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PolyHEMA and polyHEMA-poly(MMA-co-AA) as substrates for culturing Vero cells

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Poly (2-hydroxyethyl methacrylate), polyHEMA, is known to prevent cellular attachment and spreading. This hydrogel is used to culture cells not dependent on anchorage. Blending polyHEMA with a copolymer of methyl methacrylate and acrylic acid introduces negative charges to the hydrogel and improves its mechanical characteristics. PolyHEMA and the blend were tested for attachment and proliferation of Vero cells. Dense and porous samples of the hydrogels were used. Attachment assays included cellular quantification with MTT photometry and cellular morphology with the scanning electron microscopy after 2 h culture. Proliferation assays were carried out with 5 and 10 days culture. Cellular morphology included cytochemistry of resin sections and scanning electron microscope observations. Hydrogels allowed a few cells to attach and proliferate. The cells growing on the surface of hydrogels were organized in various layers and showed a differential morphology. Cells located inside the pores remained rounded. The hydrogels showed the possibility of inducing differentiated phenotypic expression.
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1. Introduction
The need for developing different materials for medical and dental applications expands the use of cell culture and tissue engineering [1–3]. Cell culture can be applied to tests of cytotoxicity, biocompability, molecular and biochemical mechanisms and cellular transformation induced by a specific biomaterial. The ability of cells to attach and grow on the biomaterial determines its use. The interaction biomaterial-cells should resemble the *in vivo* condition. The physico-chemical characteristics of a biomaterial should mimic extracellular matrix. The molecular interactions in the extracellular matrix influence cellular development, such as cell adhesion, proliferation, morphology, metabolism, differentiation and induction of cellular function [4–8].

Hydrogels are solid material formed by a cross linked hydrophilic polymer network. They have a stable three-dimensional structure that when water swollen, shows a soft and rubbery consistency, water permeability and low interfacial tension [9–11]. PolyHEMA was first reported by Woodhouse (1934) [12] and was recognized as a biomaterial by Witchterle (1960) who applied this hydrogel to contact lenses [13]. Since then, extensive research was carried out to adapt this hydrogel to other biomedical applications, such as drug delivery, dental and orthopedic implants [1, 2, 9–11]. PolyHEMA is known to prevent cellular adhesion and spreading, being used as a substrate for cells that do not depend on anchorage [1, 4].

The physico-chemical characteristics of this hydrophilic hydrogel resemble cartilage matrix and are widely used for culturing chondrocytes for the possible repair of articular cartilage [14–16]. Blending polyHEMA with a copolymer of methyl methacrylate (MMA) and acrylic acid (AA) introduces negative charges to the hydrogel due to the COOH group, and changes its mechanical characteristics [17–19].

The objective of this investigation was to establish a culture system based on the blend polyHEMA-poly(MMA-co-AA). This hydrogel was first tested *in vitro* as a substrate for Vero cell culture. Vero cells have the characteristics of fibroblastic cells, such as attachment to plastic and glass surfaces, monolayer growth and an elongated morphology [20]. This growth pattern can be altered by different factors, including substrate interaction [21]. Adhesion and morphology of Vero cells cultured on polyHEMA and on the blend were analyzed.

2. Materials and methods
2.1. Substrates
Hydrogel samples were synthesized by thermal polymerization using 2-hydroxyethyl methacrylate—HEMA (Aldrich Chemie), methyl methacrylate—MMA (Metacril) and acrilic acid—AA (Rhom Haas) monomers, with trimethylol propane trimethacrylate—TMPTMMA (Retilox) as the cross linking agent and benzoil peroxide (Laporte Chemicals) as an initiator.

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Porous samples were prepared by adding glucose crystals to the monomer solution before polymerization resulting in pore diameter from 45 μm to 125 μm [19, 22].

Samples 2 mm thick of dense and porous hydrogels were used for culturing Vero cells. Plastic plates (Corning, Fisher Scientific) and silicone adhesive samples (Rhodiastic) were used as controls.

