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Evaluation of human serum albumin as a substitute of foetal bovine serum for cell culture

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Abstract

Cell microencapsulation requires clinically approved materials for their use in pharmaceutical and/or biomedical applications. The overwhelming majority of the literature has used the classical alginate-poly-L-lysine-alginate (APA) capsules for cell immobilization. Although alginate is granted with the medical approval, some of the remaining components such as foetal bovine serum (FBS), an essential ingredient of cell culture media, are not in accordance with the guidelines affirmed by the American Society for Testing and Materials (ASTM) and Food and Drug Administration (FDA). In this paper, human serum albumin (HSA), a medically approved substance, was evaluated as a potential substitute of FBS. The effect of different percentages of FBS and HSA was studied on the proliferation rate, viability and protein production of two different cell lines (C2C12 and baby hamster kidney (BHK) cells), maintained in culture and immobilized in APA microcapsules. Results show that substitution of FBS by HSA reduced the functionality of both non-encapsulated and encapsulated BHK cells. However, immobilized C2C12 cells presented the highest level of viability and a reduction in protein production of 25% when 1% HSA was used. It can be concluded that HSA might be a possible substitute of FBS in order to maintain or transport encapsulated C2C12 cells for short periods of time before implantation.

Keywords: Encapsulation; Human serum albumin; Foetal bovine serum; C2C12 cells; BHK cells; Culture medium

1. Introduction

The aim of encapsulated cell technology (ECT) is to restore or improve native tissue function without immunosupression. Other therapeutic applications of the ECT are the development of artificial organs such as pancreas or liver, and the eradication of cancer. Although the potential of this technology is not totally developed, the efforts of the past years have made ECT become into a realistic alternative to the traditional therapies for the treatment of a wide variety of chronic diseases such as diabetes (Calafiore et al., 2004), renal and hepatic failure (Chang, 2004), cancer (Cirone et al., 2004) and hemophilia (Hortelano et al., 1999). Since the introduction of encapsulation concept in 1964 by T.M.S. Chang (Chang, 1964), a wide range of cell lines have been immobilized within these semipermeable and biocompatible devices. The membrane of the microcapsules permit the secretion of de novo produced therapeutic proteins and the elimination or unless the reduction of immunosuppressive drugs (Orive et al., 2003a).

The unique and attractive properties exerted by ECT have driven the attention of many scientists and biotechnology companies (Orive et al., 2002). However, ECT has found some obstacles including ethical considerations, technological and biological challenges and government regulations, assuming that this cell-based therapy must overcome the same quality controls required for conventional therapies. In effect, ECT has to standardize the manufacturing process so that the regulatory authorities would accept this cell-based approach (Orive et al., 2004).

One challenge of this technology is that all the components used in the elaboration and maintenance of cells and microcapsules must meet the safety criteria of the American Society for Testing and Materials (ASTM) and US Food and Drug Administration (FDA). Although alginate, the most frequently employed material in microcapsule elaboration, is granted for its use in pharmaceutical and/or biomedical application (Dornish et al., 2001), some of the remaining components are not in accordance with the guidelines of these regulatory

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institutions. One of these components is foetal bovine serum (FBS), an essential ingredient of cell culture media used for the maintenance of immobilized cells until being used in vivo. This is especially important in the case of multicentric clinical trials where the transportation of microcapsules among centers would be necessary. Therefore, a cell culture medium supplemented with medically approved ingredients would be required for these short periods of time such as in vivo pre-implantation and during the delivery of microcapsules from manufacturing place to the clinical centers. In 2003, the Committee for Proprietary Medical Products integrated in the European Agency for the Evaluation of Medical Products formulated a note for guidance that established the general principles which should be applied to the control of the quality and safety of bovine serum used during the manufacturing of human biological medical products including biotechnological products. Although using FBS is allowed, this committee recommends the replacement of FBS with non-animal origin material or the reduction of its use (<http://www.emea.eu.int/pdfs/human/bwp/179302en.pdf>). Thus, several products free of animal components have been used for cell cultivation. However, none of these products have been utilized for encapsulated cell maintenance. Furthermore, since these non-animal origin products are not present in human body, the use of these products for capsule incubation during the pre-implantation period could be a reason for transplant rejection.

