



MatrigelBD, Un Andamiaje Biocompatible que Estimula la Proliferación de Celulas Troncales Mesenquimales Derivadas del Tejido Gingival

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MatrigelBD, A Biocompatible Scaffold that Improves Gingival Mesenchymal Stem Cells Proliferation

MatrigelBD, Un Andamiaje Biocompatible que Estimula la Proliferación de
Células Troncales Mesenquimales Derivadas del Tejido Gingival

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BRIZUELA, C.; SAINTJEAN, N.; GARCHITORENA, N.; RODRIGUEZ, I. & INOSTROZA, C. MatrigelBD, a biocompatible scaffold that improves gingival mesenchymal stem cells proliferation. *Int. J. Morphol.*, 34(2):692-698, 2016.

SUMMARY: MatrigelBD is a hydrogel scaffold with three-dimensional intercrossed networks of hydrophilic polymers with high water content. Human gingival tissue might represent a better source of MSCs, allowing these cells to be easily obtained in a relatively non-invasive way. The objective of this study was to evaluate the biocompatibility of MatrigelBD with GMSCs *in vitro*. Gingival connective tissue samples were obtained from healthy donors. Fresh tissue was minced and cultured during two weeks, after which cells at passage fourth were analyzed for their immune phenotype by flow cytometry. Differentiation into osteogenic, chondrogenic, and adipogenic lineages was induced and evaluated by culture staining. The "construct" was made of MatrigelBD with GMSC. To assess the biocompatibility, an MTT cellular proliferation assay was performed. The differentiation potential of the cells toward the osteogenic, adipogenic, and chondrogenic lineages was analyzed after 21 days of growth in MatrigelBD with induction differentiation media. The MTT analysis showed that MatrigelBD stimulated cell proliferation; the GMSCs maintained the expression of MSC markers. Importantly, the growth of GMSCs within the MatrigelBD did not interfere with the cell differentiation potential. These findings indicate that MatrigelBD is biocompatible with GMSCs, and this matrix improves cell proliferation *in vitro*.

KEY WORDS: Adult stem cell research; Hydrogels; Tissue scaffolds; Regeneration; Materials testing.

INTRODUCTION

MatrigelBD is a natural hydrogel scaffold mainly composed of laminin, collagen IV, heparin sulfate proteoglycans, and entactin/nidogen. Several growth factors, such as TGF-, epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), neural growth factor (NGF), tissue plasminogen activators, and others are also included in its composition. MatrigelBD is a solubilized basement membrane extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Hughes *et al.*, 2010). In preliminary studies, MatrigelBD has been used in cardiomyocyte progenitor transplantation experiments, in a cardiac infarction animal model, and to promote *in vivo* angiogenesis (Ou *et al.*, 2011; Bhat *et al.*, 2012), and stimulates embryonic stem cells (ESC) to survive and differentiate *in vitro* (Uemura *et al.*, 2010). One report showed that dental pulp stem cells (DPSC) co-cultured within MatrigelBD maintained their osteogenic

differentiation potential, suggesting possible applications for use in skeletal defects (van Gastel *et al.*, 2012).

Stem cells are characterized by their ability to renew themselves and to differentiate into a diverse range of specialized cells types (multipotent capabilities) (Hermann *et al.*, 2006). Over the last few years, it has become possible to isolate and characterize human mesenchymal stem cells (MSCs) from diverse sources, such as bone marrow, fat tissue, the placenta, and even the oral cavity (Sonoyama *et al.*, 2006). MSCs have been found from different niches, including (DPSC) (Suchanek *et al.*, 2009), stem cells from the human exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003), periodontal dental stem cells (PDSCs) (Wada *et al.*, 2011), apical papilla stem cells (APSCs) (Sonoyama *et al.*, 2008), dental follicle stem cells (DFSCs) (Morsczeck *et al.*, 2005) and gingival mesenchymal stem cells (GMSCs) (Mitrano *et al.*, 2010). MSCs are characterized according to

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the minimal criteria established in the position statement of the International Society for Cellular Therapy (ISCT) (Dominici *et al.*, 2006).

