Evaluation of castor oil-based polyurethane membranes in rat bone-marrow cell culture[1]

Avaliação de membranas à base de óleo de polímero de mamona em culturas de células de medula óssea de ratos

Sofia de Amorim Cerejo[I], Sheila Canevese Rahal[II], João Ferreira de Lima Neto[III], Fabiana Azevedo Voorwald[IV], Fernanda da Cruz Landim e Alvarenga[V]

[1] Research performed at the Advanced Reproduction and Cell Therapy Laboratory (Lança), Department of Animal Reproduction and Veterinary Radiology, Faculty of Veterinary Medicine and Animal Science, Sao Paulo State University (UNESP), Botucatu-SP, Brazil.
[I] Scientific Initiation Student, Department of Veterinary Surgery and Anesthesiology, School of Veterinary Medicine and Animal Science, UNESP, Botucatu-SP, Brazil. *Main author. Acquisition of data, helped with technical procedures, collection and processing of study informations, manuscript writing.*
[II] Full Professor, Department of Veterinary Surgery and Anesthesiology, School of Veterinary Medicine and Animal Science, UNESP, Botucatu-SP, Brazil. *Mentor, conception and design of the study, manuscript writing and critical revision.*
[III] Fellow PhD degree, Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Animal Science, UNESP, Botucatu-SP, Brazil. *Acquisition of data, helped technical procedures, collection and processing of study informations.*
[IV] Fellow Master degree, Department of Clinical and Veterinary Surgery, Faculty of Agrarian and Veterinary Sciences, UNESP, Jaboticabal-SP, Brazil. *Helped technical procedures, collection and processing of study informations.*
[V] Full Professor, Department of Animal Reproduction and Veterinary Radiology, School of Veterinary Medicine and Animal Science, UNESP, Botucatu-SP, Brazil. *Conception and design of the study, manuscript writing and critical revision.*

ABSTRACT
PURPOSE: To evaluate three methods to isolate rats MSCs and to analyze the potential of a castor oil polyurethane base membrane as a scaffold for MSCs.
METHODS: Four male Wistar rats, aged 20-30 days were used. Bone marrow aspirates from femur and tibia were harvested using DMEM high glucose and heparin. The cell culture was performed in three different ways: direct culture and two types of density gradients. After 15 days, was made the 1st passage and analyzed cell viability with markers Hoerscht 33342 and propidium iodide. The MSCs were characterized by surface markers with the aid of flow cytometry. After this, three types of castor oil polyurethane membranes associated with the MSCs were kept on the 6-well plate for 5 days and were analyzed by optical microscopy to confirm cell aggregation and growth.
RESULTS: Separation procedures 1 and 2 allowed adequate isolation of MSCs and favored cell growth with the passage being carried out at 70% confluence after 15 days in culture. The cells could not be isolated using procedure 3. When the 3 castor oil polyurethane membrane types were compared it was possible to observe that the growth of MSCs was around 80% in membrane type 3, 20% in type 2, and 10% in type 1.
CONCLUSION: Both Ficoll-Hypaque densities allow isolation of rat MSCs, and especially castor oil-based membrane type 3 may be used as a scaffold for MSCs.
Key words: Mesenchymal Stem Cells. Castor Oil. Rats.