In this work, human serum albumin (HSA), a substance with the medical approval of the FDA and also present in the human body, was evaluated as a potential substitute of FBS in the culture medium. Previous reports have addressed that HSA significantly enhances cell survival and insulin secretion of encapsulated rat islets over a time period of 3 weeks (Schneider et al., 2003). However, little research has involved the study of FBS replacement by HSA on stable and genetically modified cells frequently employed in somatic gene therapy. To address this issue, the effect of different percentages of FBS (10%, 1% and 0%) or the replacement of FBS by HSA (1%) on the proliferation rate, viability and protein production was studied for two different genetically modified cell lines, C2C12 myoblast cells and baby hamster kidney (BHK) fibroblast cells. Firstly, the influence of culture medium composition on these functional parameters was assayed for non-encapsulated cells. Finally, both cell lines were immobilized in alginate-poly-L-lysine-alginate (APA) microcapsules, and the effect of the different culture media on the viability and therapeutic protein production at different post-encapsulation periods was evaluated.

2. Materials and methods

2.1. Cells and materials

C2C12 myoblasts genetically engineered to secrete erythropoietin (EPO) were kindly provided by the Laboratoire D'etude de la Neurodegenerescence of Institut des Neurosciences (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland). Baby hamster kidney fibroblasts genetically engineered to produce brain-derived neurotrophic factor (BDNF) were kindly provided by the Department of Cellular Biology and Pathological Anatomy of the Universidad Autónoma de Barcelona (IDIBAPS, Barcelona, Spain). Firstly, both cell lines were cultured under standard conditions, maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere and passed every 2–3 days. C2C12 cells were grown in Dulbecco's modified eagle medium (DMEM) with high glucose (4500 mg/L) supplemented with 10% FBS and 1% penicillin/streptomycin. For cultivation of BHK cells, a DMEM with high glucose (4500 mg/L) supplemented with 2 mM L-glutamine, 1 mM sodium-pyruvate, 240 mg/L geneticin, 10% FBS and 1% penicillin/streptomycin was used. The proliferation, viability and production assays were undertaken maintaining both cell lines in different culture media. These culture media were prepared by adding to DMEM different percentages of foetal bovine serum (FBS; 0%, 1% and 10%) or replacing FBS by 1% human serum albumin. The remaining components of the culture medium were maintained unaltered for both myoblasts and fibroblasts. All the components of culture media were purchased from Gibco BRL/Invitrogen Life Technologies (Barcelona, Spain), except HSA that was obtained from Sigma–Aldrich (Madrid, Spain).

Low viscosity and high guluronic (LVG) alginate (250 cps at 2% solution and 25 °C) was purchased from NovaMatrix/FMC Corporation (Oslo, Norway); poly-L-lysine (PLL; hydrobromide MW: 15,000–30,000) and the remaining chemicals were obtained from Sigma–Aldrich.

2.2. Microcapsule elaboration

Microcapsules were prepared at room temperature and under sterile conditions using an electrostatic droplet generator. A 2% (w/v) solution of low viscosity and high guluronic alginate containing each of the two cell lines was extruded into 0.05 M solution of $CaCl_2$. Two cell lines were immobilized: C2C12 myoblast cells and BHK fibroblast cells, the former was encapsulated at a density of 2×10^6 cells/mL and the latter, at a density of 4×10^6 cells/mL. The alginate droplets were coated with PLL 0.05% for 5 min. Subsequently, a second coat with alginate LVG 0.1% for 5 min was applied. For each cell line one batch of cell-loaded microcapsules was prepared and divided into four parts in order to maintain the same capsules in the different culture media (FBS: 10%, 1% and 0%, and HSA: 1%). During the period of the assays, immobilized cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and the medium was replaced every 2–3 days.

2.3. Proliferation assay

A 96-well plate was used for the proliferation assay. Firstly, wells were seeded with myoblast or fibroblast cells (5000 cells were placed into each well), and then the different culture media were added. The plate was maintained at 37 °C in a 5% CO_2 humidified atmosphere during 3 days. After the incubation period, the cells from each well were harvested using trypsin-EDTA (Gibco BRL/Invitrogen Life Technologies, Barcelona, Spain) and then counted using a hemacytome-

ter. Results are expressed as mean (cells/mL) ± S.D. for three replicates.