MSCs derived from human oral mucosa and gingiva, are easy for isolation, accessible tissue source, and rapid ex vivo expansion, with maintenance of stable stem-cell like phenotypes, render oral mucosa- and gingival derived MSCs a promising alternative cell source for MSC-based therapies (Zhang *et al.*, 2009, 2012; Tomar *et al.*, 2010; Xu *et al.*, 2013). However, there is no information about GMSCs within MatrigelBD related to cell survival and differentiation potential. The aim of this study was to evaluate the biocompatibility of MatrigelBD with GMSCs in vitro by analyzing the cell viability and differentiation potential towards different cellular types. We demonstrate that MatrigelBD is biocompatible with GMSCs, and this matrix improves cell proliferation in vitro.

MATERIAL AND METHOD

Human gingival derived mesenchymal stem cell culture:

All of the procedures were approved by the Ethics Committee of the Faculty of Medicine, Universidad de los Andes. Samples corresponding to human healthy oral mucosa were obtained from the Dentistry Faculty, Universidad de los Andes, Santiago, Chile. Considering the ethical principles expressed in the statement of the declaration of Helsinki. All donors agreed to participate in this study and provided their written informed consent. The fresh explants were minced and placed on culture plates in basal media composed of 90% alpha-MEM (Minimum Eagle Medium, Invitrogen) and 10% fetal bovine serum (FBS) (Fetal Bovine Serum, HyClone), supplemented with 1% penicillin-streptomycin (Pen Strep, Invitrogen), and cultured at 37°C in a 5% CO₂ fully humidified atmosphere.

The basal media were replaced every 3 days until cellular migration outside of the explants was observed. The explants were removed, and the cells continued to grow until they reached 80% confluence. The cells were harvested by treatment with 0.25% Trypsin/EDTA (GIBCO, Invitrogen) (passage 0th), and the process was repeated until passage 4th, at which the immunophenotyping cell characterization was performed.

Immunophenotyping of GMSCs

GMSC: Immunophenotyping was carried out at passage 4th using different antibodies conjugated with fluorescein isothiocyanate-(FITC), phycoerythrin-(PE), or peridinin

chlorophyll protein (PerCP) by flow cytometry. The antibodies were anti-CD34 (Beckmann Coulter San Jose, CA, USA), anti-CD45 (Beckmann Coulter), anti-CD73 (BD Pharmingen, San Jose, CA, USA), anti- CD90 (BD Pharmingen), and anti-CD105 (Caltag, USA). Sample acquisition and analysis was performed using a Coulter Epics-XL (Coulter Corporation, Florida, and USA) flow cytometer.

Differentiation potential assays: GMSCs at 80% confluence from the 4th passage, were cultured with specific induction media for osteogenic, adipogenic, and chondrogenic lineages. The cells were cultured in specific induction media for the adipogenic, osteogenic, and chondrogenic lineages. The cell culture images were captured with phase contrast, on an Olympus CKX41 (Tokyo, Japan) inverted light microscope.

Osteogenic Differentiation: A total of 35,000 cells/cm² were resuspended in 4-well culture plates. The cells were grown in basal medium supplemented with 0.1 mM dexamethasone (Sigma- Aldrich Inc., St. Louis, MO, USA), 10 mM B-glycerol phosphate (Sigma-Aldrich Inc., St. Louis, MO, USA), and 50 mg/mL ascorbate-2 phosphate (Sigma-Aldrich Inc., St. Louis, MO, USA). Ascorbate-2 phosphate was added every day during the whole differentiation process. This medium was replaced every 3-4 days for 21 days. The specimens were stained for the formation of birefringent crystals with Alizarin Red (Sigma-Aldrich Inc., St. Louis, MO, USA).

Chondrogenic Differentiation: The cells were cultured at a density of 30,000 cells/cm², 10 mL were placed in 4-well culture plates to achieve a microcellular mass formation. The cells were incubated for 1.5 h and induced by adding 0.1 mM dexamethasone (Sigma-Aldrich Inc., St. Louis, MO, USA), 5 mg/mL insulin (Sigma-Aldrich Inc., St. Louis, MO, USA), 10 ng/mL TGF- beta -1 (BioVision Incorporated 155 S. Milpitas Boulevard, Milpitas, CA 95035 USA), and 50 mg/mL ascorbate-2-phosphate (Sigma-Aldrich Inc., St. Louis, MO, USA). This medium was replaced every 3-4 days during 21 days. The microcellular masses were stained for the presence of glycosaminoglycan with Safranin-O (Sigma-Aldrich Inc., St. Louis, MO, USA).

