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Nested-PCR multiplex test with increased sensitivity for detection of allogeneic cells transplanted from male to female mice

Nested-PCR *multiplex* com aumento da sensibilidade de detecção de células alogênicas transplantadas de camundongos machos para fêmeas

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ABSTRACT

Cell therapy has shown encouraging perspectives for human and veterinary medicine. Experimentally, genetic manipulation allows to mark and locate allogeneic cells. However, this makes their genotype/phenotype different from non-marked cells used clinically. Alternatively, the presence of the Y-chromosome enables male donor cells detection in female organisms. However, the concentration of engrafted cells may be minimal in tissues, due to systemic distribution. In this study, a nested-PCR multiplex test was developed, aiming to increase the sensitivity of the presence/absence diagnosis of male mice adipose-derived (ADSC-Y) and bone marrow mononuclear (BMNC-Y) cells in samples of blood and lungs from females, after endovenous transplantation. Four females received placebos; four females received ADSC-Y from two males; and four females received BMNC-Y from two males. The PCR first-step included two primer sets (multiplex): one for amplification of a Y-chromosome fragment (SRYout; 300bp); the other for amplification of an X-chromosome (DXNds3 gene) fragment. In the PCR second-step, one primer set (SRYinn) was used for amplification of a 110bp fragment, restrained in the SRYout amplification product. The PCR internal control (DXNds3 gene) was detected in all DNA samples, whereas the SRY gene external fragment (300bp) was detected exclusively in ADSC-Y and BMNC-Y pure DNA samples. The SRY gene internal fragment (110bp) was detected in 100% of the blood and lung samples from the ADSC-Y and BMNC-Y female recipients. The nested-PCR technique increased sensitivity and reliability for molecular diagnostic of presence or absence of male mice cells in body fluids and tissues of female recipients after endovenous transplantation.

Key words: *cellular therapy, transplantation, allogeneic cells, diagnosis, Y-chromosome.*

RESUMO

A terapia celular traz perspectivas encorajadoras à medicina humana e veterinária. Experimentalmente, a manipulação genética permite a marcação e a localização de células alogênicas. Porém, isso torna seu genótipo/fenótipo diferente daquelas usadas clinicamente, sem marcação. Alternativamente, a presença do cromossomo Y possibilita detectar células de doadores machos no organismo de fêmeas. Todavia, a concentração de células transplantadas pode ser mínima em certos tecidos, pela distribuição sistêmica. Neste estudo, foi desenvolvida uma nested-PCR multiplex, visando a aumentar a sensibilidade do diagnóstico de presença/ausência de células derivadas do tecido adiposo (CDTA-Y) e derivadas da fração mononuclear da medula óssea (CFMO-Y) de camundongos machos, em amostras de sangue e de pulmões de camundongos fêmeas, após transplante endovenoso. Quatro fêmeas receberam placebo; quatro fêmeas receberam CDTA-Y de dois machos; e quatro fêmeas receberam CFMO-Y de dois machos. A primeira fase da PCR teve dois pares de primers (multiplex): um para amplificação de fragmento do cromossomo Y (SRYout; 300pb); outro para amplificação de fragmento do cromossomo X (gene DXNds3). Na segunda fase da PCR, foi usado um par de primers para amplificação de fragmento de 110pb (SRYinn) interno ao produto amplificado pelo SRYout. O controle interno da reação (gene DXNds3) foi detectado em todas as amostras de DNA testadas, enquanto que o fragmento externo do gene SRY (300pb) foi detectado apenas nas amostras puras de DNA de CDTA-Y e CFMO-Y. O fragmento interno do gene SRY (110pb) foi detectado no sangue e nos pulmões de 100% das receptoras de CDTA-Y e CFMO-Y. A técnica de nested-PCR aumentou a sensibilidade e a segurança do diagnóstico molecular de presença ou ausência de células de camundongos machos em fluidos e tecidos de receptoras fêmeas após transplante endovenoso.