2.4. Viability measurement

The tetrazolium assay (MTT assay) of Uludag and Sefton (1990) was applied to determine the metabolic activity of the encapsulated cells after 3 and 10 days of incubation in the different culture media. For non-encapsulated cells, the MTT assay was carried out after the 3-days incubation period. Results are expressed as mean ± S.D. for four replicates.

2.5. Production of the therapeutic proteins

The influence of cell culture medium composition over therapeutic protein production for non-encapsulated and encapsulated cells was studied. The two cell lines used in this work were C2C12 myoblast cells and BHK fibroblast cells, which produced EPO and BDNF, respectively.

In the case of non-encapsulated cells, after the 3-days incubation period in the different culture media, supernatants from each well were removed to measure the protein level. On the other hand, for immobilized cells protein production was measured after 3 and 10 days of incubation in the different culture media. A 24-well plate was used to evaluate EPO and BDNF production by encapsulated cells. Briefly, 150–200 microcapsules incubated in the different media (10% FBS, 1% FBS, 0% FBS and 1% HSA) were placed per well. After 24 h of incubation (37 °C in a 5% CO_2 humidified atmosphere) supernatants were removed and protein level was measured. EPO concentration was determined using an enzyme linked immunosorbent assay (ELISA) kit (Quantikine® IVD®, R&D Systems, Minneapolis, MN). BDNF concentration was calculated applying E_{max}® ImmunoAssay System (Promega Biotech Ibérica, SL, Barcelona, Spain). Results were expressed as mean ± S.D. for four replicates.

2.6. Microscopic observation

The morphology of C2C12 and BHK cells was observed using an inverted optical microscope (Nikon TMS) equipped with a camera (Sony CCD-Iris). The images showed for non-encapsulated cells correspond to day 3 of incubation, and the images for encapsulated cells, to day 10 of incubation.

2.7. Statistical analysis

Comparisons of cell proliferation, cell viability and protein production between 10% FBS DMEM and the remaining culture media were analyzed by the Student's *t*-test or the Mann–Whitney test according to the result of the Levene test of homogeneity of variances. When more than two groups were compared one-way ANOVA followed by Scheffé or Tamhane post hoc test depending on the homogeneity of variances was applied. SPSS 11.0 computer program (SPSS, Inc., Chicago, IL) was used for the statistical analysis. Significance was set at $P < 0.05$ for all analyses.

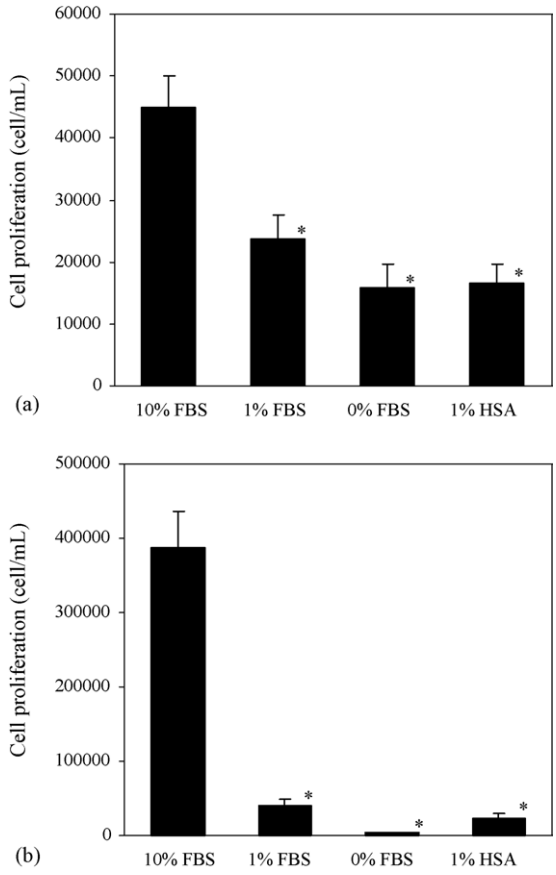

Fig. 1. Proliferation rate of (a) C2C12 cells and (b) BHK cells, after 3 days of culture with different culture medium compositions (FBS: 10%, 1% and 0%, and HSA: 1%). Results are expressed as mean ± standard deviation for three replicates ($^{*}P < 0.05$ vs. 10% FBS).