2.2. Cell culture

Vero cells were obtained from the Adolfo Lutz Institute, São Paulo. Cells from passage number 235 were cultured in HAMF10 medium (Sigma Chemical Company) supplemented with 10% fetal calf serum (FCS) (Nutricell). Cultures were incubated at 37 °C.

2.3. Attachment assay

For the attachment assay 100 μl of Vero cells, at the density of $5,4 \times 10^5$ cells/ml, were allowed to adhere in 96-wells culture plates containing samples of the hydrogels and silicone adhesive. After 2 h the medium containing unattached cells was removed. Attached cells were quantified using the photometry of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT) (Sigma), at 540 nm filter [23]. Cell morphology was analyzed using scanning electron microscopy.

2.4. Proliferation assay

For the proliferation assay Vero cells at the initial density of $2,1 \times 10^5$ cells/ml were cultured in 24-well culture plates (Corning) containing samples of the hydrogels and silicone adhesive. Cell morphology was evaluated after 5 and 10 days, with light microscopy and scanning electron microscopy.

2.5. Light microscopy

For light microscopy the cultures were fixed in paraformaldehyde 1% and processed for resin inclusion (Technovit 7100, Kulzer GmBH). Resin sections of 4 μm were made with an ultramicrotome (LKB Ultratome III). Sections were stained with Toluidine Blue (pH 4.0) for extracellular matrix demonstration, Xylidine Ponceau (pH 2.5) for nuclear morphology and periodic acid-Schiff reaction (PAS) at neutral pH for demonstration of acidic and neutral polysaccharides, hematoxylin was used to evidence the nucleus. Observations were made with an Olympus inverted photomicroscope IX50 (Japan).

2.6. Scanning electron microscopy

Samples of hydrogels, silicone adhesive and the plastic plates containing Vero cells were fixed with glutaraldehyde 2.5% (Sigma) in 0.05 M cacodylate, pH 7.4 (Sigma), followed by post-fixation in osmium tetroxide 1% (Sigma), in the same buffer. After dehydratation in graded ethanol, samples were dried at critical point dried (CPD030 Balzers) and gold sputtered (SCD050, Balzers). The observations were made in Jeol JXA-840A (Japan).

3. Results

3.1. Attachment assay

Vero cells adhere intensely to the culture plates (Fig. 1), no cells were found in the supernatant (data not shown). The plastic plates were considered the positive controls for cellular attachment and proliferation. Almost no cells were attached to silicone adhesive, as determined by the absorbance (Fig. 1). Cellular cytotoxicity of this substrate was confirmed [19].

Attachment of Vero cells to polyHEMA and to the blend occurred in a lower proportion if compared with the adhesion of Vero cells to the culture plates in MTT analysis. Cellular attachment to the hydrogels showed a similar MTT value, but the blends carried less cells than the polyHEMA samples. And the porous samples were less favorable to attachment, for both hydrogels tested (Fig. 1).

The results of MTT absorbance were confirmed by microscopy. Only a few cells attached to the hydrogels surfaces as clusters. Dense samples of polyHEMA were more favorable to attachment. A three-dimensional morphology of cells could be observed on dense hydrogels (Fig. 2C,E). Cells attached to porous hydrogels showed a rounded morphology and were preferentially located inside the pores (Fig. 2D,F).

A large amount of cells were attached to the culture plates and showed elongated morphology (Fig. 2A). No cells were found on silicone adhesive samples, only cellular debris were observed, confirming the low absorbance determined by MTT. (Fig. 2B).