Palavras-chave: *terapia celular, transplante, células alogênicas, diagnóstico, cromossomo Y.*

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INTRODUCTION

Cell therapy continues to evolve as one of the most promising frontiers of scientific knowledge applied to human and veterinary health (FORTIER & TRAVIS, 2011; OTSURU et al., 2012; TABAR & STUDER, 2014). However, its efficacy and safety are not fully established either for animals or humans, in view of the risks concerning tumor induction and transplantation rejection (DJOUAD et al., 2003; KRSTEVSKA et al., 2011). The cell therapy has been tested for several purposes such as: repair and functional regeneration of organs as heart, lungs, liver, kidneys and skin; bone tissue remodeling; bone marrow transplantation and bone marrow cancer therapy; treatment of degenerative neurological diseases; and treatment against autoimmunity and graft-versus-host disease, among others (BARRY & MURPHY, 2004; LAU et al., 2009; LINDVALL & KOKAIA, 2010; FORTIER & TRAVIS, 2011; MIELCAREK et al., 2012; ZANETTI et al., 2013). The allogeneic cells can be administered systemically or locally, by injection, infusion or controlled releasing systems of the cells or their chemical messengers (SHIM et al., 2013; LIU et al., 2014).

Cell therapy can act by paracrine or systemic signaling, and has been used to modulate immune response and promote cellular differentiation, depending on the cell type (BARRY & MURPHY, 2004). The pattern of distribution of the allogeneic cells throughout the recipient organism depends on such factors as the cell type, administration route and the immune response to transplantation (GAO et al., 2001; LIAO et al., 2008; LINDVALL & KOKAIA, 2010). Molecular or histological detection of allogeneic cells in body fluids and tissues from recipients demonstrates the organic distribution of these cells after transplantation, whereas their effect may be related to the clinical course of the disease. However, this also depends on the interaction of other factors inherent to the environment, the patient and the disease etiology. Therefore, knowing the fate of transplanted allogeneic cells is essential for understanding their function, which may be due to the allogeneic cells' location in the tissues or due to mediators secreted by these cells.

In general, genetically manipulated cells are used to allow detection of these cells after experimental transplantation (LIAO et al., 2008; OTSURU et al., 2012). However, this kind of manipulation of the cell population is not desirable in clinical trials, as the genotype/phenotype of the marked cells is different from non-marked cells. Consequently, the effects of the therapy and risk factors previously verified with genetically manipulated cells may be not repeatable in clinical therapy with non-marked cells.

Detection of specific DNA fragments of the Y-chromosome can be used to localize cells from a male donor into female recipient tissue (WULF et al., 2003; TAKEHARA et al., 2013; LIU et al., 2014). However, detection sensitivity is a key-factor in these cases, since the concentration of allogeneic cells may be minimal in the blood and tissue samples from recipients, due to a broad systemic distribution after transplantation (GAO et al., 2001; LIAO et al., 2008). In a single-step PCR, one pair of primers is used for exponential amplification of the targeted DNA fragment (amplicon). Thus, in a second-step reaction with another specific pair of primers (nested-PCR), an internal sequence of DNA restrained in the first-step amplicon can be exponentially amplified. This step increases the sensitivity of molecular detection due to the increased number of the targeted DNA fragment copies. Besides, the internal fragment amplification depends on the complementarities between primers and DNA sequences in the two steps of the nested-PCR, thus increasing the specificity of molecular diagnosis (KUNIEDA et al., 1992).

The aim of this study was to develop a nested-PCR test to improve the certainty of the diagnosis for presence/absence of allogeneic DNA in the blood and tissue of recipients, after male-female transplantation of adipose-derived (ADSC-Y) and bone marrow mononuclear-derived (BMNC-Y) cells. An additional detection of a DNA fragment belonging to the X-chromosome was included as an internal control of viability of each sample in the first step of the PCR (multiplex), constituting a nested-PCR with the first-step multiplex.

MATERIAL AND METHODS

The products used were provided by Sigma-Aldrich, USA, except where explicitly mentioned.

Animals, procedures and facilities

The adult eight weeks old Balb/c mice used, four male donors and 12 female recipients, were kept in controlled environmental conditions throughout the experimental period. Euthanasia of donors and recipients and cell therapy procedures described below were done according to the regulations of the CONCEA, Brazil.

Adipose-derived cells from male donors (ADSC-Y)

The ADSC-Y were isolated from adipose tissue of the inguinal region from two male donors,

after euthanasia. Tissue samples were washed with PBS, digested with 1 mg ml^{-1} type II collagenase in PBS solution (37ºC; 30min) and centrifuged (800*g*; 10min). The pellet was resuspended in DMEM-F12 and the cells were cultured in cell culture flasks (TPP, Switzerland) at 37ºC, 5% CO_2 and saturated humidity, with DMEM-F12 supplemented with 10% bovine fetal serum (Invitrogen, Brazil), penicillin (100 UI ml^{-1}), streptomycin (100µg ml^{-1}) and amphotericin B (0.25µg ml^{-1}). The first medium change was performed at 72h after culture, removing non-adherent cells from the culture surface. Cell passages were performed when the culture confluence was 80-90%, using 0.25% trypsin solution diluted in Hank's Balanced Salt Solution.