3. Results

3.1. Effect of culture medium composition over non-encapsulated cells

3.1.1. Cell proliferation assay

The proliferation rate of non-encapsulated C2C12 and BHK cells was evaluated after an incubation period of 3 days in the different culture media (10% FBS, 1% FBS, 0% FBS and 1% HSA). In the case of C2C12 cells, the proliferation rate declined nearly 50% ($P < 0.05$) when the classical culture medium (10% FBS) was changed by 1% FBS DMEM (Fig. 1a). However, for BHK cells, this proliferation reduction reached approximately 90% ($P < 0.05$) (Fig. 1b). The substitution of 10% FBS by 1% HSA reduced by more than 50% ($P < 0.05$) the C2C12 cell proliferation. On the contrary, BHK cell proliferation in the presence of 1% HSA decreased by more than 90% ($P < 0.05$). These results showed that BHK cell proliferation was more dependable on the presence of FBS in the culture medium than C2C12 cell proliferation.

3.1.2. Viability measurement

The viability of C2C12 and BHK cells after 3 days of incubation in the four different media (10% FBS, 1% FBS, 0% FBS

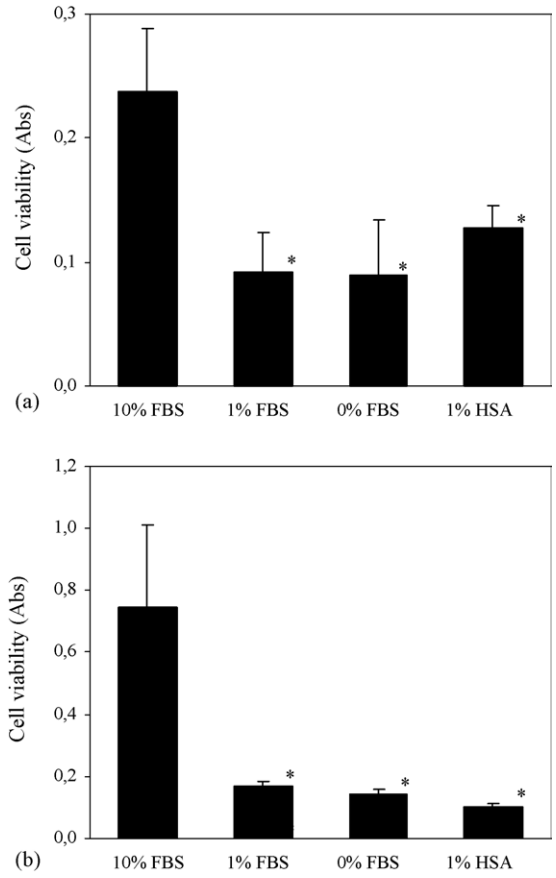

Fig. 2. Metabolic activity of (a) C2C12 cells and (b) BHK cells, after the 3-days incubation period in the different media (FBS: 10%, 1% and 0%, and HSA: 1%) using the MTT assay. Results are expressed as mean ± standard deviation for four replicates ($^{*}P < 0.05$ vs. 10% FBS; Abs: absorbance).

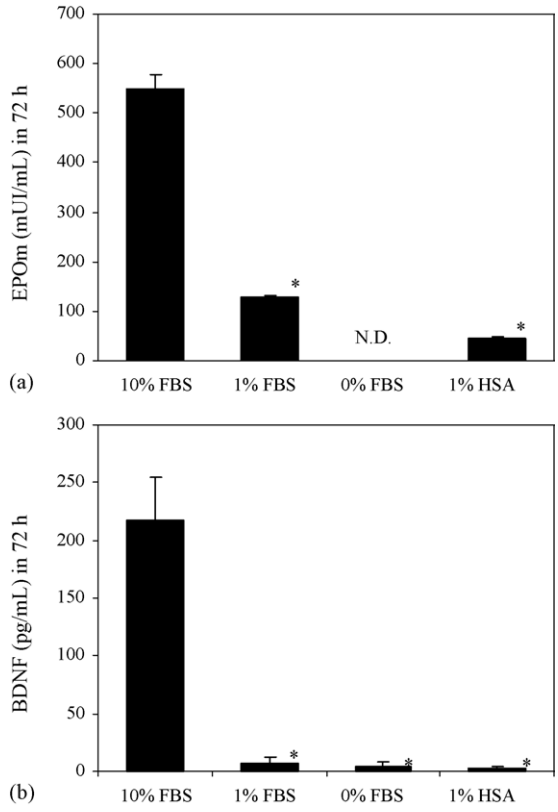

Fig. 3. Concentration of the therapeutic protein released from (a) C2C12 cells and (b) BHK cells, after 3 days of culture with the different media (FBS: 10%, 1% and 0%, and HSA: 1%). Results are expressed as mean ± standard deviation for four replicates ($^{*}P < 0.05$ vs. 10% FBS; N.D.: not detectable).