3.2. Proliferation assay

The proliferation assay confirmed the results obtained at the adhesion tests. Cells showed a tendency to become confluent during the culture. Growing on plastic, cells remained flattened in a monolayer (Fig. 3A). On dense hydrogels cells showed an elongated morphology and were organized in various layers (Fig. 3C,E). On porous hydrogels cells located inside the pores remained rounded and formed aggregates (Fig. 3G,H). Cells growing on the surface of porous hydrogels showed prolongation crossing certain pores (Fig. 3D,F). No cells were found on silicone adhesive, just some debris was observed (Fig. 3B).

The analyzes made on semi-thin sections of cells growing on hydrogels and stained with Toluidine Blue

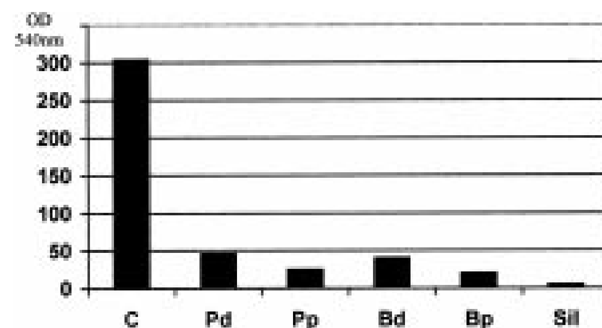

Figure 1 MTT photometry for quantification of attached Vero cells after 2 h culture. Absorbance filter: 540 nm. C: culture plate; Pd: dense polyHEMA; Pp: porous polyHEMA; Bd: dense blend; Bp: porous blend; Sil: silicone adhesive.

