs have been associated with significant therapeutics benefits [10].

On the other hand, the administration of the cells to the patient is not unmediated. After obtaining the cells, the formulation of the finished medicinal product is carried out, all quality checks must be performed before approving its release, hence cells must be stored and transported in the best conditions to maintain stability [19]. The finished medicinal product of a CTMP includes an active ingredient (hMSCs) and the selected excipients (packaging medium), which are different from the expansion media. The cell stability of the finished medicinal product is determinant for its therapeutic applications in clinical use since baseline characteristics of cells should be maintained [15].

The stability information should include biological (sterility including mycoplasma, endotoxin and adventitious viral agents, identity, purity and potency) and physicochemical tests including those related to the design of the dosage form such as cell sedimentation rate and resuspension [20]. Alternatively, these products are likely to have a short shelf-life, which often means that these products are administered to patients before current sterility test results are available [17]. Due to cells are highly fragile and sensitive to their surrounding environment, and in order to maintain their quality, their environment needs to be strictly controlled during the time gap between cell harvesting and administration. Therefore, for the formulation of a cell suspension, important key factors must be taken into account, such as selection of the excipients of the packing medium, which must be protein-free, to avoid inflammatory responses affecting the efficacy and safety [21]. Temperature and time conditions of storage as well as transport should be also studied.

Even though characterization of hMSCs has been extensively studied for their *in vitro* expansion, however there are not data about the cell characterization in the finished

medicinal product. The present work studies the stability of MSCs from human adipose tissue elaborated in a cell suspension for intra-arterial application.

The choice of the packing medium and storage conditions for a hMSCs suspension have been studied through stability studies, with the purpose of formulating a finished medicinal product that assured the maintenance of the characteristics more similar as possible to those of native hMSCs. hMSCs have been characterized before and after formulation, studying cell viability, immunophenotypic and genotypic characterization, differentiation, microbiology and physicochemical properties.

2. MATERIALS AND METHODS

2.1. GMP environment

This study was performed in the context of a clinical trial, in phase I/II under GMP conditions. All procedures were performed in a certified clean room at the Center for Molecular Biology and Regenerative Medicine (CABIMER), it was the first laboratory accredited by the Spanish Agency for Medicines and Medical Devices for production of stem cells as medicines in Andalucía (Spain).

All starting materials and reagents required for this study were according to GMP guidelines. All equipment was validated.

2.2. Human mesenchymal stem cell isolation and culture from adipose tissue

The donor source of the hMSCs was appropriately screened and tested for human pathogens. Procedures were performed at the San Lazaro Hospital (Spain). The therapeutic protocol was approved by the hospital ethics committee in accordance with Spanish law. All patients signed a detailed informed consent form before intervention and gave their consent for publication of the study results. This study was conducted in

accordance with the ethical standards of the Helsinki Declaration (1975). In particular, the presence of Human Immunodeficiency Virus (HIV), hepatitis B and hepatitis C virus were analyzed. On the other hand, all starting materials and reagents required for the expansion were analyzed to certify that they were sterile and endotoxin-free. Autologous hMSCs were isolated from adipose tissue by enzymatic digestion with collagenase (2 mg collagenase/1 g adipose tissue) (Roche Farma, Reinach, BL, Switzerland). Briefly, the sample was centrifuged at 400 g for 10 min, filtered and washed with Phosphate Buffered Saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). The isolated cells were suspended and plated at medium density (passage 0) of 12–20 $\times 10^4$ cells/cm^2 in culture flasks (Nunc, Roskilde, Denmark) with expansion medium composed by Dulbecco´s Modified Eagle´s Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% 100 U/mL Penicillin – 100 µg/mL Streptomycin, 2% 200 mM L-alanine solution and 1% 2 mM L-glutamine, (all from Sigma-Aldrich, St. Louis, MO, USA). After 24 h non adherent cells were removed by replacing the expansion medium. Cells were harvested upon reaching 80% confluence, and subcultured using 0.25% trypsin (Gibco, Invitrogen, Grand Island, NY, USA) on expansion medium, and plated at medium density of 3500–5500 cells/cm^2. Cells were cultured in a 95% relative humidity, incubator at 37 °C in 5% CO_2. The medium was completely replaced every 2 or 3 days a week.

2.3. Cell viability and counting assay

This study was conducted before and after the preparation of the finished medicinal product, in the supernatant and in each packaging media.

Cell viability was determined by trypan blue dye exclusion staining [22] and posterior counting of cells in a Neubauer chamber. Each sample was counted three times

and the average was calculated. The percentage of viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100 \qquad (1)$$

2.4. Assessment of stability

2.4.1. Influence of the packaging medium

hMSCs from passage 4 were used. Cells were harvested using 0.25% trypsin (Gibco, Invitrogen, Grand Island, NY, USA) and centrifuged at 400 g for 10 min with expansion medium. The cell pellet was resuspended at concentration of 1×10^6 cells/mL with 1 mL of packaging medium. For this study, different excipients (Grifols, Barcelona, Spain) were selected to analyze the hMSCs viability during the storage period of the finished cell medicinal product. Four packaging media were prepared, the excipients for 50 mL of each such media is reported in Table 1.

hMSCs were packed in 10mL Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA). 11 syringes for each medium were prepared and stored at 4 ºC in a normal atmosphere. Every 6 h for 60 h, cell viability was tested.

2.4.2. Influence of the temperature of storage

hMSCs were packed in 24 10mL-Luer-lock syringes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 1×10^6 cells/mL with 1 mL of the most stable packing medium after the study described above. Samples were stored at different temperature: 4 ºC, 8 ºC, 25 ºC and 37 ºC in normal atmosphere (6 syringes each temperature). Cell viability was tested every 12 h for 60 h.

2.5. hMSCs immunophenotypic characterization

At passage 4 and after stability study of the finished medicinal product, immunophenotyping study of hMSCs was performed in order to identify the presence of specific surface antigens. Between 2.5 and 5×10^5 cells were separated in 1.3 mL of expansion medium. The following markers were analyzed: CD13-PE, CD29-PE, CD90-FITC, CD105-PE, CD31-FITC, CD34-PE, CD45-FITC and HLA II-FITC (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Mouse antibodies served as control: Isotype-FITC IgG1-k, Isotype-FITC IgG2a-k, Isotype-PE IgG1-k (all from Becton Dickinson and Company, Franklin Lakes, NJ, USA). 100 µL of cell suspension were prepared with 5 µL of each of the following reagents: fluorescein isothiocyanate (FITC), phycoerythrin (PE) antibody and control, and incubated at 4 °C for 20 min in the dark. Then, 3 mL of PBS (Sigma-Aldrich, St. Louis, MO, USA) were added to each cell suspension and centrifuged at 400 g for 10 min. Finally each cell pellet was diluted in 300 µL of PBS and 5000 labeled cells were acquired and analyzed using a FACSCalibur analyzer flow cytometer system running CellQuest Pro software (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the percentage of viable cells positive for each marker were determined.

2.6. *In vitro* osteogenic and adipogenic differentiation

The differentiation of hMSCs into osteoblasts and adipocytes was analyzed on passage 4 and after stability study of the finished medicinal product. For osteogenic and adipogenic induction, cells were seeded at 16×10^4 cells/plate in two 35 mm plastic Petri dishes (Nunc, Roskilde, Denmark) and cultured for one week with expansion medium. Briefly, the medium was removed and Osteogenic Differentiation Medium and Adipogenic Induction Medium (Lonza, Walkersville, MD, USA) were added in each

plate respectively [23]. For adipogenic differentiation, two weeks after the Adipogenic Induction Medium was changed by Adipogenic Maintenance Medium (Lonza, Walkersville, MD, USA). New medium was replenished every 3 to 4 days. After 21 days cells were fixed in 4% paraformaldehyde (VWR International, Radnor, PA, USA) for 5-10 min at room temperature and stained with Oil Red-O adipocytes (Sigma-Aldrich, St. Louis, MO, USA) and Alizarin Red S sodium salt osteoblast (Alfa Aesar, Ward Hill, MA, USA). Images were captured using a microscope (Olympus 1×71, Tokyo, Japan) and a CCD camera (Olympus DP70).

2.7. hMSCs karyotyping analysis

The hMSCs were analyzed before and after the stability study. Karyotype analysis was performed by G band techniques [24]. In order to obtain chromosomal preparations the hMSCs were treated with 0.8 μg/mL colchicines (KaryoMAX®-Colcemid™; Gibco, Invitrogen, Grand Island, NY, USA) and incubated at 37 ºC for 1.15 h. Briefly the cells were washed with 1 mL trypsin (Gibco, Invitrogen, Grand Island, NY, USA) twice. Then, 2 mL trypsin was added at 37 ºC for 2 min and centrifuged at 400 g for 10 min with 1 mL FBS. The pellet was suspended in 5 mL 75 mM KCl (Merck, Darmstadt, Germany) at 37 ºC for 20 min and centrifuged at 400 g for 10 min. The cells were fixed with 5 mL methyl alcohol-acetic acid mixture (3:1 v/v) (Merck, Darmstadt, Germany) and centrifuged at 400 g for 10 min., this process was repeated twice more. For each karyotype 30 metaphases were analyzed. The final result was described to account the recommendations from the International System for Human Cytogenetic Nomenclature [25]. Chromosomal aberrations were considered when at least two metaphases showed the same alteration (additions, deletions, inversions and translocations).