and 1% HSA) was determined using the MTT assay. The viability of C2C12 cells declined by nearly 50% ($P < 0.05$) when the classical cultured medium was changed by 1% FBS and 0% FBS DMEM. This reduction reached approximately 80% ($P < 0.05$) when BHK cells were studied. On the other hand, when 1% HSA DMEM was used instead of 10% FBS DMEM, the viability of C2C12 cells decreased by no more than 50% ($P < 0.05$) while the BHK cell viability was reduced by at least 80% ($P < 0.05$). These viability results confirmed the highest dependency of BHK cell growth on the presence of 10% FBS in the culture medium, whereas C2C12 cell growth appeared to be less dependable on the use of completed culture medium as viability and proliferation results showed (Fig. 2(a and b)).

3.1.3. EPO and BDNF production

The same plate seeded with C2C12 and BHK cells to evaluate the influence of culture medium over cell viability was used to measure the protein production after the 3-days incubation period. Before carrying out the cell viability assay, the supernatants were removed from all the wells to measure EPO and BDNF levels applying the appropriate assay.

In the case of C2C12 cells, EPO production decreased severely when 10% FBS DMEM was changed by the other culture media. This reduction was more important than the reduction in cell viability. EPO production was decreased by approximately 80% ($P < 0.05$) when 1% FBS DMEM was used instead of serum-complete medium. On the other hand, a reduction higher than 90% ($P < 0.05$) was obtained when cells were maintained in 1% HSA DMEM, and an undetectable level of EPO was achieved from cells cultivated in 0% FBS DMEM. These EPO production results differed in some manner from the viability results. Thus, C2C12 cell viability in 1% HSA DMEM decreased by less than 50% ($P < 0.05$), while EPO production was reduced by at least 90% ($P < 0.05$) (Fig. 3a).

When BHK cells were studied, BDNF production was importantly reduced (more than 95%) with the change of 10% FBS medium by the other culture media. Furthermore, the level of BDNF in the supernatants removed from cells cultivated in 1% HSA was very close to the detection limit of the ELISA assay. This production profile was similar to the one observed in the viability assay for BHK cells (Fig. 3b).

3.1.4. Growth profile of C2C12 and BHK cells

The results obtained in the proliferation and viability assays were checked by the growth profiles observed in the images taken after the 3-days incubation period in the four different media composition (10% FBS, 1% FBS, 0% FBS and 1% HAS) (Fig. 4(a–h)). For both cell lines, the best growth profile was observed in the cells cultivated with 10% FBS DMEM, and also in both cases the cell growth slowed and nearly stopped when

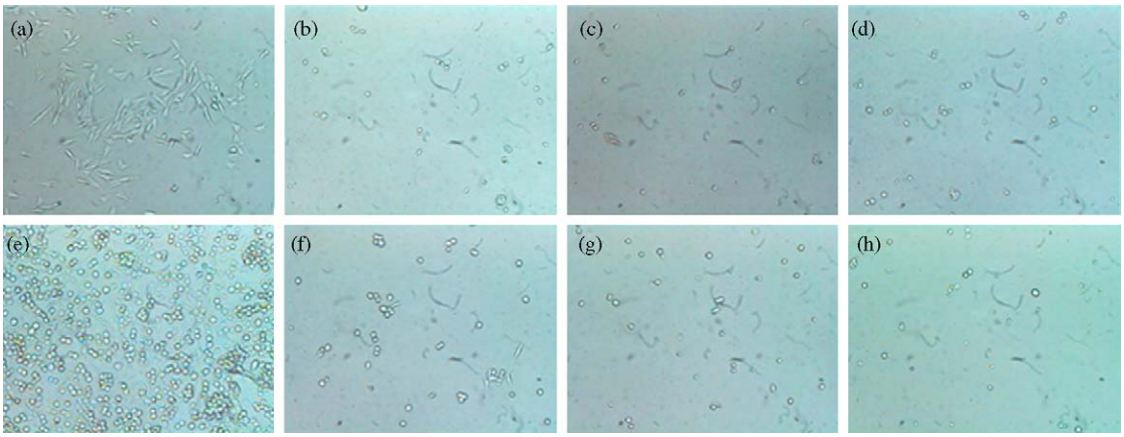

Fig. 4. Morphological evaluation of C2C12 cells (a–d) and BHK cells (e–h), after the 3-days incubation period in the different culture medium compositions: 10% FBS (a and e), 1% FBS (b and f), 0% FBS (c and g) and 1% HSA (d and h). Photographs were taken at a magnification of 20× with an inverted optical microscope equipped with a camera.