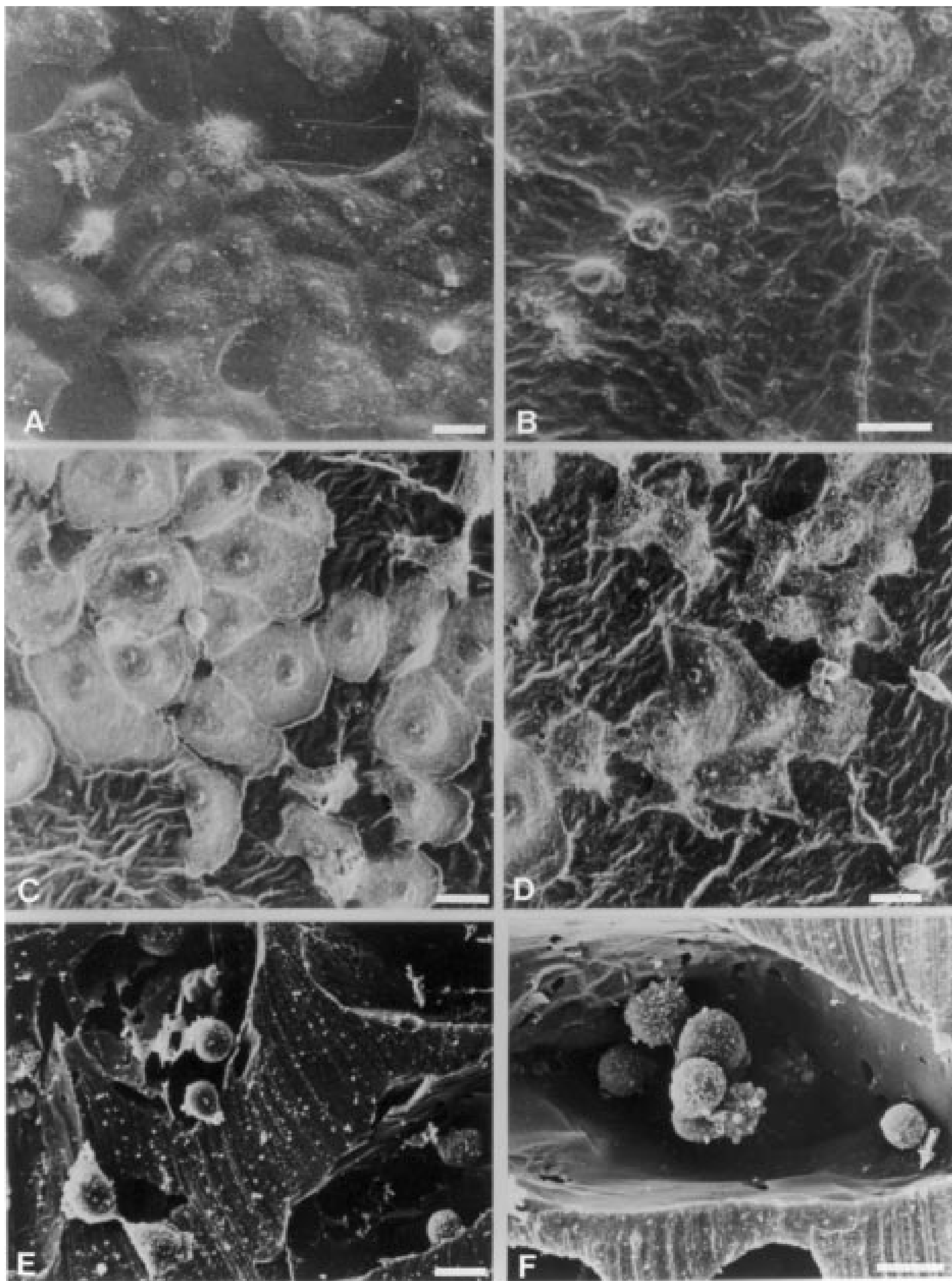

Figure 2 Adhesion of Vero cells—scanning electron microscopy. (A) Vero cells attached to plastic plates. Elongated cells with prolongation. (B) Cellular debris on silicone adhesive. No cells were found attached. (C) Vero cells attached to dense polyHEMA. (D) Vero cells attached to dense blend. (E) Vero cells attached to porous polyHEMA. A round shape was observed in cells inside the pores or on the surface of the hydrogel. (F) Vero cells attached to porous blend. Cells remained rounded inside the pores. The bars represent 10 μm.

confirmed the results of scanning electron microscopy. Cells on the surface of the hydrogels were organized in various layers. Cells in the layers in contact with the hydrogels were more flattened, and cells in the upper layer were more rounded, suggesting cellular arrangement (Fig. 4A). The cytochemical tests showed no reaction for extracellular matrix with Toluidine Blue, pH 4,0 (Fig. 4A,B). Cells inside the pores showed an