2.8. Microbiological studies

Microbiological analysis was based on the study of the sterility of the cells before being packaged and sterility of the finished medicinal product after stability study. The test was carried out as described in the European Pharmacopoeia [26], by direct inoculation of 1mL of sample (supernatant of the cells in the last passage and the final cell suspension with the selected packaging medium) in the microbiological medium to test for the growth of yeast, fungi, aerobic, and anaerobic bacteria. Two microbiological media were used: Thioglycollate Penase Broth 9 mL (TPB), to detect anaerobic and aerobic bacteria and Tryptic Soy Penase Broth 9 mL (TSPB) (VWR International, Radnor, PA, USA), which it is a soybean casein digest medium to detect fungi and aerobic bacteria. For each media (TPB and TSPB), sterility test and growth promotion test of aerobes, anaerobes and fungi were previously verified. The inoculated media were incubated for 14 days at 35 ºC and 22 ºC for TPB and TSPB respectively. After 14 days, if there had been microbial growth, the medium would have shown turbidity. Negative controls were established by inoculating 1 mL of 0.9% sterile NaCl (bioMérieux, Marcy l'Etoile, France) in duplicate for each medium. The inoculated media were incubated for 14 days at 35 ºC and 22 ºC for TPB and TSPB respectively. After 14 days, if there had been microbial growth, the medium would have shown turbidity. This assay was performed in aseptic conditions with an isolator HPI-4PI-S (Esco Technologies, Inc., Hatboro, PA, USA).

2.9. Physicochemical characterization of the finished medicinal product

2.9.1. Rheological studies

The rheological characterization was conducted in order to evaluate changes induced by cells. Properties of the formulations were studied, such as viscosity, a parameter closely

related with physical and structural stability allowing us to estimate the behaviour of the formulations.

The rheological characterization of the formulations was performed at 25 ºC using a rotational rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Karlsruhe, Germany) equipped with a parallel plate geometry set-up with a fixed lower plate and an upper plate (Haake PP60 Ti, 6 cm diameter). Different gaps between plates were tested and a separation of 0.105 mm was selected. The rheometer was connected to a computer provided with the software HAAKE Rheowin® Job Manager V. 3.3 to carry out the test and Rheowin® Data Manager V. 3.3 (Thermo Electron Corporation, Karlsruhe, Germany) to carry out the analysis of the obtained data. Viscosity curves and flow curves were recorded for 1 min during the ramp-up period from 0 to 100 s^{-1}, 1 min at 100 s^{-1} (constant share rate period) and finally 3 min during the ramp-down period from 100 to 0 s^{-1}. All determinations were performed in triplicate.

Data from the flow curve (when resulted to be non-newtonian) were fitted to different mathematical models, equations (2) – (6) using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA):

$$\tau = \tau_0 + \eta_p \times \gamma \qquad \text{Bingham} \qquad (2)$$

$$\tau = k \times (\gamma)^n \qquad \text{Ostwald-De-Waele} \qquad (3)$$

$$\tau = \tau_0 + k_1 \times (\gamma)^n \qquad \text{Herschel-Bulkley} \qquad (4)$$

$$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{k_1 \times \gamma} \qquad \text{Casson} \qquad (5)$$

$$\frac{\eta - \eta}{\eta 0 - \eta \infty} = \frac{1}{\left(1 + (\lambda\gamma)^m\right)} \qquad \text{Cross} \qquad (6)$$

Where is the τ is the shear stress (Pa), η is the viscosity (Pa·s), γ is the shear rate (1/s), τ_0 is the yield shear stress (Pa), η_p is the consistency index (Pa·s^n), k is the consistency (s),

λ is the time constant calculated from the point on the viscosity versus shear rate curve where the flow changes from the lower Newtonian region to the Power law region, η_0 and η_∞ are asymptotic values of viscosity at zero and infinite (very low and very high shear stress), respectively, *m* is a dimensionless exponent with a typical range from 2/3 to 1, and *n* is the flow index, the different values of *n* indicate the fluid behaviour. For a Newtonian fluid, $n = 1$. If $n < 1$, the fluid is called pseudoplastic; if $n > 1$, the fluid is dilatant.

2.9.2. Morphological analysis

Morphologic characteristics of hMSCs in each passage and after of stability study were observed by a microscope (Olympus 1×71, Tokyo, Japan), and captured by a CCD camera (Olympus DP70). Also cell suspension of finished medicinal product was observed at 4 ºC, 8 ºC, 25 ºC and 37 ºC, each 12 h for 48 h in the medium more stable to analyze to cell aggregation.

2.9.3. pH measurements

pH test was conducted on the supernatant before to pack hMSCs , and on the finished medicinal product stored at 4 ºC at times 0 h and 48 h. pH values were measured in triplicate by immersing the probe directly into the sample using a digital pH-meter Basic 20 (Crison Instruments S.A., Barcelona, Spain) with the electrode for liquid samples.

2.9.4. Optical characterization of the stability

Light scattering methods are often used to study the stability of suspensions; an analysis of multiple dispersion of light was used to predict and confirm the physical

stability of the cell suspensions by using the TurbiScanLab® (Formulaction, L'Union, France). The light source is a pulsed near infrared light source (λ = 880 nm). Two synchronous optical sensors receive respectively light transmitted through the sample (0 º from the incident radiation, transmission sensor), and light back-scattered by the sample (135 º from the incident radiation, backscattering detector). Each undiluted formulation (20 mL) was placed and kept on a cylindrical glass measuring cell which was completely scanned by a reading head.

2.10. Statistical analysis

Tests for significant differences between means were performed by Student's *t*-test or one-way ANOVA using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Differences where $p < 0.05$ were considered statistically significant. Experiments were repeated on three different samples and the results were expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

Clinical application of hMSCs requires a concentration approximately between 1 and 2 $\times 10^6$ cells/kg [23], therefore hMSCs should be expanded in long-term culture that can affect their characteristics [27, 28], specially their immunogenicity or a lack of safety of the medium components, resulting in chromosomal aberrations [29–32].

For intra-arterial administration of hMSCs the formulation of a suitable and safe finished medicinal product is a need absolutely vital. The storage conditions study is a key factor to assure the viability of cells and hold their properties in the moment of administration [33–35]. For this reason, different excipients at different temperatures were studied in the finished medicinal product.

3.1. Assessment of stability

3.1.1. Influence of the packaging medium

On passage 4, the average viability of the hMSCs cultured before to be packed was 98.1 ± 2.1%. The same cells were packed in four different media and the average viability decreased throughout the assay. Viability measurements are indicated in Figure 1. Different effects of packing media were observed on cell viability. Medium 1 showed that viability was greater than 80% for 48 h after packaging (82.2 ± 2.7%). Media 2, 3 and 4 maintained the viability above 80% for less time. Medium 2 for 36 h (84.4 ± 1.9%), medium 3 until 30 h (81.3 ± 3.3%), and medium 4 maintained viability of cells only for 12 h (82.7 ± 2.5%).

Although generally NaCl 0.9% is the vehicle of choice for any parenteral suspension, our study demonstrated that it was the medium in which viability decreased, and thus the least suitable. These results indicated greater cell viability in media with albumin and nutrients. Whilst, media 3 and 4 were solutions lacking of essential nutrients causing a rapid and significant decreased of cell viability.

In general there was a progressive loss of viability but the results indicated that hMSCs had better survival rate when they were packed in medium 1 as compared to the packaging media 2, 3 and 4.

3.1.2. Influence of the temperature of storage

Taking into account results above described, the effect of storage temperature was tested on medium 1. The hMSCs were maintained in culture until passage 5, their average viability before the study was 95.1 ± 2.4%. All syringes were stored at 4 ºC, 8 ºC, 25 ºC and 37 ºC, for 60 h and their viability was calculated each 12 h, results are depicted in Fig. 2. Our results indicated that during storage, the viability of hMSCs had

decreased considerably at 37 ºC compared to other temperatures analyzed, decreasing from 90.5 ± 0.3% at 0 h to 60.1 ± 1.1% at 12 h. Among 4 ºC, 8 ºC and 25 ºC the viability mean values were similar throughout the study, approximately 80% up to 36 h at three temperatures (88.3 ± 0.4%, 85.7 ± 0.8% and 80.4 ± 1.9% respectively). However at 4 ºC the viability of hMSCs remained above 80% (83.4 ± 1.1%) up to 48 h, being the temperature most suitable for maintaining the viability of the cells for longer.