the classical cultured medium was changed by the other media. However, this cell growth reduction was more important for BHK cells than for C2C12 cells. This difference was supported by the proliferation and viability results. Regardless to incubation of cells in 1% HSA DMEM, C2C12 cells presented a better growth profile in this medium than BHK cells.

3.2. Effect of culture medium composition over encapsulated cells

3.2.1. Cell viability assay

The viability of immobilized cells was evaluated after 3 and 10 days of incubation in the different culture media using the MTT assay.

In general, the viability results obtained for encapsulated cells differed in some manner from those reached for non-encapsulated cells (Fig. 5(a and b)). In the case of immobilized C2C12 cells, the highest level of viability at both time points checked was found in cells maintained in 1% HSA DMEM, while non-encapsulated cells presented the best viability in 10% FBS DMEM ($P < 0.05$). Moreover, no differences were found in C2C12 cell viability level between day 3 and day 10 for all culture media.

On the contrary, this behavior was not observed for encapsulated BHK cells, presenting a similar pattern to the one observed for non-encapsulated cells. Thus, a progressively decrease in the cell viability was obtained from 10% FBS DMEM to 1% HSA DMEM, being the viability in 0% FBS and 1% HSA very similar (no statistically significant; $P < 0.05$). Statistical differences ($P < 0.05$) were found in the level of BHK cell viability obtained at day 3 and day 10 between completed medium and the rest culture media tested.

3.2.2. Production of the therapeutic proteins

The therapeutic protein production from encapsulated cells was measured after 3 and 10 days of incubation in the different media. In the case of myoblast cells, EPO concentration after 3 days of incubation declined by 25% ($P < 0.05$) when the classical culture medium was changed by 1% HSA. However, at day 10 of incubation EPO concentration was reduced by more than 90% ($P < 0.05$). Furthermore, no level of EPO was detected after 3 and 10 days of culture in 1% and 0% FBS (Fig. 6a).

On the other hand, the concentration of BDNF released from encapsulated BHK cells decreased progressively from 10% FBS

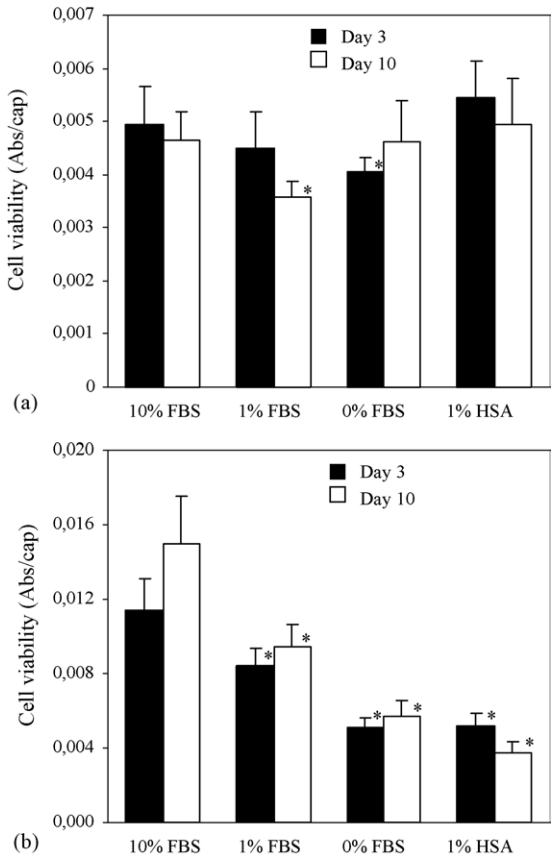

Fig. 5. Metabolic activity of (a) C2C12 cells and (b) BHK cells, both cell lines immobilized in APA microcapsules at a cell density of 2×10^6 cell/mL and 4×10^6 cell/mL, respectively, after 3 and 10 days of culture with the different media (FBS: 10%, 1% and 0%, and HSA: 1%) using the MTT assay. Results are expressed as mean ± standard deviation for four replicates ($^*P < 0.05$ vs. 10% FBS at day 3 and day 10; Abs: absorbance; cap: capsule).