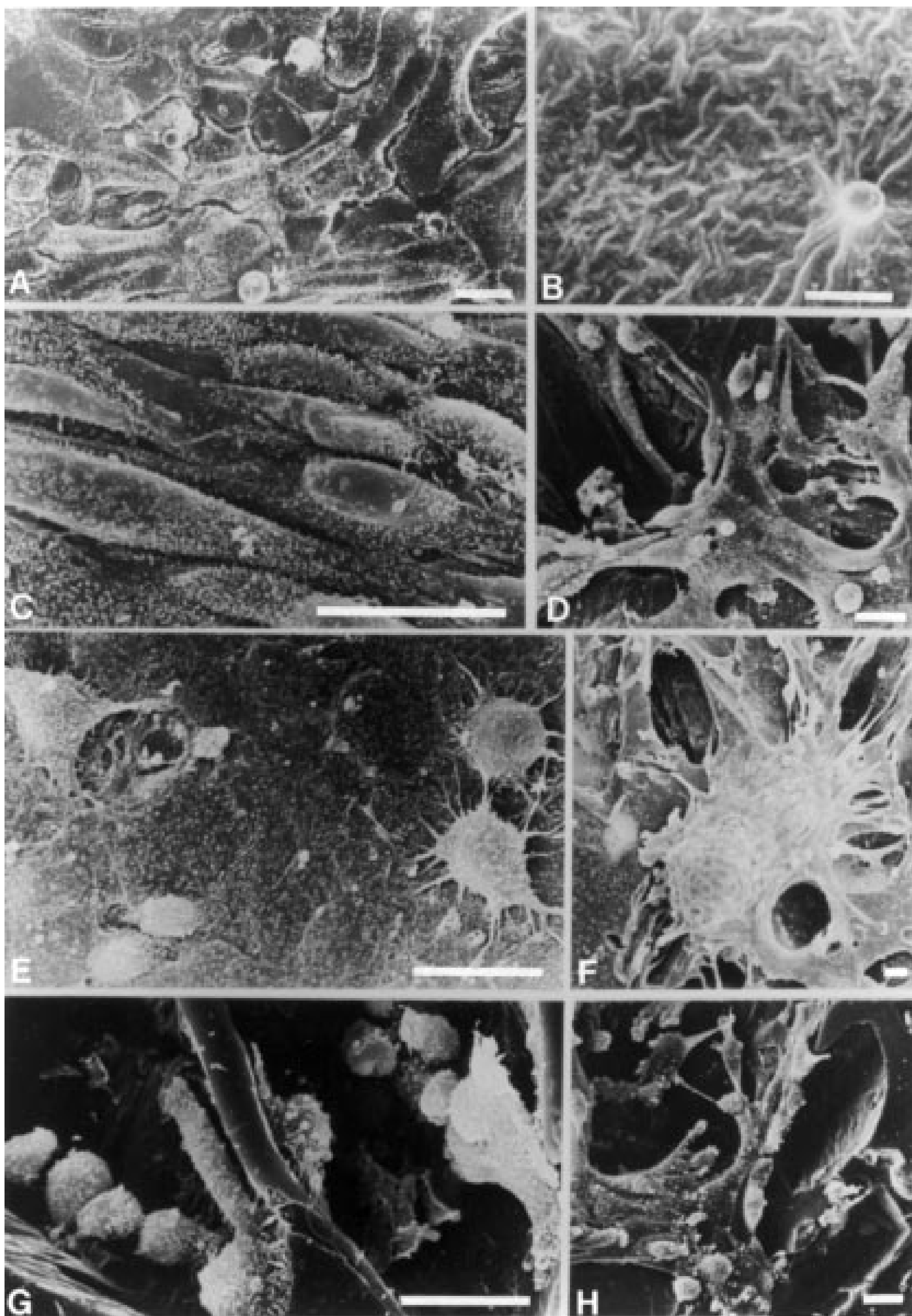

Figure 3 Proliferation of Vero cells on hydrogels—scanning electron microscopy. (A) Vero cells on plastic plates, 10 days culture. Flattened cells reached the confluency. (B) Vero cells on silicone adhesive, 5 days culture. Only debris were found. (C) Vero cells on dense polyHEMA, 10 days culture. Cells reached the confuency in certain areas of the hydrogel. Cells remain less flattened and show irregular surface (microvilli). (D) Vero cells on porous polyHEMA, 10 days culture. Some flattened cells growing on the surface cover the pores. Cells inside the pores show a rounded morphology. (E) Vero cells on dense blend, 10 days culture. Some rounded cells with prolongations can be distinguished from confluent cells. (F) Vero cells on porous blend, 5 days culture. Some cells form aggregates on hydrogel surface, and prolongations extend over the pores. (G) Vero cells on porous polyHEMA, 5 days culture. Cells inside the pores are rounded forming aggregates. Cellular surfaces show irregularities, such as microvilli. (H) Vero cells on porous blend, 10 days culture. Cells inside and on the surface of the hydrogel remain rounded. The bars represent 10 μm.

intense cytoplasmatic staining with Xylidine Ponceau, indicating cytoplasmatic condensation (Fig. 4C). Differential glycoprotein synthesis was observed with PAS staining (Fig. 4D). Cells located inside the pores or on layers in contact with the hydrogels showed a stronger reaction.