RESUMO
OBJETIVO: Avaliar três formas de cultivo de células-tronco mesenquimais de ratos; e analisar o potencial do polímero de mamona na forma de membrana como arcabouço para CTMs.
MÉTODOS: Foram utilizados quatro ratos machos Wistar, de 20 a 30 dias de idade. Aspirados da medula óssea do fêmur e da tíbia foram colhidos com DMEM alta glicose e heparina. As células foram isoladas de três formas diferentes: cultivo direto e com dois tipos de gradientes de densidade. Após 15 dias, foi feita a 1ª passagem e analisada a viabilidade celular com os marcadores Hoerscht 33342 e Iodeto de Propídio. As CTMs foram então caracterizadas por marcadores de superfície, com o auxílio de citômetro de fluxo. Após, três tipos de membrana à base de óleo de polímero de mamona associadas com as CTMS foram mantidas em cultivo por cinco dias, e analisados por microscópio ótico para confirmar o crescimento e a adesão celular.
RESULTADOS: Após 15 dias, Os procedimentos que utilizaram gradientes de densidade permitiram o isolamento das CTMs e favoreceram o crescimento celular com a passagem, sendo obtido 70% de confluência após 15 dias em cultura. O procedimento direto não se mostrou eficaz para o isolamento das células. O crescimento das CTMs foi aproximadamente 80% sobre a membrana tipo 3, 20% na tipo 2 e 10% na membrana tipo 1.
CONCLUSÃO: Os dois gradientes de concentração Ficoll-Hypaque permitem isolar CTMs de ratos; e especialmente a membrana de polímero de mamona tipo 3 pode ser usada como um bom arcabouço para as CTMs.
Descritores: Células-Tronco Mesenquimais. Óleo de Rícino. Ratos.

Introduction

Bone marrow contains a type of stem cell denominated mesenchymal stem cell (MSC) that possesses a high degree of self-renewal and is capable of differentiation into several lineages of connective tissue[1,2]. MSCs are present in the bone marrow of newborns as well as that of adults, but there is evidence that these cells present an age-related decline in frequency[1]. Under defined culture conditions the MSCs may originate a spectrum of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat and a variety of other connective tissues[2-4].

In this context, tissue engineering has used a combination of scaffolds, of natural or synthetic origin, and cells or a combination of both to produce a three-dimensional tissue that should be structurally and mechanically similar to or better than the tissue that it was designated to replace[3,5].

Among the several different materials already tested may be cited polyurethane derived from the castor oil plant[6]; which is a polymer formed by the urethane reaction of two components, namely polyol and pre-polymer[7]. The product allows versatility of formulations[8], with the commercial presentations being especially moldable as biomass and pre-molded blocks[7]. This polymer has been studied to evaluate toxicity, biocompatibility, osteointegration, capacity to induce bone neoformation, among other properties[6,9-11]. Some properties are still controversial such as, the ability of polyurethane to perform as an osteoconductive agent or just as a space filler[9,11].

A prior study[6] evaluated polyurethane derived from the castor oil plant in cell culture with three different chemical compositions: pure, calcium carbonate addition or calcium phosphate addition. Bone marrow cells were cultivated under conditions that allowed osteoblastic differentiation. The results showed that addition of calcium carbonate and, even better, the addition of calcium phosphate facilitated the events that promoted matrix mineralization. Moreover, another study[12] cultivating MSCs from bone marrow with granules of castor oil polyurethane for seven days found that, besides initiating matrix deposition, the biomaterial allowed growth of MSCs that adhered and multiplied.

Since the chemical composition, consistency and format of the polymer may influence cell growth and adhesion to biomaterial, the aim of this study was to evaluate three methods to cultivate MSCs of rats and to analyze the potential of castor oil polyurethane in membrane format as a scaffold for MSCs.

Methods

Isolation of rat MSCs

This study followed the Guide for Care and Use of Laboratory Animals and was approved by the Ethics Committee of our Veterinary School (protocol nº 32/2009-CEEA). Four male Wistar rats, aged 20-30 days were used. Animals were euthanized by using halothane. Bone marrow aspirates from femur and tibia were harvested using Dulbecco's Modified Eagle Medium (DMEM) high glucose (DMEM + GlutaMAX - Gibco 10569-010) and heparin at a final concentration of 5U/mL.