Adipogenic Differentiation: A total of 25 000 cells/cm² were seeded in 4-well culture plates and induced by adding 0.1 mM dexamethasone (Sigma-Aldrich Inc., St. Louis, MO, USA), 10 mg/mL insulin (Sigma-Aldrich Inc., St. Louis, MO, USA), and 0.02 mg/mL indomethacin (Sigma- Aldrich Inc., St. Louis, MO, USA). This medium was replaced every 3-4 days for 21 days. Adipogenesis was measured by the accumulation of lipids in vacuoles, stained with Oil Red O (Sigma-Aldrich Inc., St. Louis, MO, USA).

MatrigelBD scaffold preparation: Matrigel Basement Membrane Matrix (BD Bioscience, USA, cat. 354234) (MatrigelBD), were made following the thick gel method according to the manufacturer's instructions. Each culture plate (1.9 cm²) was coated with 200 mL liquid phase MatrigelBD to achieve a 1-mm thick layer suitable for later incubation at 37 °C and 5 % CO₂ for 30 min. Then, 10,000 cell/cm² suspensions were seeded in each well treated with MatrigelBD.

Morphological analysis of GMSCs within the MatrigelBD scaffold: GMSCs were identified by histological staining with hematoxylin and eosin (H&E). A portion of the scaffold was removed and cultured in a new plate over two weeks. The morphology of the GMSCs within the MatrigelBD was assessed by phase contrast using an Olympus CKX41 (Tokyo, Japan) inverted microscope equipped with an Olympus DP12 camera.

Immunophenotypic characterization of GMSCs within the MatrigelBD scaffold: The immunophenotype of GMSCs in the MatrigelBD scaffold was determined by flow cytometry. A total of 500,000 cells at passage 4th were incubated with specific individual monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP) in 250 mL phosphate buffered saline for 30 min in the dark at room temperature. The primary antibodies used were CD13, CD90, CD73, CD45, CD34, CD38, CD105, CD54, and CD44. Cells were diluted in 4 mL phosphate buffered saline, centrifuged, and suspended with 600 mL phosphate buffered saline and 2% formaldehyde. Acquisition and analysis were performed with a flow cytometer (Coulter Epics-XL, Coulter Epics Corporation). Isotype controls used were fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

Multilineage differentiation GMSC within a MatrigelBD scaffold: Differentiation of GMSCs towards the osteogenic, chondrogenic, and adipogenic lineages in the presence of 200 mL of MatrigelBD was performed following the previously mentioned protocol. For samples submitted to the differentiation protocol, GMSCs and the MatrigelBD controls were kept in basal media without induction media for 4 to 5 weeks. Cultures were stained with the same dyes used for the differentiation assays.

MTT cellular proliferation assay: Cell Titer 96® Non-Radioactive Cell Proliferation (Promega Corporation, Madison, WI, USA) tests were carried out using GMSCs at different concentrations (5,000 cell/cm², 10,000 cell/cm², 20,000 cell/cm², 30,000 cell/cm², and 40,000 cell/cm²) with and without MatrigelBD. The cells were seeded on 96-well

plates, and the MTT activity was measured after 48 h. The experiment was performed in triplicate. Proliferation curves were generated by plotting the average of the values calculated with optical density measurements at 570 nm in a 96-well plate reader (RT-2100C Rayto Life and Analytical Sciences Co., Ltd.). Alpha-MEM culture media, MatrigelBD were used as negative controls.

Statistical analysis: Statistical analysis was performed using STATA 11.2 software. The MTT cell proliferation results were analyzed using a Wilcoxon's test. The differences were considered statistically significant for *p <0.05. The data are presented as the Means ± SD.

RESULTS

GMSC morphology, immunophenotype and multilineage differentiation: GMSCs exhibited fibroblastoid morphology and showed positive expression of CD13, CD73, CD90, and CD105 and negative expression of CD34, CD38, and CD45. In addition were able to differentiate into osteocytes, chondrocytes, and adipocytes according to the ISCT criteria (data not shown).

Morphological and immunophenotypic analysis of GMSCs in a MatrigelBD scaffold: Histological evaluation of the GMSCs within MatrigelBD revealed that cells had spread inside the scaffold itself. The GMSCs maintained their undifferentiated morphology and homogeneous cells layer (Fig. 1).