3.2. hMSCs immunophenotypic characterization

hMSCs packed in medium 1 and stored at 4 °C after 48 h were analyzed by a flow cytometer. The results were compared with previous storage test results (cell on passage 4). Analysis revealed that both populations (before and after packing) were positive (>95%) for mesenchymal markers (CD13, CD29, CD90 and CD105) and were negative (<10%) for endothelial and hematopoietic lineage markers (CD34, CD45, CD31 and HLA-II) as is shown in Fig. 3. Fluorescence cytometry between hMSCs in both cultures and finished medicinal product showed no differences when the hMScs were stored at 4 ºC for 48 h in medium 1.

3.3. *In vitro* osteogenic and adipogenic differentiation

hMSCs were seeded and cultured in osteogenic and adipogenic media again to confirm if hMSCs packed and stored in the medium 1 at 4 ºC after 48 h could differentiate into osteoblasts and adipocytes. The results were compared with differentiation conducted on passage 4, before the stability test performed (Fig. 4). The differentiation was confirmed following the standard protocols and no difference was observed in the differentiated cells indicating that storage at 4 ºC for 48 h in the medium 1 did not affect the differentiation capability of hMSCs.

3.4. hMSCs karyotyping analysis

Cells were tested for genomic stability using a conventional analysis by G band techniques to check whether the hMSCs maintained their normal karyotype during cultivation to passage 4 and during their packing and storage before their administration phase. The results showed that on passage 4, the hMSCs were normal diploid karyotype (46, XX). After packaging and storage phases in medium 1 at 4 ºC for 48 h, a sample contained in a sterile syringe Luer-lock, was taken to carry out another karyotype test. The results of these test showed no karyotype changes in the finished medicinal product, obtaining a normal diploid karyotype (46, XX) (Fig. 5).

3.5. Microbiological studies

Sterility testing was performed on the hMSCs during culture (passage 4) and packing after stability study to ensure no contamination. When the incubation period had finished all tested samples were observed and no turbidity was exhibited, and so completely free of contaminating microorganisms. These results showed that not only the expansion procedure maintained asepsis, but also package and storage in medium 1, for 48 h at 4 °C, preserved the sterility of the finished medicinal product until administration phase. All negative control tubes were negative after the required incubation period.

3.6. Physicochemical characterization of the finished medicinal product

3.6.1. Rheological studies

Rheological disturbances can play important role in the parenteral administration of cell suspensions. In fact the viscosity of suspensions is strongly influenced by factors such

as aggregation of hMSCs. Thus, the rheological properties of the finished cell suspension are fundamental in the parenteral administration in micro and macrovessels. For this reason the rheological characteristics of unstirred cell suspension were also studied. The sample exhibited Newtonian behavior and has lower viscosity value (1.11 ± 0.06 mPas), which indicated that there was migration of aggregates/flocculates. To distinguish both alternatives suspensions were studied after being shaken. Most aggregates were separated as consequence of shear forces, which will be sufficient to break-up the weak reversible flocculation. Viscosity value of the finished cell suspension in this case was 1.575 ± 0.043 mPas. This value is similar to viscosity of blood plasma of 1.2 mPa s at 37 ºC [36].

The flow and viscosity curves of the suspensions (shear stress versus shear rate in red, and viscosity versus shear rate in blue) are shown in Fig. 6. When the shear rate increases, the viscosity values decrease, and this tendency is exhibited in all samples. This rheological behaviour could be described as typical for a non-Newtonian, pseudoplastic fluid. The hysteresis area (thixotropy), a pseudoplastic natural characteristic, was observed in the rheogram obtained. Rheological parameters were fitted to mathematical models in order to identify the model that provided the best overall match of the experimentally observed rheological data based on the highest correlation coefficient of the linear regressions (r) and the lowest chi-square value. The model that best fitted the experimental data was Cross in all cases, showing that flow behaviour was not influenced by time and/or temperature with an r^2 of 0.995 and chi-square of 0.0015, the Newton model provided the worst overall prediction of rheological behaviour. Cross model is a rheological model that combines four parameters and covers the entire shear rate range [37]. In general, the four parameter models are difficult to apply because there is seldom enough data to allow good model

fitting. However, they represent the best results in predicting the behaviour of non-Newtonian fluids. These results may have important implications for the administration because the success of the injection is dependent upon limiting shear forces of the needle wall causing cell lysis mainly produced by turbulent flow.

3.6.2. Morphological analysis

To determine whether the hMSCs cultures maintained their morphologic characteristics, they were tested in each passage and before packing. The adherent cultured hMSCs exhibited a homogeneous population with a fibroblast-like morphology when observed under a light microscope before to be packed. Following the stability test, no morphological differences were observed when the cells were plated again.

On the other hand, the packed hMSCs in medium 1 at different temperatures (4, 8, 25 and 37 ºC for 48 h, were observed each 12 h to determine if cell aggregation may have occurred. Imagines showed no cell aggregation at 4 and 8 ºC at any time (Fig. 7). However, at room temperature and 37 ºC, after 24 hours cell aggregate signs could be seen. Therefore it can be concluded that the packaged finished medicinal product in medium 1, at 4 ºC for 48 h did not exhibited hMSCs aggregation in suspension and was safe to be administrated.

3.6.3 pH measurements

pH for intra-arterial administration should be equal to the blood pH, slightly alkaline to prevent potential stinging, burning, pain, irritation or tissue damage. pH in the supernatant was 7.82 ± 0.02 on passages 4 and 5. After packaging, at time 0 h pH was 7.91 ± 0.01 and 48 h was 6.86 ± 0.01. These results demonstrated that although pH

values of the finished medicinal product decreased over time, at 48 h remained alkaline and therefore acceptable for parenteral administration.

3.6.4. Optical characterization of the stability

Stability evaluation is generally a crucial point in the scenario of advanced therapies. The long-term physical stability of cell suspensions was tested by evaluating the photon backscattering profiles of the various samples. Turbiscan®Lab is considered as a device which predicts the stability, being able to detect destabilization before than the classical stability methods (microscopy, spectroscopy or turbidity). Moreover, it provides real-time information on the destabilization process based on the variation of backscattering. When sedimentation process is produced (migration of cells from the top to the bottom), a backscattering increase versus time at the bottom of the sample is observed. When the sample suffers a creaming process, an increase of transmission versus time on the top of the vial is observed. If the destabilization phenomenon occurs due to aggregation (migration of cells form the bottom to the top), a backscattering increase versus time can be observed over the whole height of the sample [38]. If backscattering profiles have a deviation of $\leq \pm 2\%$ it can be considered that there are no significant variations. Variations up to ± 10% indicate instable formulations. Fig. 8 shows backscattering profile of the final product containing hMSCs corresponding to measurements on different hours. The left side of the curves corresponds to the bottom of the vial, whereas the right side corresponds to the top.

The region below 4 mm marks the metal base and the strong decay of backscattering above 38 mm the beginning of the free surface of the sample.

For cell suspension both creaming and flocculation mechanisms, were involved. It can be observed that the initial dispersion presented a backscattering value about 5% and

there were no changes in backscattering for the first 2 h. Between 2 and 6 h, a progressive backscattering increase was observed. This phenomenon could be attributed to the sample flocculation, possibly due to the formation of aggregates, as it was previously observed in microscopy study. These result confirmed the natural preference of hMSCs to form [39]. In our samples, flocculation started at different times.

The peak that appeared at the top of the tube was indicative of creaming, presumably caused for migration of cellular components as lipid membranes from dead cells.

Finally, after about 7 h there was no evolution in backscattering, indicating that the suspension was starting to stabilize.

In this way, it could be concluded that before the future administration, the injectable cell suspension should be shacked just before using in order to get a homogeneous hMSCs suspension, avoiding thrombotic or thromboembolic events.

4. CONCLUSION

In the present study, the stability of a CTMP with hMSCs was studied to be applied in diabetic patients with critical limb ischemia in clinical trial phase I-II, based on the study of the viability of the cells before and after being packaged and stored.

Our results clearly showed that the viability of the hMSCs in a packaging medium comprising albumin 20%, glucose 5% and Ringer's lactate at a concentration of 1×10^6 cells/mL and stored at 4 °C for more than 48 h is maintained above 80%, therefore acceptable viability was obtained for a potential clinical use. Our results also demonstrated that hMSCs processed and packaged under GMP conditions environment and subsequently stored maintained their phenotypic, genotypic and physicochemical characterization. Besides their ability to differentiate into adipocytes and osteoblast was

also preserved, as well as their sterility requirements. Thus a safe medicine for clinical application was developed.

There are several papers dealing with stability studies of stem cells for clinical use. The best storage conditions for cell viability and function of hematopoietic progenitor cells have been reported to be at 4 °C for 24 h [40]. In the case of MSCs, Muraki et al. [41] demonstrated that viability of MSCs in PBS was maintained above 80% but only for 24 h. However, there is no information about which would be the best packaging medium or storage conditions (time and temperature) to maintain the viability for clinical application. For the foreseeable future, cell therapy development will be based on not only in the search for new therapies but also in the study and design of these drugs, in terms of stability and safety, to expand the margins of use over time until administration.