The cell culture was performed in three different ways. In the first one, the bone marrow cell suspension was diluted with 4mL of DMEM high glucose, and transferred slowly onto Ficoll-Paque aqueous medium 1.077g/mL density, according to Boyum[13], and the material was centrifuged at 1500rpm for 40 minutes. After this, the mononuclear cell ring was collected and washed with 2mL of DMEM high glucose once at 1500rpm for 10 minutes. The cells were then resuspended in 5mL DMEM high glucose with 20% fetal calf serum (FCS), 100UI/ml of penicillin, 100µ/ml of streptomycin and 3µ/ml of amphotericin B and plated in 25 cm^2 culture bottles.

The second method was the same as the first, except that the density gradient utilized Ficoll-Paque aqueous medium at the density of 1.084g/ml. In the third method, bone marrow cell suspension was directly transferred to 25 cm^2 culture bottles in 5mL complete DMEM high glucose medium containing 20% FCS, 100UI/ml of penicillin, 100µ/ml of streptomycin, and 3µ/ml of amphotericin B.

The medium was changed every 5 days. Once the cells achieved 70% sub-confluence, they were resuspended, and the cell viability analysis was performed. Hoerscht 33342 (Sigma Chemical Co) staining was applied for 10-15 minutes; then the culture was washed with Phosphate buffered saline (PBS), and propidium iodide (Sigma Chemical Co) was used for 5 minutes. Finally the culture was washed with PBS to maintain cell integrity. Intact cells were stained blue by Hoerscht staining, and non-viable cells were stained red by propidium iodide. Fluorescence microscopy was used to analyze 5 different fields at 200X magnification.

Characterization of MSCs

To confirm the mesenchymal stem cell lineage, CD34 and CD44-specific surface antibodies (AbD Serotec, Oxford, UK) were used to mark mesenchymal cells. After the first

passage, the isolated cell populations were prepared according to the antibody manufacturer protocols. The CD34 antibody FITC (Fluorescein Isothiocyanate) -conjugated was negative at direct immunofluorescence staining for flow cytometry (FACS Calibur–BD). The CD44 antibody associated with RPE secondary antibody (R. Phycoerythrin) was positive at indirect immunofluorescence staining for flow cytometry.

Biomaterials

The membranes evaluated in this study were provided by the manufacturer (Biomecânica Indústria e Comércio de Produtos Ortopédicos Ltda.). The membranes (1.5 x 1.5 cm length, and 0.3-0.4 mm thick) were transparent, flexible, thermoplastic, semipermeable, sterilized with ethylene oxide, and composed of isocyanate and a different percentage of castor oil (membrane type 1=18%, membrane type 2=39.2%, membrane type 3=51.3%).

Evaluation of membrane surface

For the evaluation of the biomaterial's surface, a $1cm^2$ sample of each type of membrane was submitted to scanning electron microscopy in JEOL JSM5310, with a tungsten filament at 10kV, using secondary electron detectors. For this, the membrane samples were mounted onto a metal stub with double-sided carbon tape, and liquid silver coating was used to increase the conductivity of the material. The samples were then coated with gold using a Denton Vacuum Desk II sputter.

Biomaterials in cell culture

Each type of membrane was dampened with FCS and each well was filled with 5ml of a medium containing DMEM low glucose, 20% FCS, 100UI/ml of penicillin, 100µ/ml of streptomycin, and 3µ/ml of amphotericin B before MSCs were added. The membranes associated with the MSCs were kept on the 6-well plate for 5 days and were analyzed by optical microscopy to confirm cell aggregation and growth.

Results

Scanning electron microscopy of the membrane surfaces (from x200 to x7500) showed morphological and topographical differences depending on the percentage of castor oil (Figure 1). Defects, pores of non-uniformity diameter, castor oil fibers, bubbles, sulcus and reentrances varied in size and amount according to the percentage of castor oil in the membranes.

Membrane type 3 had a rough irregular surface and presence of more defects, pores, bubbles, sulcus, reentrances, and large amount of castor oil fibers. Membrane type 2 had an aspect similar to membrane type 3, but the bubbles, pores and castor oil fibers were fewer than in membrane type 3. Membrane type 1 showed microscopic geometric irregularities on the surface characterizing a smoother pattern comparing to the others. In addition, the membrane type 1 had fewer bubbles, pores and castor oil fibers than the other types.