GMSCs grown in MatrigelBD displayed a rapid proliferation rate, fibroblast-like cell morphology, and migrated outside of the matrix at 7th day. GMSCs nearly reached 80 % cell confluence after two weeks in culture.

Immunophenotypic characterization of GMSCs within the MatrigelBD scaffold: After three weeks of culturing GMSCs within MatrigelBD, the cell surface antigens were analyzed by flow cytometry. The results showed positive expression of CD13, CD73, CD90, and CD105 and negative expression of CD34, CD38, and CD45. Both GMSCs and GMSCs within MatrigelBD showed the same expression patterns of these markers (Fig. 2).

Multilineage differentiation of GMSCs within a MatrigelBD scaffold: After 21 days of cell culture, the differentiation assays were evaluated for adipogenic differentiation, intracellular microscopic drops of fat were observed inside the GMSCs (Figs. 3A, B and C). For osteogenic differentiation, the GMSCs stained with Alizarin

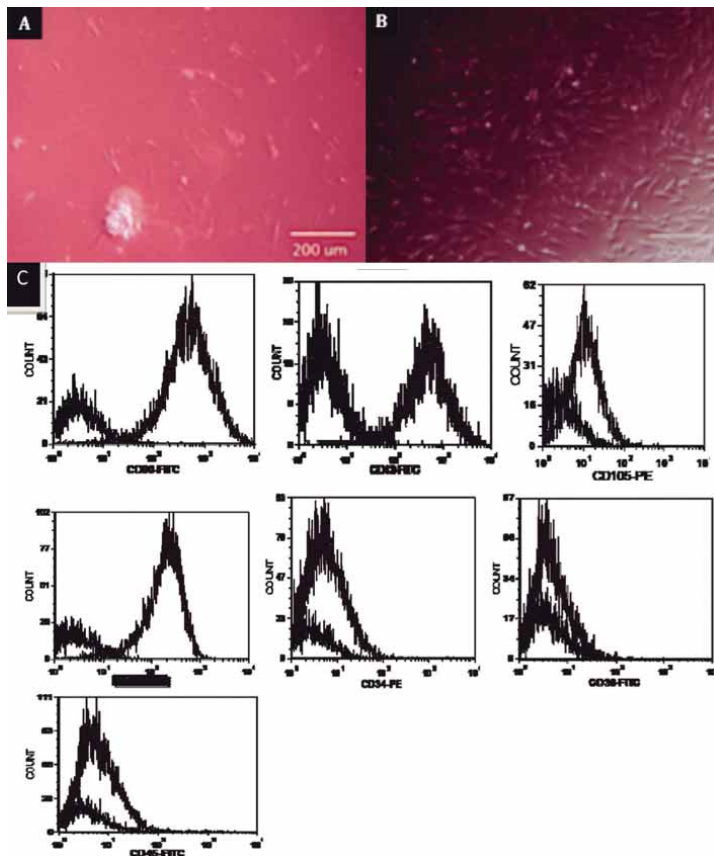


Fig. 1. GMSC characterization in MatrigelBD. (A) GMSC proliferation in Matrigel BD. GMSC grows elongated and spread out of MatrigelBD after 7 days. (B) GMSC grows to approximately 80 % cell confluence on MatrigelBD after two weeks on culture. Magnification X10, scale bar 200 μ m. (C) Phenotypic features of GMSC in presence of MatrigelBD. Positive expression for CD13, CD73, CD90, CD105 markers and negative expression for CD34, CD38, CD45 markers.