In conclusion, the stability of a medicine is a function of storage conditions and chemical properties of the active ingredients. The conditions used in the stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Our stability studies in the final medical product were based on showing that characteristics of hMSCs remained unchanged until administration.

This is the first study to examine the stability and viability of hMSCs with different excipients for cell suspension packaging at various temperatures, but further studies will be needed to improve the stability of cells for developing a cell medicine.

Acknowledgments

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Declaration of interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Table(s)

Table 1. Composition (mL) of different packaging media (50 mL final volume).

Excipients	Lactated Ringer's solution	Glucose 5%	Albumin 20%	Sodium Chloride 0.9%
Medium 1	22.5	25	2.5	–
Medium 2	47.5	2.5	–	–
Medium 3	50	–	–	–
Medium 4	–	–	–	50

Figure(s)

Fig.1. hMSCs viability (%) packed in different media and stored at 8 °C, from 0 h to 60 h. Data represent mean ± SD.

Fig. 2. hMSCs viability (%) packed in medium 1 and stored at different temperatures, from 0 h to 60 h. Data represent mean ± SD.

Fig.3. Inmunophenotipic characterization of the hMSCs after being packed in medium 1 and stored at 4 ºC for 48 h. Both of the MSCs were CD13+, CD29+, CD90+, CD105+ and CD34−, CD45−, CD31−, HLA-II−.

Fig. 4. Cell culture differentiation of adipocytes (panel A) and osteoblasts (panel B) stored in medium 1 after storage at 4 ºC for 48 h. Bar length 200 µm.

Fig. 5. Karyotyping analysis of human MSCs. The image shows the result of G-banding karyotyping of a metaphase after to study stability of the finished medicinal product. The karyotype shows a female normal karyotype (46,XX) .

Fig. 6. Cell suspension rheogram. It shows the shear stress (Pa) (in red) and the viscosity (Pa·s) (in blue).

Fig. 7. Morphology of hMSCs for stability study at different storage temperatures and times. Panel A 0 ºC and 12 h; panel B 0 ºC and 24 h; panel C 4 ºC and 12 h; panel D 4 ºC and 24 h, panel E 8 ºC and 12 h, panel F 8 ºC and 24 h; panel G 25 ºC and 12 h; panel H 25 ºC and 24 h.

Fig. 8. Backscattering profiles of suspensions. The left side of the curve corresponds to the bottom of the vial, whereas the right side corresponds to the sample behaviour on the top.

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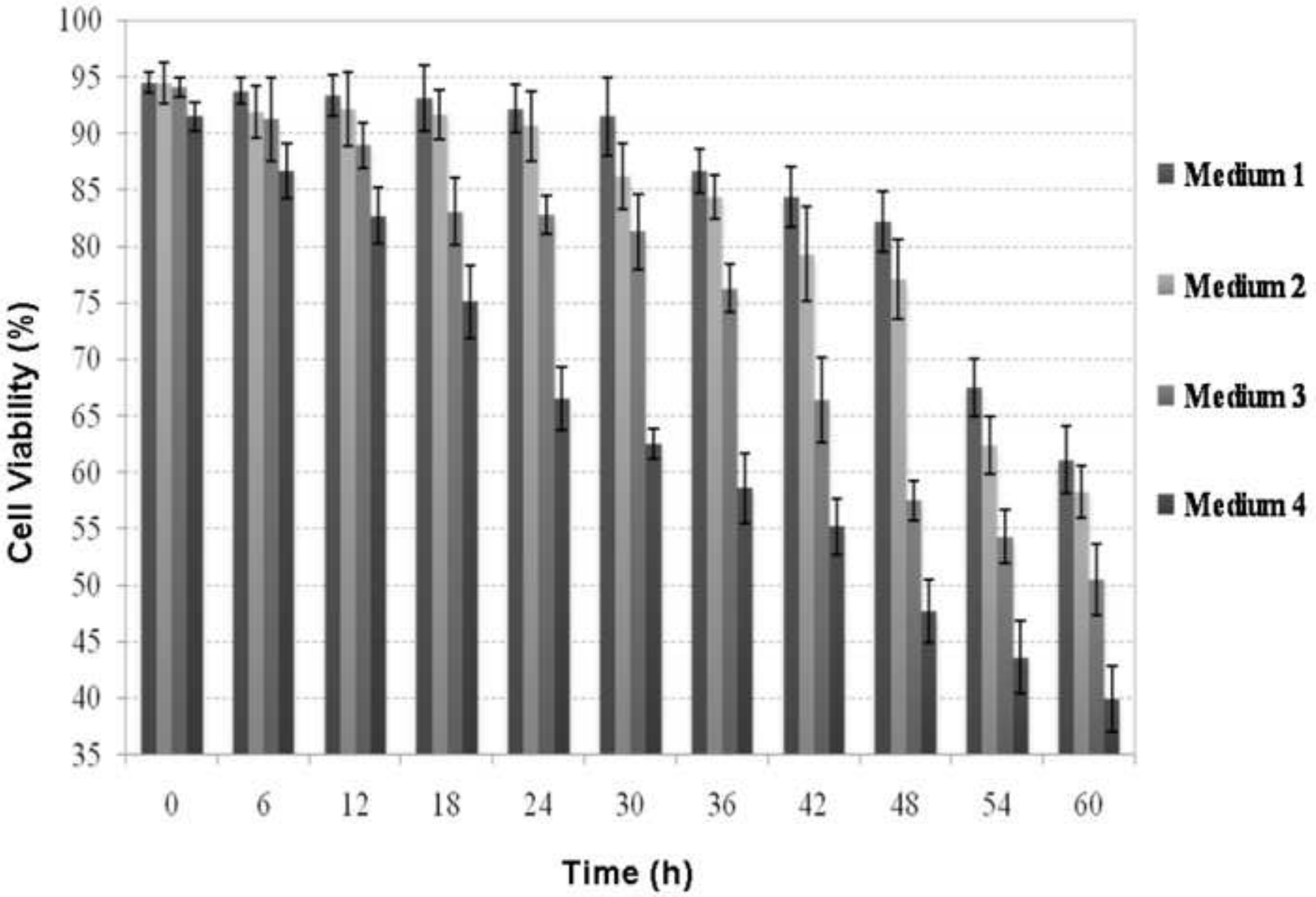

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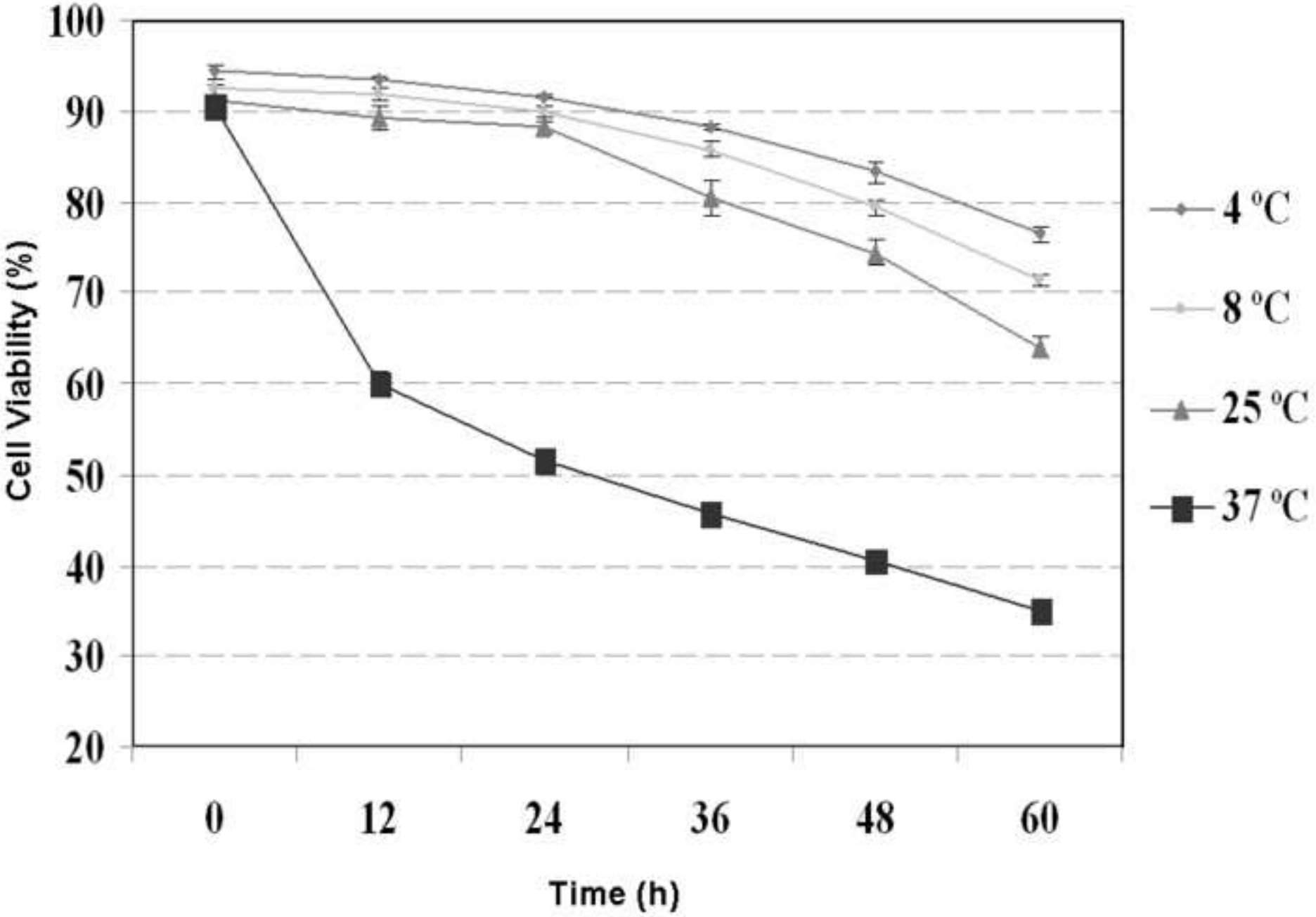