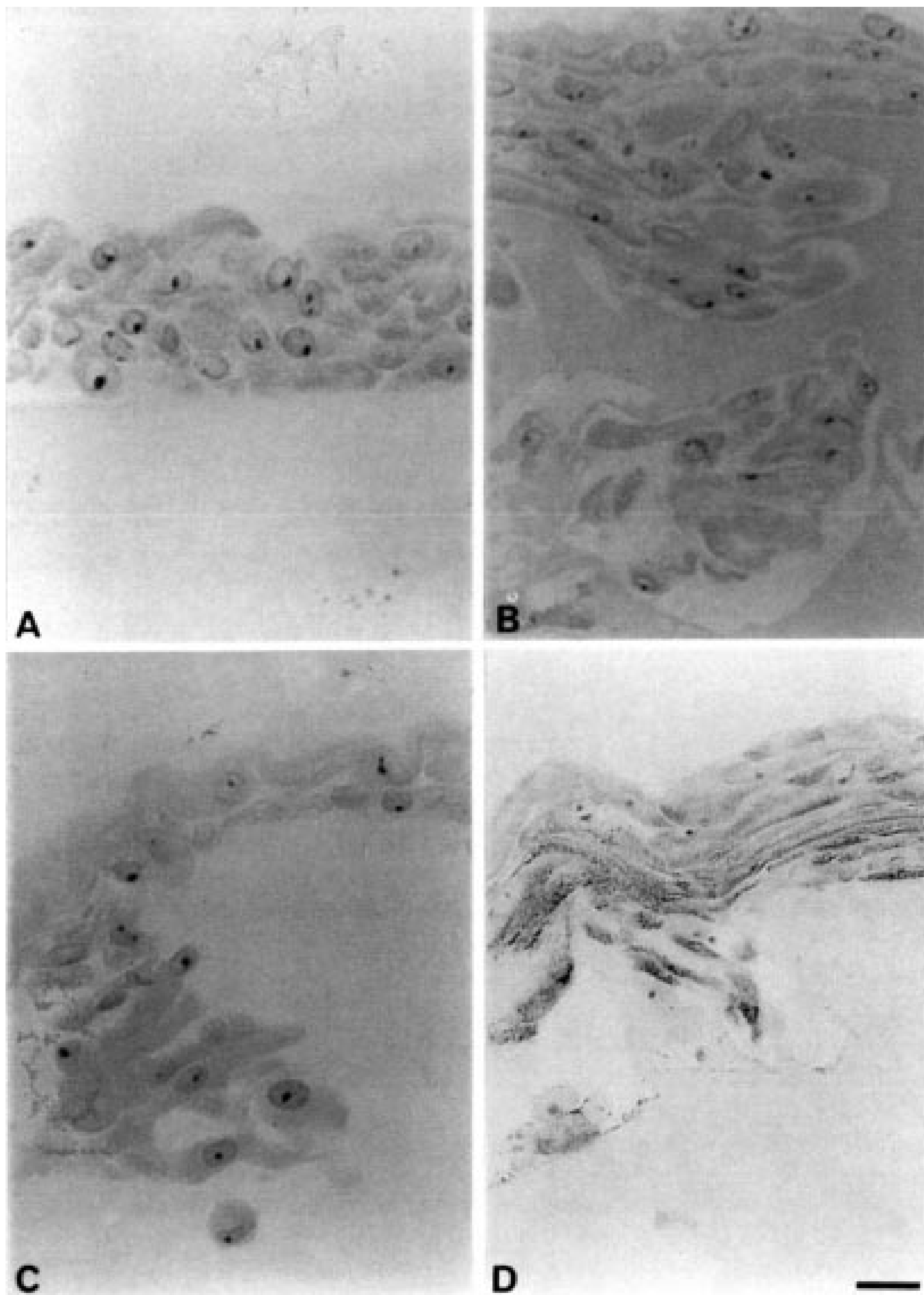

Figure 4 Proliferation of Vero cells on hydrogels—light microscopy of resin sections. (A) Dense polyHEMA, 5 days culture. Cells in contact with the hydrogel were flattened and rounded cells in upper layers. Toluidine Blue (pH 4.0) staining. (B) Dense polyHEMA, 5 days culture. No extracellular matrix was observed with staining of Toluidine Blue, pH 4.0. (C) Porous polyHEMA, 5 days culture. Cytoplasmatic condensation and nuclear rounding were observed in cells inside the pore. Xylidine Ponceau staining. (D) Porous blend, 10 days culture. Glycoprotein synthesis was more intense in layers in contact with the hydrogel. PAS/Hematoxylin staining. The bars represent 25 μm.

4. Discussion

Vero cells are obtained from the kidney of African green monkey (*Cercopithecus aethiops*) and have the characteristics of fibroblastic cells, such as elongated morphology and monolayer growth on plastic or glass [20]. This growth pattern can be modified by different factors [24, 25], such as stress conditions [26] or specific interactions on the substrate [21, 27, 28]. The cell-substrate interactions are not totally elucidated. Adhesion is followed by cellular activation, spreading and migration. This process results in cellular proliferation and biosynthesis [3, 8, 17]. The adhesion processes is related to the adsorption of adhesive proteins, and is directed by the characteristics of the substrate, such as hydrophilicity, hydrophobicity and the presence of charged groups [4, 17, 29].

Vero cells adhered and proliferate as expected when cultured on culture plates. The plastic substrate allowed

the attachment of almost all cells in suspension, and the cells proliferate up to ten days in culture with elongated morphology, characteristic of fibroblasts. Some areas of cellular detachment were observed due to confluency. The cytotoxicity of silicone adhesive was confirmed [19] and these results were not found for any other substrate tested.

Vero cells growing on hydrogels lost contact inhibition. Cells were organized in multilayers without any pronounced cellular degeneration or detachment. This pattern can be observed in Vero cells submitted to stress conditions [26] and indicates differential cellular development [4, 29, 30]. Cells in intermediary layers showed a more rounded morphology and the cells in the interface with the hydrogels had more glycoprothein synthesis. These observations suggest cellular organization inside the layers that in some areas resembled cellular nodules. No extracellular matrix was detected between the cell layers by toluidine blue method.