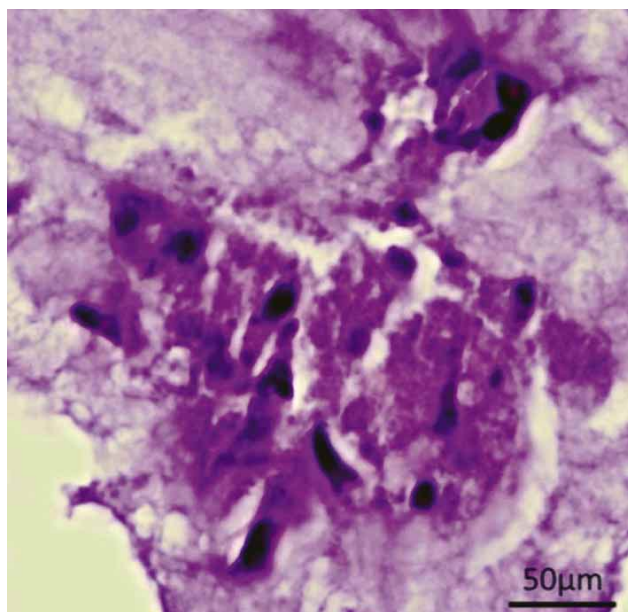


Fig. 2. Hematoxylin and Eosin (H&E) staining of longitudinal sections of MatrigelBD with GMSC. The expansion of GMSC in the hydrogel, exhibited fibroblastoid cell morphology. Magnification X40 scale bar 50 μ m.

Red showed the presence of calcium deposits, indicating functional osteoclasts (Figs. 3D, E and F). For chondrogenic differentiation, GMSCs stained with Saphranin O demonstrated the formation of a red-stained chondrogenic mass (Figs. 3G, H and I).

MTT cellular proliferation assay: The results of the MTT assay with the GMSCs grown in MatrigelBD revealed an increased cell density. MatrigelBD stimulated cell proliferation, in comparison to GMSCs alone. The absorbance obtained was highly correlated with the cell number, even at low concentrations (Fig. 4).

DISCUSSION

The potential use of MatrigelBD with GMSCs in tissue engineering is a promising alternative with many advantages in comparison with MSC derived from other human niches. The accessibility of gingival-derived mesenchymal stem cells implies minimal complications for the donor, making gingival tissue cells an excellent option