ADSC-Y were characterized by adhesion to culture surface, morphology and osteogenic differentiation (LIAO et al., 2008). A portion of the fourth-passage cells was submitted to an osteogenic differentiation protocol. Another portion was suspended with 0.25% trypsin, diluted in DMEM-F12 and prepared for transplantation, at a dose based on viable cells counting in Neubauer's chamber (aliquot diluted 1:1 with 0.4% Trypan Blue solution). An aliquot of ADSC-Y was stored at -80ºC prior to DNA extraction.

Osteogenic differentiation of the ADSC-Y

Fourth-passage ADSC-Y were plated in six-well dishes, at a concentration of 5,000 cells cm^{-2} (or $1x10^5$ cells/well), and cultured without passages for 21 days. In three of these wells, cells were cultured with complete DMEM-F12 (control), and in the other three wells, with complete DMEM-F12 supplemented with osteogenic inductive factors (differentiation medium). Both culture mediums were renewed every 72h. The differentiation medium contained ascorbic acid (5µg ml^{-1}), β-glycerophosphate (10mM) and dexamethasone (10nM). At 21 days, each well was washed with PBS and the cell monolayer was fixed with 4% paraformaldehyde for 20min at room temperature, followed by washing with deionized water and incubation with 2% Alizarin Red dye at pH 4.1 in aqueous solution for 5min, finishing with five washings with deionized water and evaluation of the calcium matrix deposition (MEIRELLES & NARDI, 2003; LIAO et al., 2008).

Bone marrow-derived mononuclear cells from male donors (BMNC-Y)

The BMNC-Y were harvested from two donors euthanized at transplantation day. Femurs and tibias were isolated and their extremities were cut for bone-marrow collection as described by TROPEL et al. (2004). Briefly, DMEM-F12 was injected using a 1ml syringe with 25G needle attached to the marrow cavity, exerting pressure for bone-marrow removal embedded in culture medium. Subsequently, harvested bone-marrow cells were resuspended by repeated pipetting and were centrifuged for 30min at 400*g* in Ficoll-Paque™ PREMIUM gradient with density of 1.077±0.001g ml^{-1} (GE Healthcare, Sweden). The mononuclear cells fraction, separated by density, was recovered, washed twice in PBS (two cycles of resuspension and centrifugation at 400*g* for 5min), resuspended in 2ml of DMEM-F12 and prepared for transplantation, at a dose based on viable cells counting, as described above for ADSC-Y. An aliquot of BMNC-Y was stored at -80°C prior to DNA extraction.

Experimental design

The females were separated into three groups with four animals in each: group 1. 100µl of DMEM-F12 endovenous (e.v.; placebo); group 2. $1x10^5$ ADSC-Y diluted in 100µl of DMEM-F12 (e.v.); group 3. $1x10^5$ BMNC-Y diluted in 100µl of DMEM-F12 (e.v.). Approximate cell viabilities of ADSC-Y and BMNC-Y injected were 97% and 92%, respectively. The doses of the cells injected were adjusted to $1x10^5$ viable cells per recipient. Each female received the cell transplantation (or placebo) by injection in the caudal vein, using 1ml syringe and 25G needle.

Four days after the injection of allogeneic cells, which was considered as sufficient time for the distribution of these cells in the recipient organism (GAO et al., 2001), each female was anesthetized by isoflurane inhalation (Virbac, Brazil) and a sample of 300µl of blood was collected by submandibular puncture. This was followed by euthanasia by anesthetic deepening and cervical dislocation. Since transplanted cells could be located in lungs (GAO et al., 2001; LIAO et al, 2008), besides blood sampling, a piece of approximately 15mg was collected from a random region of the lungs from each female. Thus, reliability of the nested-PCR multiplex technique could be evaluated in body fluids and organs samples. The samples of blood and lungs were stored at -80ºC prior to DNA extraction by the Wizard Genomic DNA Purification Kit (Promega, USA). Each lung sample was cut, macerated and homogenized on sterile polystyrene plate (Corning, USA), using scalpel blade (Advantive, China) and serological tip (Bio-Centrix, USA) following the DNA extraction protocol.