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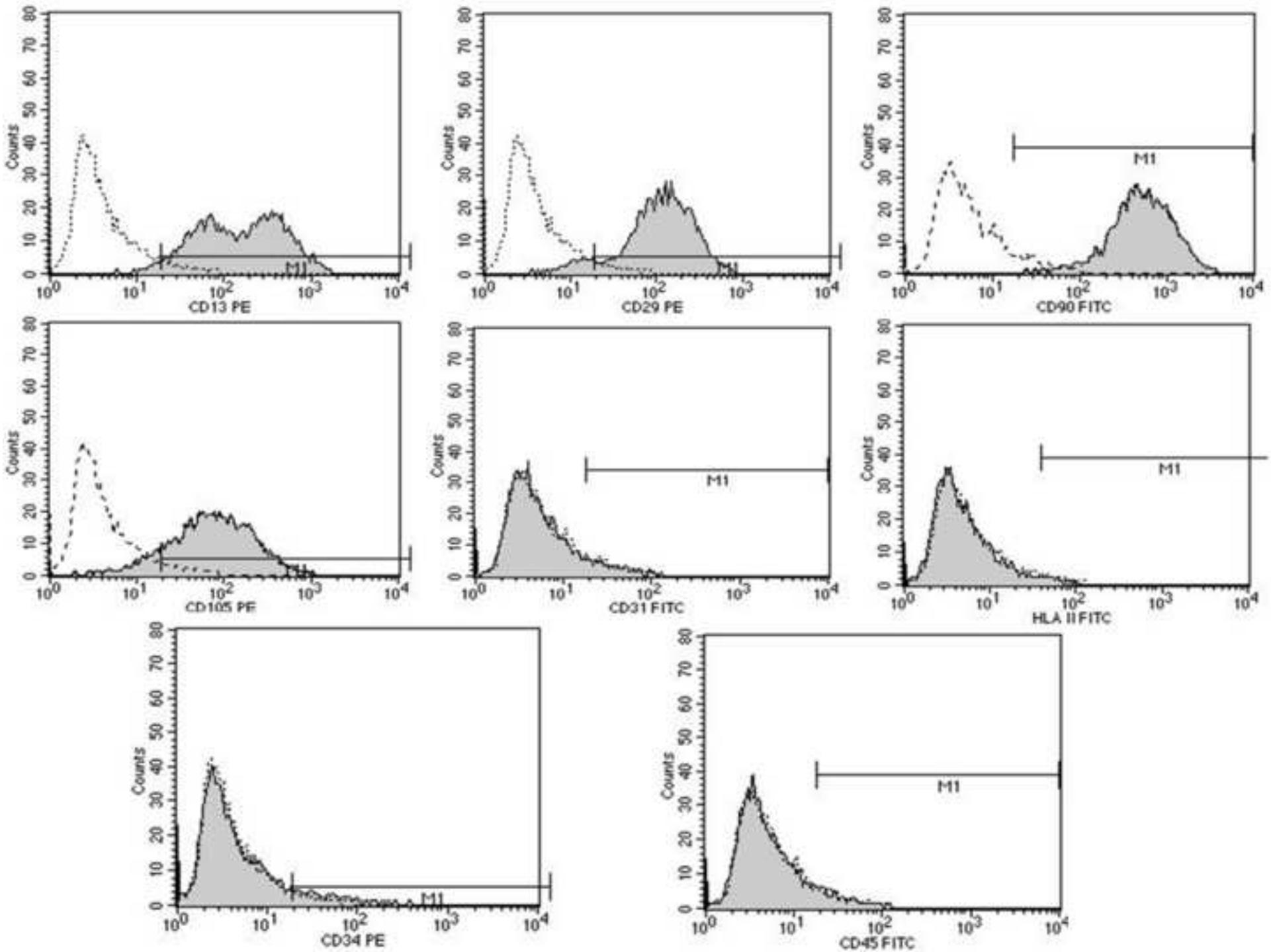

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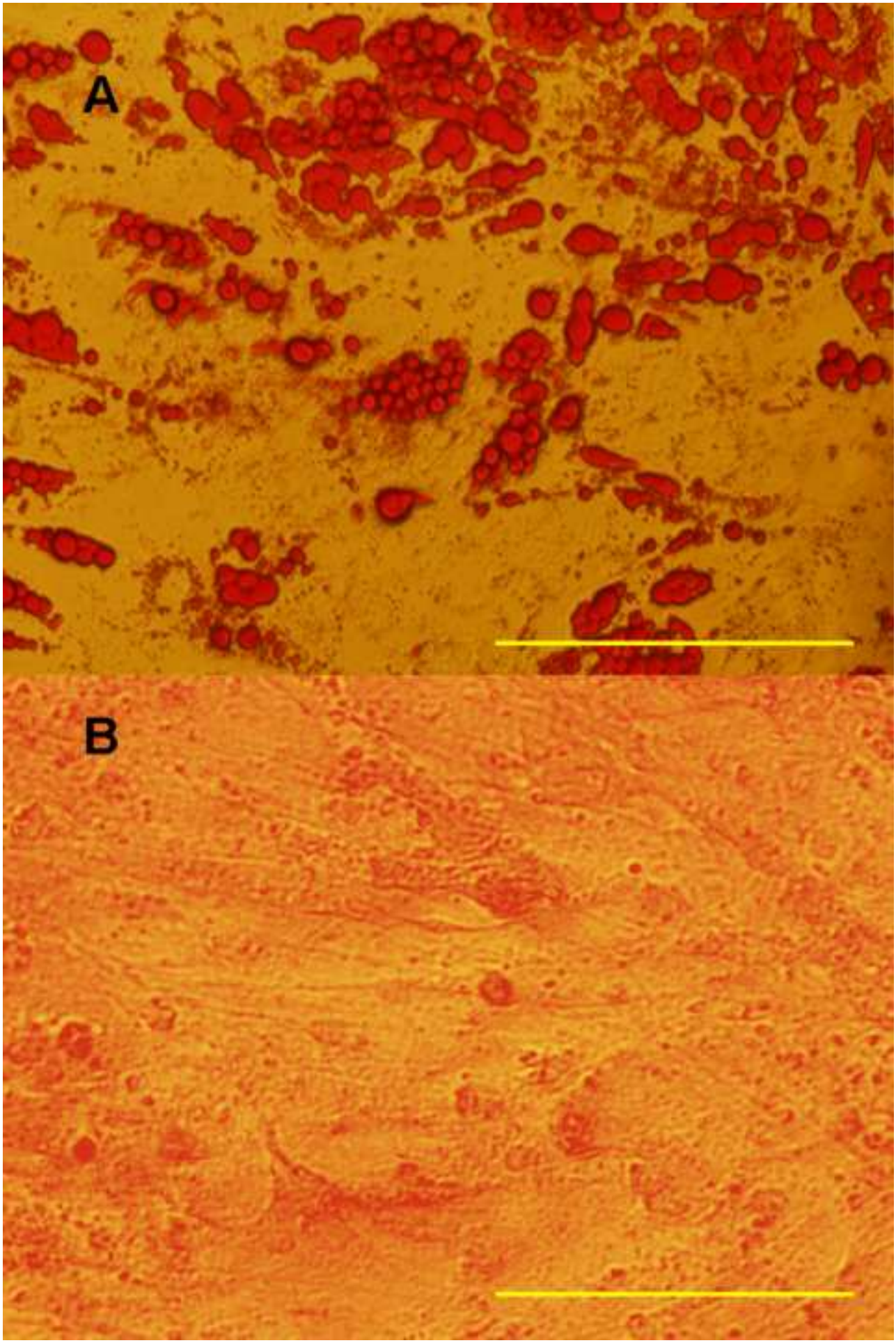