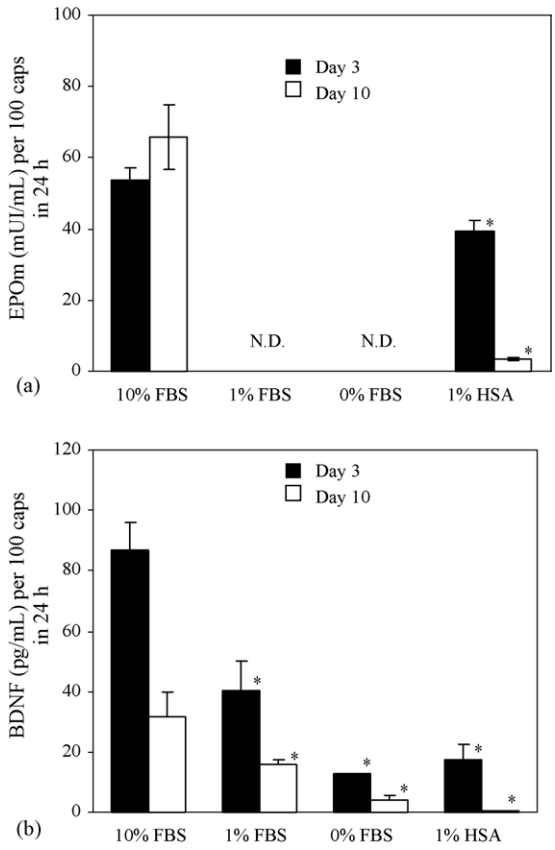

Fig. 6. Therapeutic protein production from encapsulated (a) C2C12 cells and (b) BHK cells, after an incubation period of 3 and 10 days in the different culture media (FBS: 10%, 1% and 0%, and HSA: 1%). Results are expressed as mean ± standard deviation for four replicates ($^{*}P < 0.05$ vs. 10% FBS at day 3 and day 10; N.D.: not detectable; cap: capsule).

to 1% HSA at both time points checked. This behavior was similar to the one observed for the viability of encapsulated BHK cells (Fig. 6b).

3.2.3. Morphological appearance

The growth profile of encapsulated cells was followed along the incubation period. Firstly, no signs of cellular lysis or necrosis were observed from the beginning until the last day of incubation (the images showed correspond to day 10 of incubation, Fig. 7(a–h). The numerical results obtained in the cell viability and protein production assays were completely supported by the growth profiles observed in the images. For both cell lines, the best growth profile was obtained in 10% FBS DMEM. In addition, it was shown the dependency of BHK cell growth on the presence of FBS in the culture medium. That dependency is less remarkable for C2C12 cells.

4. Discussion

The final clinical success of encapsulated cell technology requires the optimization of the cells or tissues to be immobilized and the use of clinically proven materials for microcapsule elaboration (Orive et al., 2003b). One of the most common devices for cell immobilization has been the well-known APA microcapsule in which alginate is the main biomaterial. Although the use of alginate for biomedical applications is approved, some of the remaining materials necessary for microcapsule preparation have not been granted with the medical approval. One of these materials is FBS, a common substance in culture medium composition.

In this work, the use of HSA as a potential substitute of FBS was evaluated since HSA is an agent for which the medical approval is granted. Revising the scientific literature, HSA has been applied for microcapsule membrane formation using a transacylation reaction between ester groups of propylene glycol alginate and amino groups of HSA (Levy and Edwards-Levy, 1996). A modified procedure consists in addition of HSA both to the initial solution of alginate and propylene glycol alginate and to the calcium chloride solution was applied to microencapsulate hepatocytes (Joly et al., 1997). In addition, HSA has already been tested with other encapsulated cells including islets of Langerhans (Schneider et al., 2003) and 293 cells (Shinya et al., 1999). Results obtained in these studies showed that HSA-stabilized alginate capsules were a suitable immobilization device for the mentioned cell lines. In the study of pancreatic islets mentioned above, rat islets were immobilized in HSA-