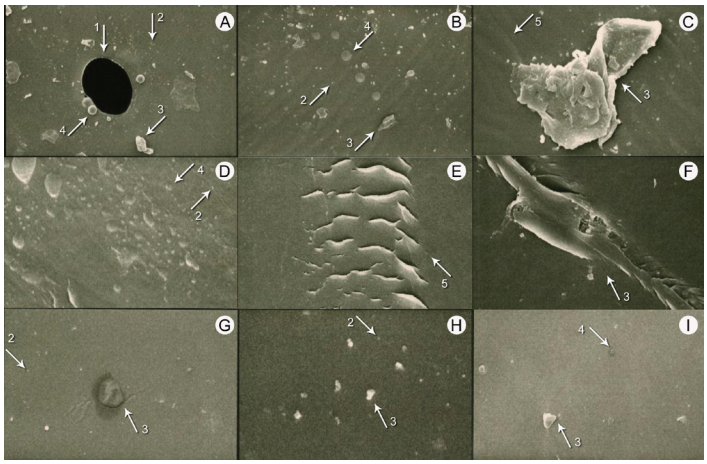

FIGURE 1 – Scanning electron microscopy of castor oil-based polyurethane membranes. Membrane type 3: x350 (**A**), x1500 (**B**), x2000 (**C**). Membrane type 2: x5000 (**D**), x2000 (**E**), x1500 (**F**). Membrane type 1: x1000 (**G**), x7500 (**H**), x200 (**I**). White arrows are showing defects (1), porous (2), castor oil fibers (3), bubbles (4), and sulcus and reentrance (5).

Separation methods 1 and 2 allowed adequate isolation of MSCs and favored cell growth with the passage being carried out at 70% confluence after 15 days in culture. Cell viability was the same in both methods with an average of 93% of cells intact and 7% non-viable (Figure 2). The cells could not be isolated using method 3.

Flow cytometry showed 83% CD 44-positive/34-negative (mesenchymal) and 17% CD 34-positive/44-negative (hematopoietic stem) markers.

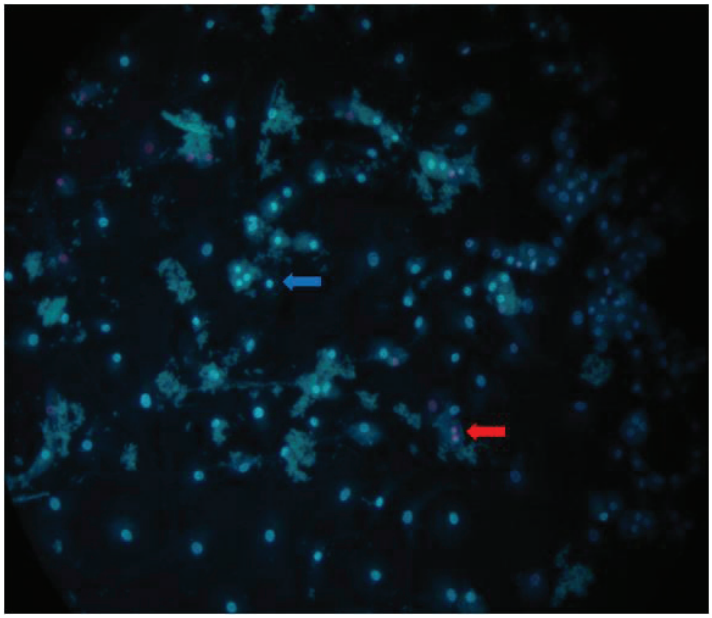

FIGURE 2 - Fluorescence microscope showing the cell viability: viable cells (blue arrow) and damaged cells (red arrow). (x200).

By optical microscopy, it was observed that the cell growth around the membranes was similar in the three types of membranes. However, the growth of MSCs on the membrane tops was approximately 80% in membrane type 3, 20% in type 2 and 10% above type 1 (Figures 3, 4 and 5).