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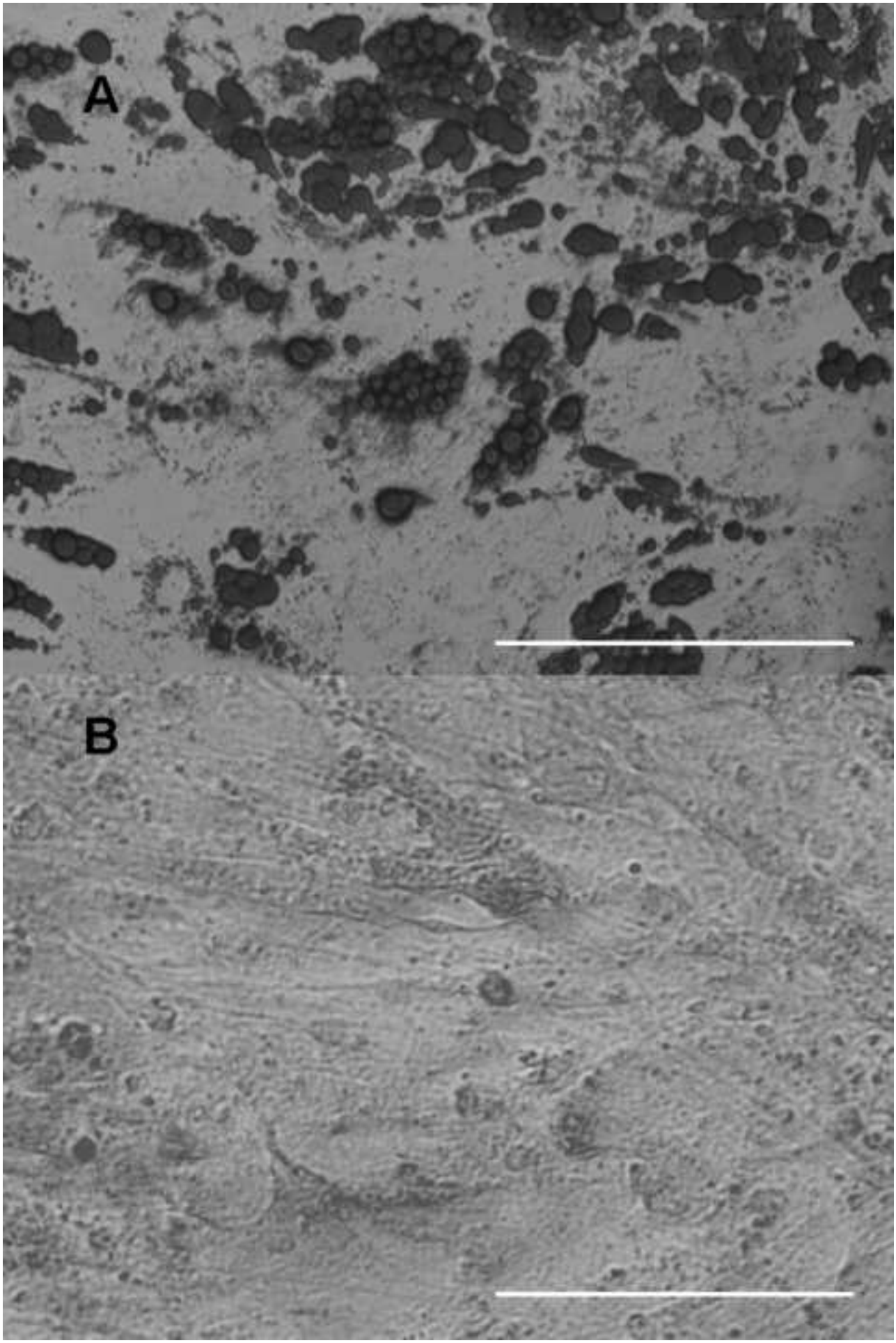

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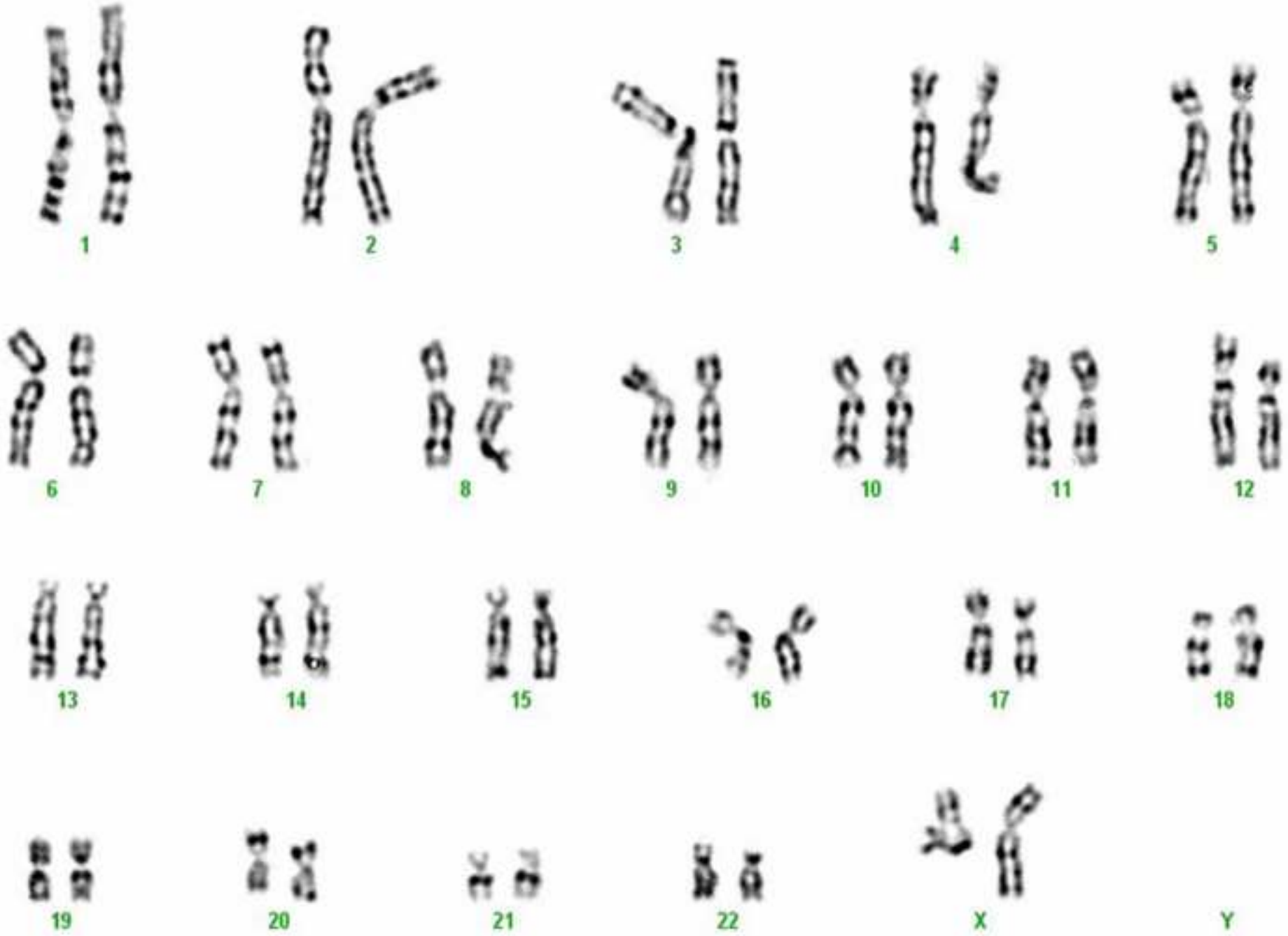