The induction of cellular differentiation by covering the culture surfaces with polyHEMA is well known [4] and has been applied to culture cells not dependent on anchorage, such as chondrocytes [1, 15, 31]. These cells need to maintain a differential rounded shape. If chondrocytes are cultured on glass or plastic they assume a fibroblastic-like morphology. This morphologic differentiation is accompanied by metabolic alterations [4, 31]. To maintain differentiation it is necessary to grow in three-dimensional cultures, such as suspension culture, agar, agarose or collagen gel [8, 14, 30].

The importance of a porous structure of the substrate has already been discussed [32]. The pores induced differential morphology. Round cells were often observed inside the pores. These cells were less proliferative, as they did not occupy all the pore. Some cells in direct contact with the pore wall were more elongated, but this was an exception. The pore structure offers less contact surface for the cells that acquire a rounded morphology. This should be the ideal substrate for cultured cells not dependent on anchorage such as the chondrocytes.

The results obtained from polyHEMA and blend samples were similar. The adhesion was quantitatively lower for blend samples. The proliferative pattern also shows a preferential differentiation of cells growing on blend samples. This may result from the increased negative charges. As previously discussed the cellular attachment occurs preferentially in positively charged substrata, for the adsorption of adhesive molecules [4, 17, 29].

Our results showed that hydrogels induced a differential adhesion pattern for Vero cells. Hydrogels also induced morphological differentiation of these cells during the culture period. The porous structure of the substrate favored cellular differentiation and made cellular attachment difficult. Porous blend was the most effective substrate for inducing differential shape in Vero cells.

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