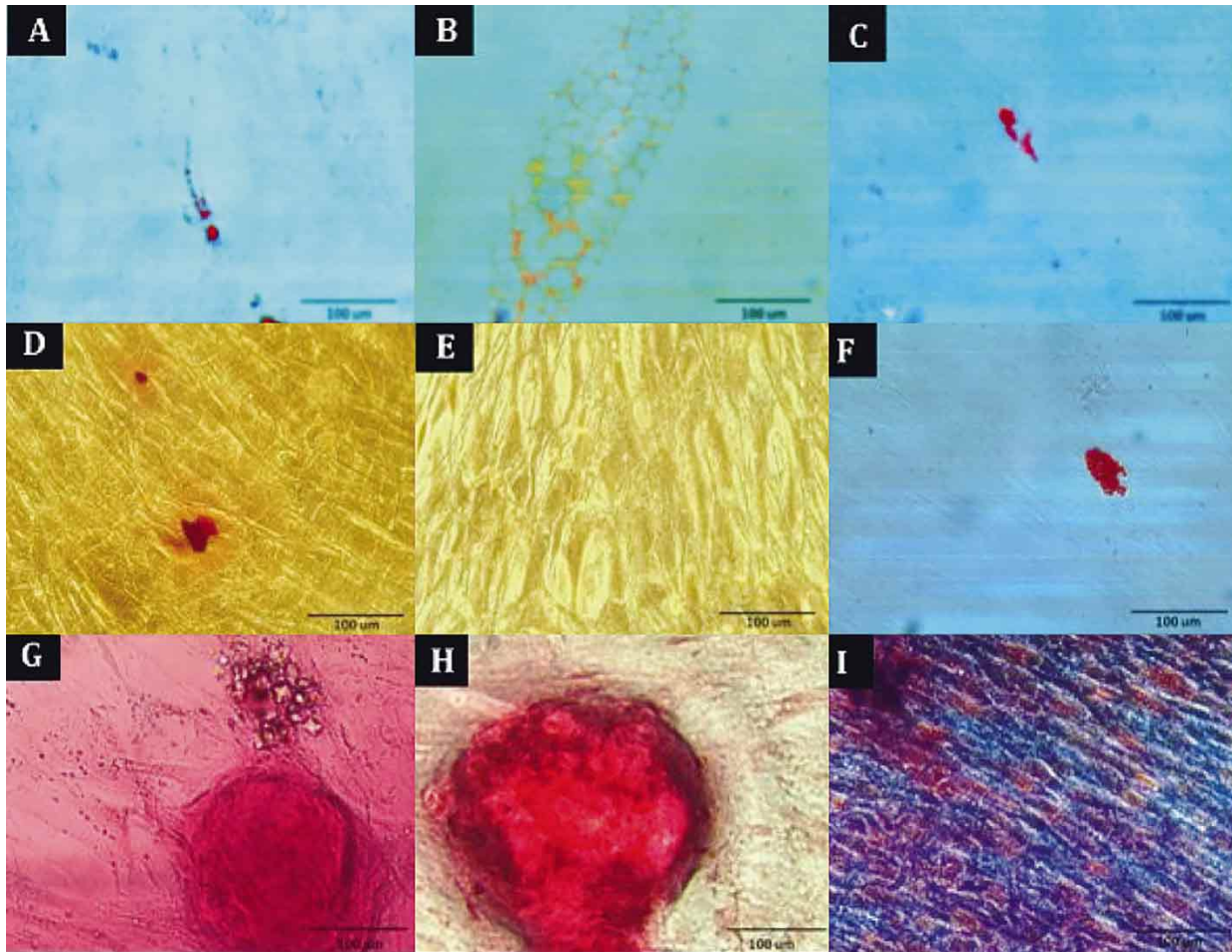


Fig. 3. Multilineage differentiation potential of GMSC within MatrigelBD. (A) Oil red staining of localized adipose deposits, (D) Alizarin Red staining for calcium deposits of functional osteocytes and (G) Safranin O staining of chondrogenic mass. (B), (E) and (H) MatrigelBD respective staining negative controls. (C), (F) and (I) GMSC respective differentiation positive controls. Magnification X10, scale bar 100 µm.

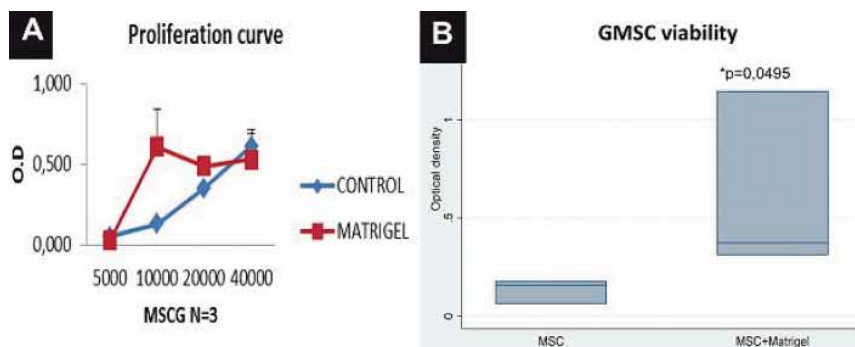


Fig. 4. MTT Assay. (A) Proliferation curve of GMSC within MatrigelBD. (B) Cell viability of 10 000 GMSC within MatrigelBD. MTT proliferation assay was carried out using GMSC at different concentrations within or without MatrigelBD. MTT activities were measured in triplicate. The proliferation curves were illustrated by plotting the average of triplicated values calculated by optical density measurements at 570 nm in a 96-well plate reader. GMSC viability graph shows extreme values samples means, the differences between means was significant applying Wilcoxon's test with a 5 % significance probability, $p=0.0495$.