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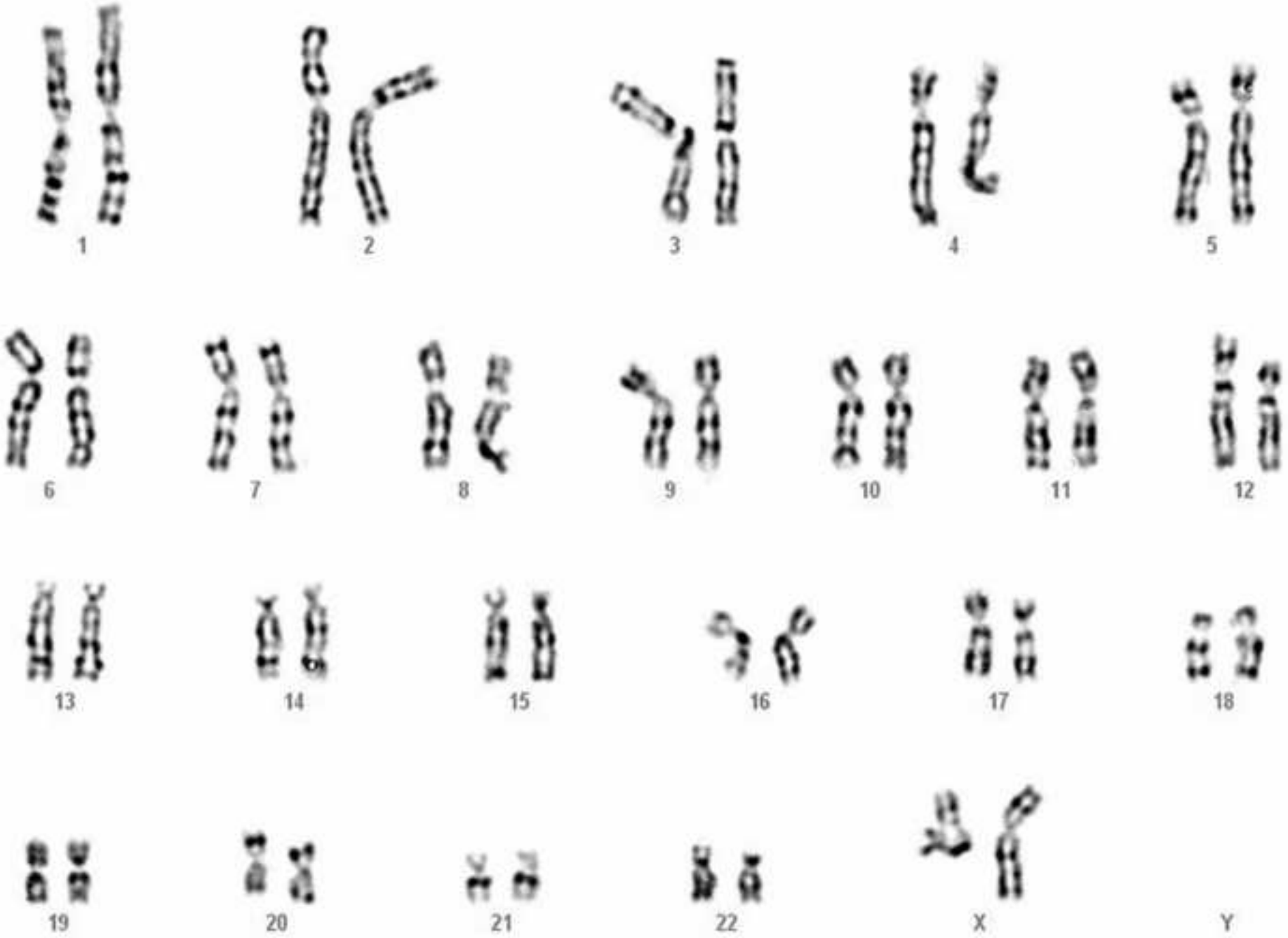

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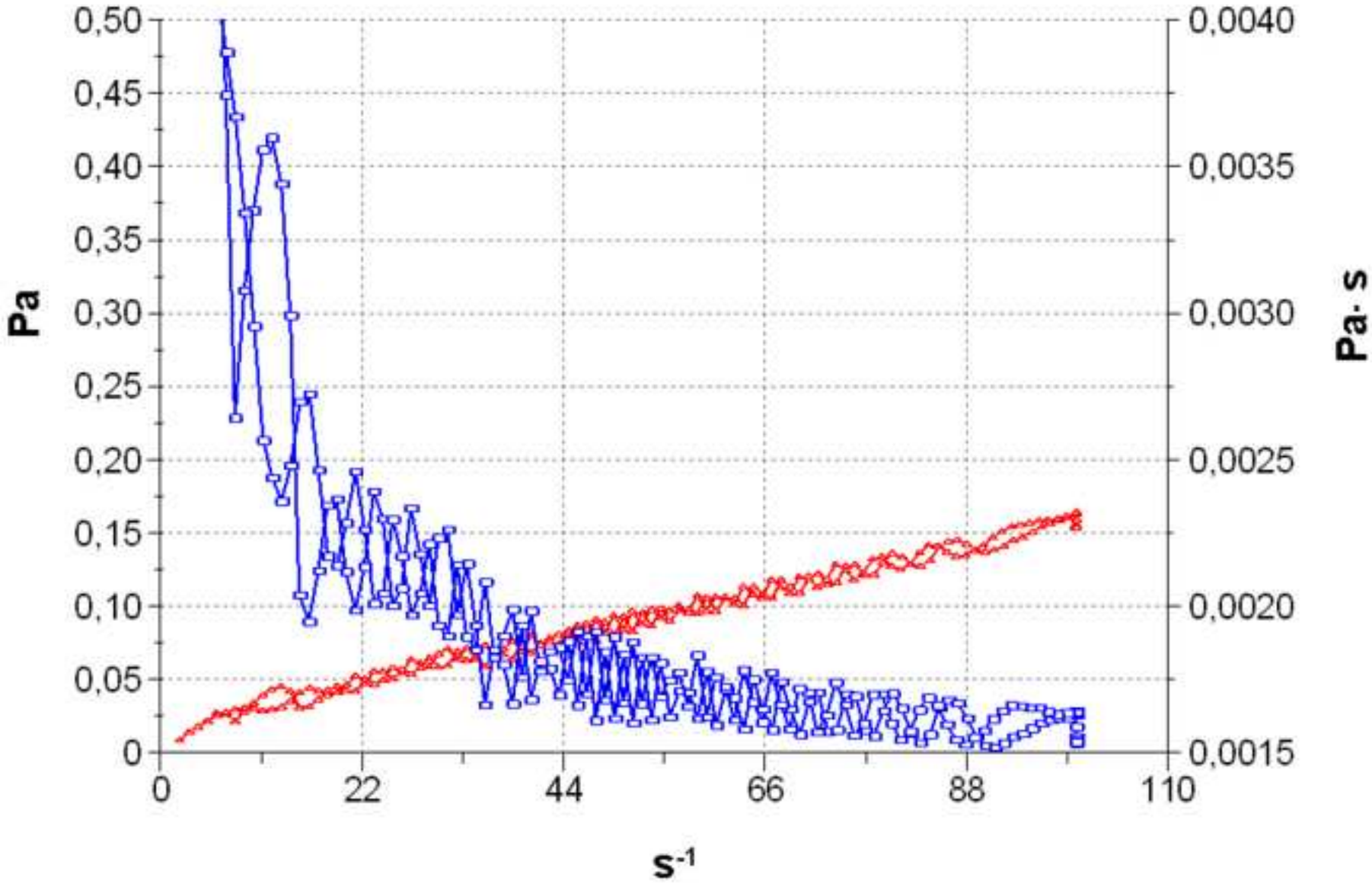

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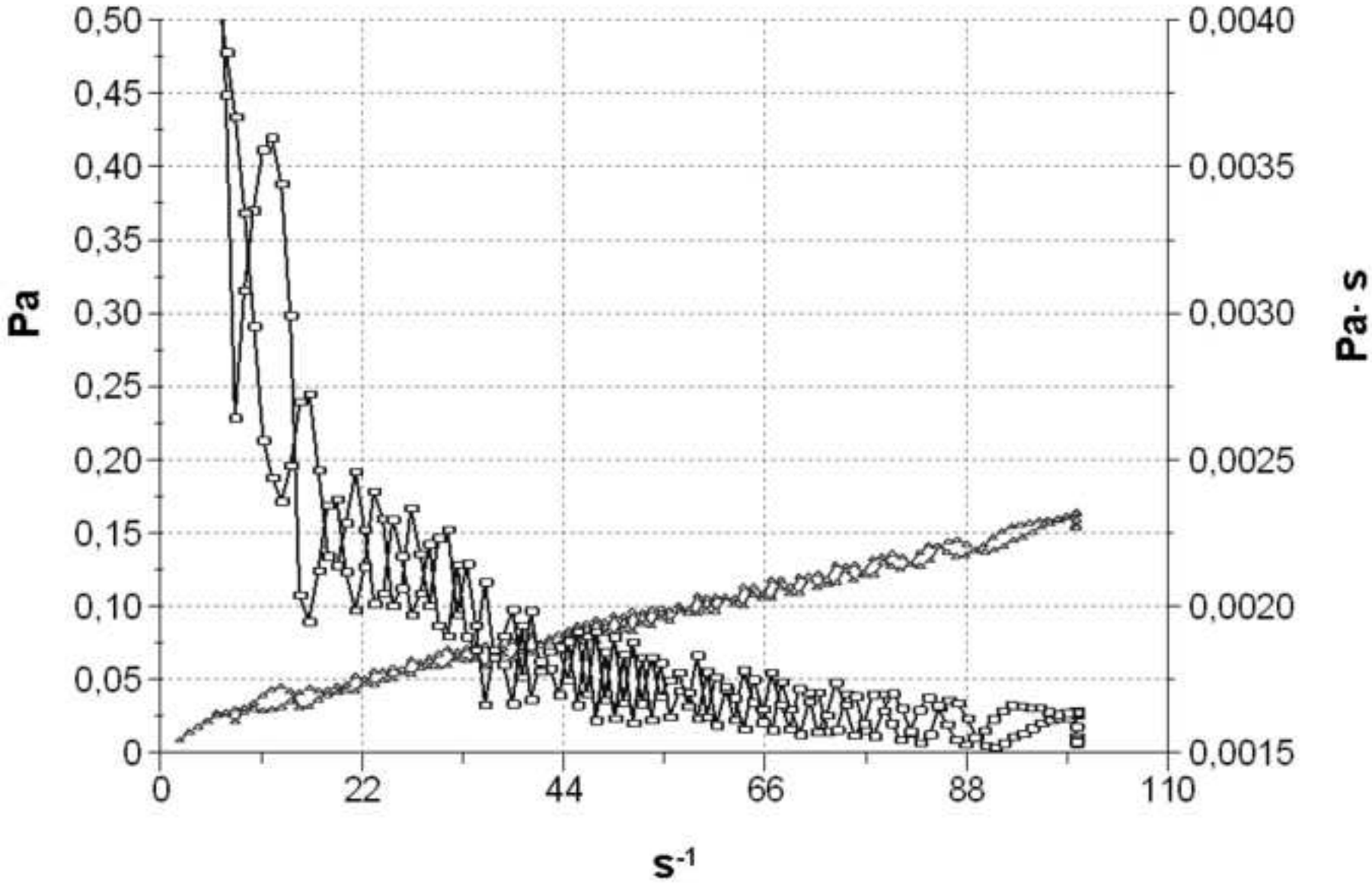