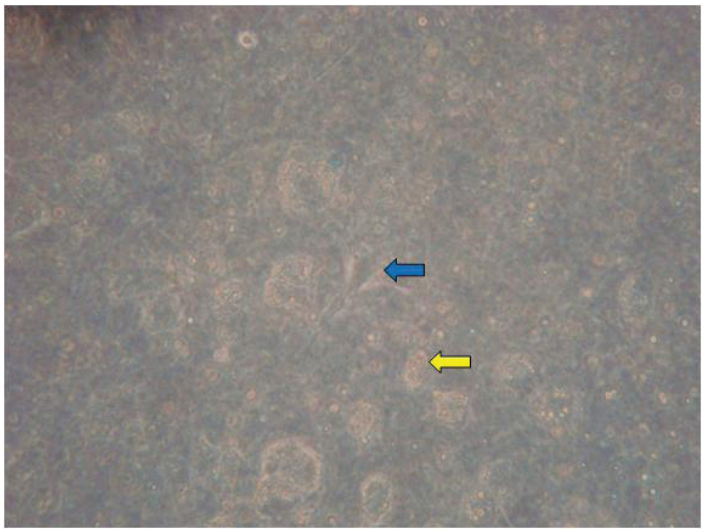

FIGURE 3 – Optical microscope showing the cells growth (blue arrow) and the apoptosis cells (yellow arrow) on the membrane 1 (x200).

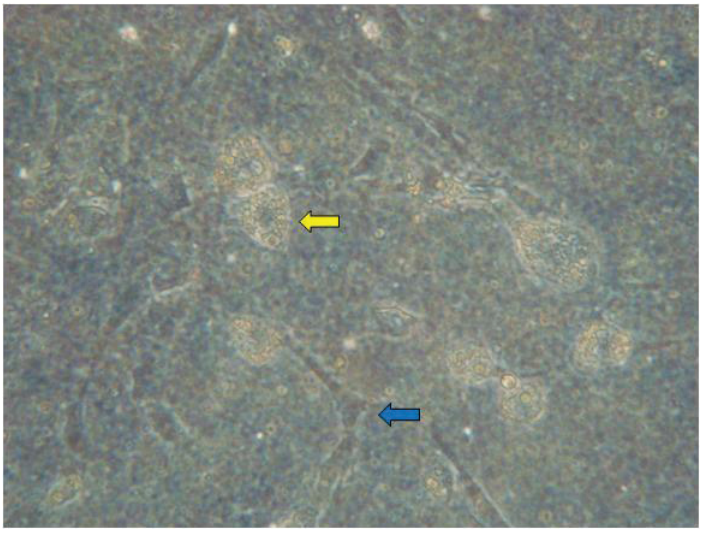

FIGURE 4 – Optical microscope showing the cells growth (blue arrow) and the apoptosis cells (yellow arrow) on the membrane 2 (x200).

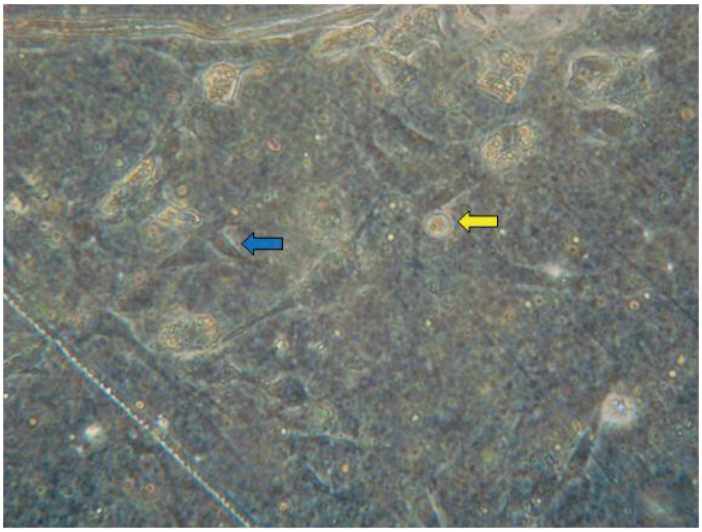

FIGURE 5 – Optical microscope showing the cells growth (blue arrow) and the apoptosis cells (yellow arrow) on the membrane 3 (x200).