for the collection of explant samples (Xu *et al.*). Our results show that cells isolated from gingival tissue under culture conditions exhibited a spindle-shaped fibroblast appearance, similar to bone marrow-derived MSC (BMSC) the cells adhered to the surface of tissue culture flasks and expressed different cell-surface markers, which is indicative of a mesenchymal phenotype (Tomar *et al.*; Zhang *et al.*, 2012; Fournier *et al.*, 2013). A recent study showed that gingiva contain both neural-crest- and mesoderm-derived MSCs, with distinctive stem cell properties useful for cell therapy (Xu *et al.*). The physical and chemical property of a hydrogel, such as polymer chains and water insolubility, provides a high degree of flexibility, very similar to natural tissue (Drury & Mooney, 2003). Hydrogels also have the ability to respond to changes in pH, temperature, or metabolite concentrations, and they are able to release their loads in response to these changes. When hydrogels were used in combination with MSCs, they offered a suitable alternative for tissue regeneration (Salinas & Anseth, 2009). In this study we cultured GMSCs within MatrigelBD, the scaffold allowed cellular proliferation and preserved the typical morphological features of the cells. Our results are consistent with those reported by Kohen *et al.* (2009), who demonstrated that cells grown on MatrigelBD retained all pluripotent stem cells features. Similar results reported by Chan *et al.* (2011) demonstrated the biocompatibility of Puramatrix TM with DPSCs and SHEDs, showing that this hydrogel is compatible and allows cellular proliferation (Drury & Mooney; Chan *et al.*). Here we revealed that MatrigelBD is easy to manipulate, it allows the inclusion of GMSCs, and it incorporates several growth factors that stimulate cellular proliferation, hence creating a suitable environment for the potential use in tissue reparation or regeneration. In conclusion, the casting method used for the MatrigelBD scaffolding preparation did not affect the cell morphology. A proteomic complete analysis of MatrigelBD, demonstrated that this hydrogel is a complex protein mixture ideal for growth of cell culture (Hughes *et al.*). We found that the H&E analysis of the matrix confirmed that the GMSCs were within the MatrigelBD. After performing GMSCs culture within MatrigelBD, cells retained the positive expression of CD13, CD73, CD90, and CD105 as well as the negative expression of CD34, CD38, and CD45. Patterns of surface antigens suggests that the presence of MatrigelBD in culture did not alter the cell phenotype of GMSCs. GMSCs grown in MatrigelBD with a specific induction media were able to differentiate to the osteogenic, chondrogenic, and adipogenic lineages. This study is consistent with the results obtained by Demarco *et al.*, 2011 and by Chan *et al.*, who used DPSCs. Riccio *et al.* (2010) developed co-cultures of DPSCs with MatrigelBD and showed a high osteogenic differentiation potential, with a promising potential for use in the repair of skeletal defects (Riccio *et al.*). In our analysis GMSCs within MatrigelBD demonstrated a constant proliferation rate. This

hydrogel induces an increase in cellular density compared to the control. This result is in agreement with that obtained by Hughes *et al.*, who demonstrated that MatrigelBD improves the proliferation of Schwann and bone marrow stem cells. One explanation of the results could be the presence of growth factors, which play an important role in the generation and maintenance of angiogenesis and vascularization, which are needed for the formation of tissues (Zhang *et al.*, 2009).

In conclusion, MatrigelBD is a biocompatible matrix that allowed GMSC differentiation and did not modify the cellular phenotype. Under the conditions of this study, this investigation provides new evidence for the potential use of MatrigelBD and GMSCs in tissue regeneration. However, the disadvantages of MatrigelBD is that it cannot be used in human stem cell therapies, because it is derived from Engelbreth-Holm-Swarm mouse sarcomas.

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BRIZUELA, C.; SAINTJEAN, N.; GARCHITORENA, N.; RODRIGUEZ, I. & INOSTROZA, C. MatrigelBD, un andamiaje biocompatible que estimula la proliferación de células troncales mesenquimales derivadas del tejido gingival. *Int. J. Morphol.*, 34(2):692-698, 2016.

RESUMEN: MatrigelBD es un andamiaje de hidrogel con redes tridimensionales entrecruzadas de polímeros hidrófilos con un alto contenido de agua. El tejido gingival humano podría representar una mejor fuente de MSCs, estas células pueden obtenerse fácilmente de una manera relativamente no invasiva. El objetivo de este estudio fue evaluar la biocompatibilidad de MatrigelBD con GMSCs *in vitro*. Muestras gingivales de tejido conectivo se obtuvieron de donantes sanos. El tejido se trituró y se cultivó durante dos semanas, y cuando las células se encontraban en el cuarto pasaje se les analizó su fenotipo inmunológico utilizando citometría de flujo. Se indujo la diferenciación hacia los linajes osteogénico, condrogénico y adipogénico, evaluándose con tinciones. El "constructo" se hizo de MatrigelBD con GMSC. Para evaluar la biocompatibilidad, se realizó un ensayo de proliferación celular: MTT. Se analizó el potencial de diferenciación de las células hacia los linajes osteogénico, adipogénico y condrogénico después de 21 días de cultivo en MatrigelBD con medio de diferenciación de inducción. El análisis de MTT mostró que MatrigelBD estimula la proliferación celular; GMSCs mantiene la expresión de marcadores de MSC. Es importante destacar que el crecimiento de GMSCs en MatrigelBD no interfirió con el potencial de diferenciación celular. Estos hallazgos indican que MatrigelBD es biocompatible con GMSCs, y esta matriz mejora la proliferación celular *in vitro*.

PALABRAS CLAVE: Células troncales adultas; Hidrogeles; Andamiajes; Regeneración; Ensayo de materiales.

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