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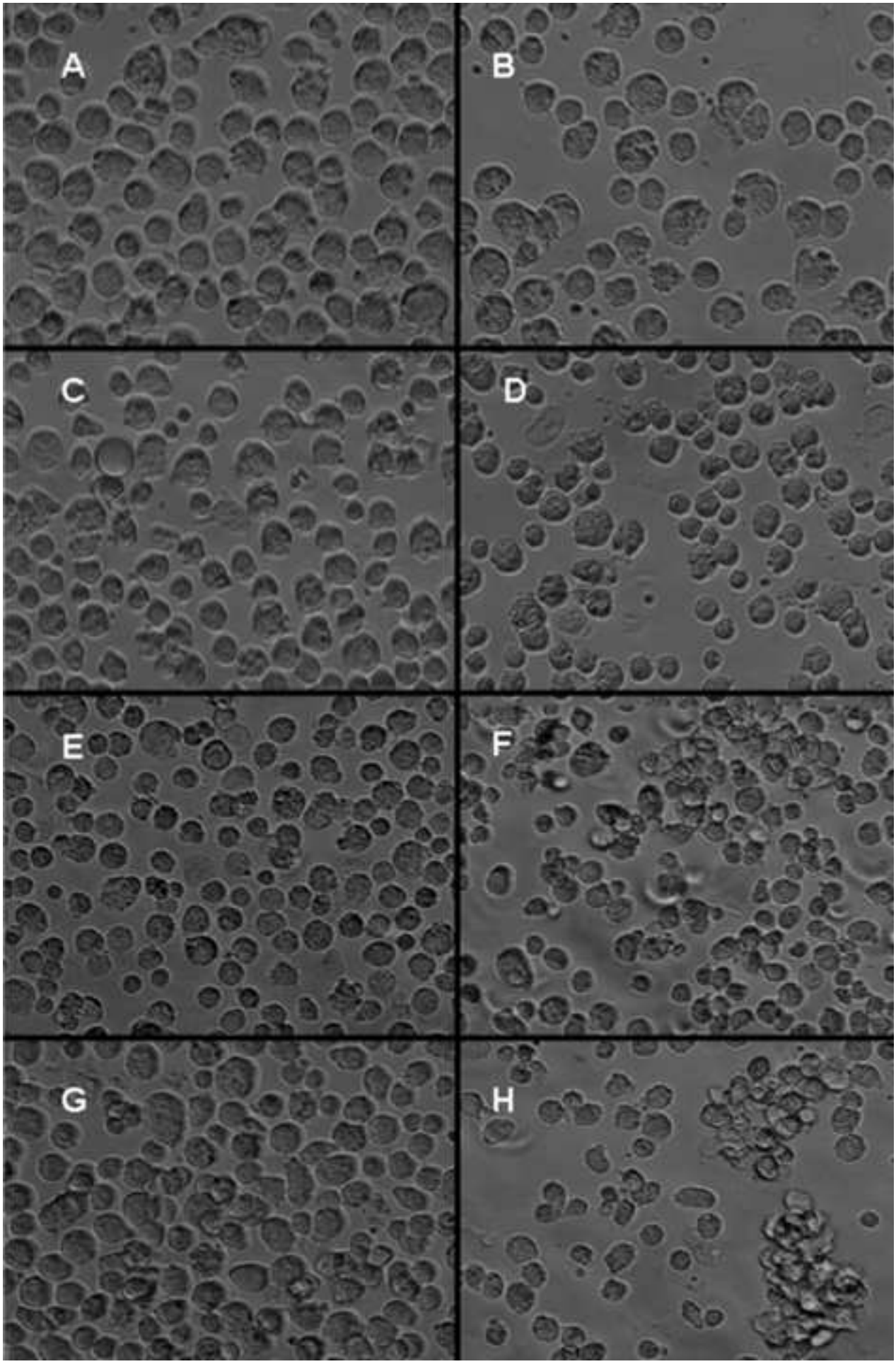

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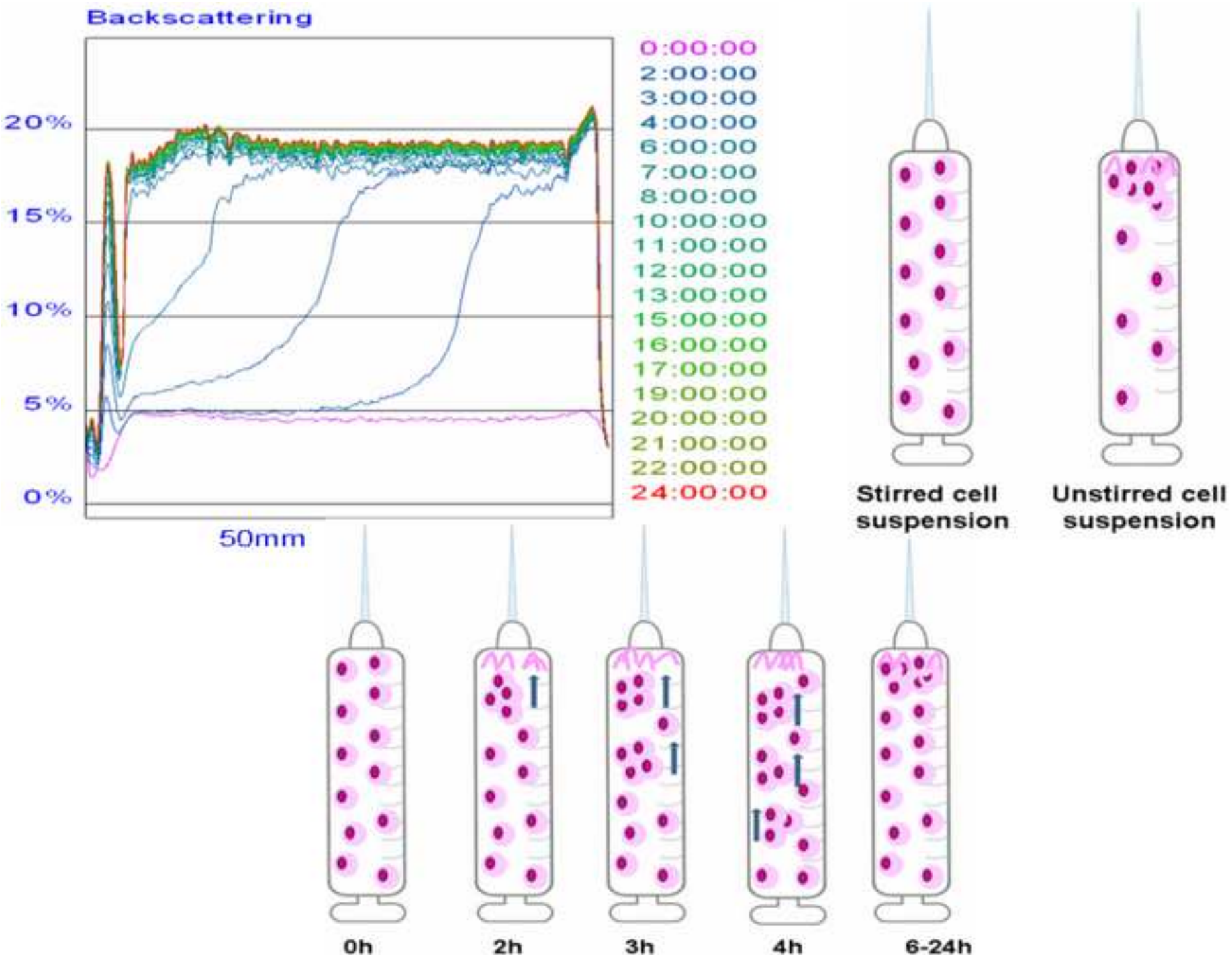

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