Discussion

MSCs are a minor fraction of the total nucleated cell population in bone marrow and thus they require isolation and expansion in culture to obtain sufficient numbers for clinical or experimental use[2,3]. In general, the sample of whole bone marrow is submitted to fractionation in solution based on density gradient, after which the cells are plated at densities from 1 x 10^4 cells/cm^2 to 0.4 x 10^6 cells/cm^2 [2]. The present study revealed that both 1.084 g/mL and 1.077 g/mL density gradients were good to obtain cell isolation, which was evidenced by the high percentage of CD 44-positive cells. The separation of erythrocytes and granulocytes

present in the sample was facilitated by the fact that after dilution the bone marrow aspirate had a lower density than the reagents[14]. On the other hand, the direct culture was demonstrated to be inadequate. As cited by other authors, purification of MSCs only by their adherence to plastic may not be sufficient[2].

Scaffolds for MSCs should allow cell adhesion, be porous, permit bioactive molecules to have contact with cells, integrate in the neotissue, supply cellular cueing and be mechanically adequate for the site, among other properties[3]. Scaffolds composed of various classes of biomaterials have been tested, each one with their advantages and disadvantages. However, they should provide mechanical and structural properties similar to the tissue to be replaced[3,15].

The castor oil polyurethane in membrane format could be used, for example, in promoting guided bone regeneration. A barrier membrane serves the function of excluding unnecessary cell lines and allowing the growth of required tissues[16,17]. For this purpose, factors such as composition, morphology, surface texture, presence of pores, and reabsorption capacity are determinate for membrane function[17,18]. In the present study the differences in castor oil concentration influenced the presence of pores, bubbles, sulcus, reentrances, and castor oil fibers among the membranes.

Concerning the MSC behavior on polyurethane membranes, it was possible to prove the growth and adhesion of cells by optical microscopy, a similar finding was observed in two other studies[6,12]. In the first study[6] the castor oil polyurethane was co-cultured with rat bone marrow cells, but under conditions that allowed osteoblastic differentiation, while the second one[12] used dog's MSCs. Both studies used compact polyurethanes discs[6] or granules[12] that may have contributed to cell adhesion. Additionally, the granules had calcium carbonate in their composition, what, according to the author, contributed to the initiation of matrix deposition by MSCs, as observed by electron microscopy[12]. In contrast, cell attachment and ALP activity were not affected by the chemical composition of the discs, but the events that promoted matrix mineralization were favored by the addition of calcium carbonate or calcium phosphate, both of which are considered more osteoconductive than pure polyurethane[6].

In the present study the membranes with a higher percentage of castor oil showed the greatest growth of MSCs. Theses membranes (types 2 and 3) had more surface pores than membrane type 1 as observed by scanning electron microscopy. These pores are formed by incorporation of air bubbles during the mixture of the basic polyurethane components[7,19] and were associated with higher castor-oil concentrations. Pore size and surface characteristics of a biomaterial are important for cell attachment and growth[20]. Therefore, in addition to porousness, the presence of the bubbles, sulcus, reentrances and castor oil fibers in membranes types 2 and 3 may have contributed to increasing the contact surface for the cells, allowing adhesion, growth and multiplication of the MSCs.

Conclusion

Both Ficoll-Hypaque densities allow isolation of rat MSCs; and especially castor oil-based membrane type 3 may be used as a scaffold for MSCs.