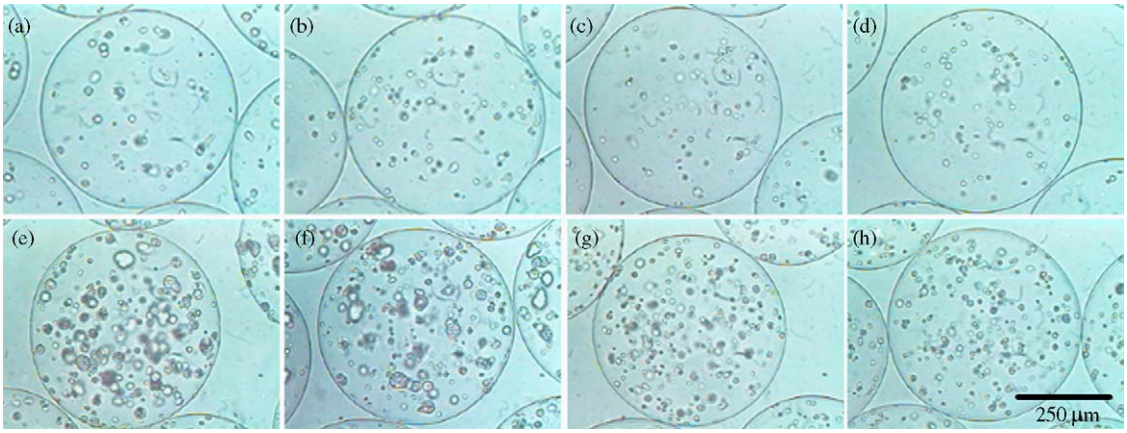

Fig. 7. Growth profile of C2C12 cells (a–d) and BHK cells (e–h) immobilized in APA microcapsules at a cell density of 2×10^6 cell/mL and 4×10^6 cell/mL, respectively. The photographs correspond to day 10 of incubation in the different media (FBS: 10%, 1% and 0%, and HSA: 1%) and were taken at a magnification of 20× with an inverted optical microscope equipped with a camera.

stabilized Ba-alginate microcapsules and maintained in medium supplemented with a concentration of HSA matched to the one inside the microcapsules. The results of this study showed that 1% HSA is sufficient to enhance stability, viability and insulin secretion of encapsulated islets.

In the first part of our study, the effect of different percentages of FBS or the replacement of FBS by HSA on the functionality of two different cell lines was evaluated. The results obtained in the proliferation rate, viability and protein production assays showed the higher dependency of BHK cells on the presence of FBS in the culture medium. In the majority of the literature, the effect of HSA is analyzed in immobilization devices, but not over non-encapsulated cells. However, one work evaluated the effect of HSA on cholesterol efflux from cultured endothelial cells (Ha et al., 2003) and the results showed that HSA promoted cholesterol efflux in a dose-dependent manner reaching a plateau at 10 mg/mL (1 g/100 mL or 1%) which was the percentage of HSA evaluated in our study. This percentage was also selected considering a study that analyzed the beneficial effects of HSA on the functionality of immobilized islets and found that 1% of HSA is enough to improve long-term stability of the device under in vitro conditions (Schneider et al., 2003). Furthermore, this concentration of HSA is in the same order of magnitude of serum proteins, so that physiological conditions were simulated.

In the second part of this work, the effect of culture medium composition was studied on the functional properties of two immobilized cell lines. Encapsulated C2C12 cells in 1% HSA presented a similar viability level to the one observed for cells maintained in 10% FBS at day 3 and day 10 of incubation. In contrast, immobilized BHK cells showed a similar viability pattern to the one presented by non-encapsulated cells. Furthermore, at day 3 of incubation in 1% HAS, EPO production from myoblast cells was only reduced by 25%, suggesting that HSA might be used for maintenance of immobilized C2C12 cells before implantation or for transportation of the microencapsulated cells. Oppositely, BDNF secretion from fibroblast cells decreased in more than 75%. However, both protein productions were importantly reduced after 10 days post-incubation. These results suggest that the possible use of HSA for encapsulated C2C12 cell maintenance should be limited for short periods of time such as pre-implantation incubation or multicentric transportation. Obviously, a final substitution of FBS by HSA cannot be generally applied before evaluation of more cell lines and their in vivo testing were performed.

Acknowledgements

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