References

1. Fibbe WE. Mesenchymal stem cells. A potential source for skeletal repair. Ann Rheum Dis. 2002;61 Suppl 2:ii29-31.
2. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol. 2004;36:568-84.
3. Caplan AI. Mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005;11(7/8):1198-1209.
4. Conrad C, Huss R. Adult stem cell lines in regenerative medicine and reconstructive surgery. J Surg Res. 2005;124(2):201-8.
5. Stock UA, Vacanti JP. Tissue engineering: current state and prospects. Ann Rev Med. 2001;52:443-51.
6. Beloti MM, Hiraki KR, Barros VM, Rosa AL. Effect of the chemical composition of *Ricinus communis* polyurethane on rat bone marrow cell attachment, proliferation, and differentiation. J Biomed Mater Res A. 2003;64(1):171-6.
7. Bioosteo. 2010. Disponível em: http://www.biomecanica.com.br Acessado em: 10 jul 2010.
8. Ohara GH, Kojima KE, Rossi JC, Telles M, Soares TVC, Salomão C, Sanda M. Estudo experimental da biocompatibilidade do polímero poliuretano da mamona implantada intra-óssea e intra-articular em coelhos. Acta Ortop Bras. 1995;3(2):62-8.
9. Ignácio H, Mazzer N, Barbieri CH, Chierice GO. Use of compact and porous castor oil polyurethane in the filling of bone defects. Experimental study in dogs. Rev Bras Ortop. 2002;37(5):187-94.
10. Barros VM, Rosa AL, Beloti MM, Chieriche G. In vivo biocompatibility of three different chemical compositions of *Ricinus communis* polyurethane. J Biomed Mater Res A. 2003;67(1):235-9.
11. Pereira-Junior OCM, Rahal SC, Felisbino SL, Iamaguti P, Pavan PT, Vulcano LC. Comparison between polyurethanes containing castor oil (soft segment) and cancellous bone autograft in the treatment of segmental bone defect induced in rabbits. J Biomater Appl. 2007;(21):283-97.
12. Pereira-Junior OCM. Biomateriais como arcabouço para sustentação de células-tronco mesenquimais (Tese de Doutorado). Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu; 2009.
13. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest. 1968;97:77-89.
14. Costa PCS, Muccillo FB. Obtenção de células mononucleares de medula óssea (CMMO). Instituto Nacional de Cardiologia de Laranjeiras (INCL), 2005. Disponível em: http://www.terapiacelular.hcl.gov.br. Acesso em: 28 jul 2010.
15. Bruder SP, Fox BS. Tissue engineering of bone. Cell based strategies. Clin Orthop Relat Res. 1999;(367 Suppl):S68-83.

16. Zellin G, Linde A. Treatment of segmental defects in long bone using osteopromotive membranes and recombinant human bon morphogenetic protein-2. Scand J Plast Reconstr Hand Surg. 1997;31:97-104.
17. Takata T, Wang HL, Miyauchi M. Migration of osteoblastic cells on various guided bone regeneration membranes. Clin Oral Implants Res. 2001;12:332-8.
18. Dongieux JW, Block MS, Morris G, Gardiner D, Dean K. The effect of different membranes on onlay bone graft success in the dog mandible. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998;86(2):145-51.
19. Claro Neto S. Caracterizações fisicoquímica de um poliuretano derivado de óleo de mamona utilizado para implantes ósseos (Tese de Doutorado). São Carlos: Instituto de Química de São Carlos, Universidade de São Paulo; 1997.
20. Kose GT, Ber S, Korkusuz F, Hasirci V. Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) based tissue engineering matrices. J Mater Sci Mater Med. 2003;14(2):121-6.

Correspondence:
Sheila Canevese Rahal
Faculdade de Medicina Veterinária e Zootecnia, UNESP
Rubião Júnior, s/n
18618-000 Botucatu – SP Brasil
Tel: (55 14)3811-6054
sheilacr@fmvz.unesp.br

Received: January 26, 2011
Review: March 24, 2011
Accepted: April 20, 2011
Conflict of interest: none
Financial source: CNPq