Allogeneic cells DNA detection

The first-step of the PCR was multiplex, using 50ng of DNA template with a ratio of absorbance

≥1.7 (260/280nm) measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). At this step, two pairs of primers were used: one to amplify a fragment of DXNds3 gene, a polymorphic microsatellite in Balb/c (244/270bp; forward: 5'GAGTGCCTCATCTATACTTACAG3'; reverse: 5'TCTAGTTCATTGTTGATTAGTTGC3'; KUNIEDA et al., 1992), which allows the evaluation of DNA template viability in the first electrophoretic run; and another (SRYout), designed to amplify the external fragment (300bp) of the SRY gene ENSMUSG00000069036; (forward: 5'CGCCCCATGAATGCATTTAT3'; reverse: 5'CCTGTCCCACTGCAGAAGGT3'), being accepted that this fragment does not appear at the first electrophoretic analysis, considering that a low concentration of allogeneic cells may be present in the samples of blood and lungs from recipients.

The second-step of the PCR was performed using the first-step generated amplicon as DNA template and one pair of primers (SRYinn; forward: 5'GAAAAGCCTTACAGAAGCCGAAA3'; reverse: 5'CCCTCCGATGAGGCTGATATT3'), designed to amplify a 110bp (internal) fragment, restrained in the 300bp (external) fragment amplified in the first-step reaction. SRYout and SRYinn were designed using the Primer Express software (Applied Biosystems, USA) and all the primers used were synthesized by IDT, Brazil.

Each nested-PCR step was performed using 2μl of DNA sample (initial concentration of 50ng), 200nM of each pair of primers and 2.5U of the enzyme Platinum® TaqDNA Polymerase (Invitrogen, Brazil) in a total mix volume of 25μl. The mix was submitted to 40 cycles of 95°C for 30s, 60°C for 60s and 72°C for 2min, in a PTC 100™ Thermal Cycler (MJ Research, USA). Genomic DNA samples from pure populations of ADSC-Y and BMNC-Y were used as positive controls and deionized water was used as a negative control. The amplified products resulting from each PCR step were analyzed by electrophoresis in 10% polyacrylamide gel stained with silver nitrate for DNA bands visualization.

RESULTS AND DISCUSSION

Figures 1A and 1B show the undifferentiated and differentiated ADSC-Y monolayers, respectively. Adherence and morphology of ADSC-Y in culture, besides calcium deposition after induction of osteogenic differentiation, were compatible with adipose-derived mesenchymal cells (LIAO et al., 2008).

Figures 1C and 1D show the main results observed by electrophoresis after the first and second steps of PCR, respectively. The DXNds3 gene, belonging to the X-chromosome, was detected in 100% of the DNA samples (groups 1, 2 and 3 and both ADSC-Y and BMNC-Y positive controls). This amplification (244/270bp; Figure 1C) ensures the viability of the DNA template and confirmed the appropriate conditions for the PCR, acting as an internal control for each sample. Therefore, amplification of DXNds3 gene fragment (244/270bp) was tested in the first step of the PCR (multiplex), along with SRYout.

The 300bp fragment (amplified by SRYout) appeared exclusively in positive controls (Figure 1C), which contained DNA extracted from pure populations of ADSC-Y and BMNC-Y. Despite this, SRY gene was present in 100% of the blood and lung samples from females which received ADSC-Y (group 2) or BMNC-Y (group 3), as proven by visualization of the internal fragment (110bp) by electrophoretic analysis after the PCR second-step (Figure 1D).

The results showed that both the sensitivity and specificity of the PCR first-step were 100% reliable, considering only the positive and negative controls, and all the samples from group 1 (placebo). However, comparing the electrophoretic analysis of the first- and second-step PCR, it was evident that the results were false-negative for the presence of SRY gene in samples from groups 2 and 3 in the first-step reaction. This was probably due to the low concentration of allogeneic DNA in proportion to the total genomic DNA extracted from each sample of blood and lung. Thus, the first-step PCR was inefficient for molecular diagnosis of allogeneic cells distribution after transplantation, justifying the use of a nested-PCR.

In this study, a low concentration of $1x10^5$ allogeneic cells in single dose was systemically transplanted into each recipient. If higher doses and/or other routes of administration are used, allogeneic cells may be present at higher concentration in the body fluids and tissues of recipients. Therefore, these cells could be detectable by single-step PCR as showed by LIU et al. (2014). However, the results of the present study clearly showed that nested-PCR can provide molecular detection of allogeneic cells at low concentrations, in samples which would be considered negative using a single-step PCR.

A highly sensitive molecular diagnosis is beneficial for *in vivo* studies in humans or animals, since it allows the use of smaller samples for DNA extraction, which diminishes risks to the patients, facilitates collections by biopsies and enables a higher frequency of sampling without euthanasia in animal models (WULF et al., 2003; MEYERROSE et

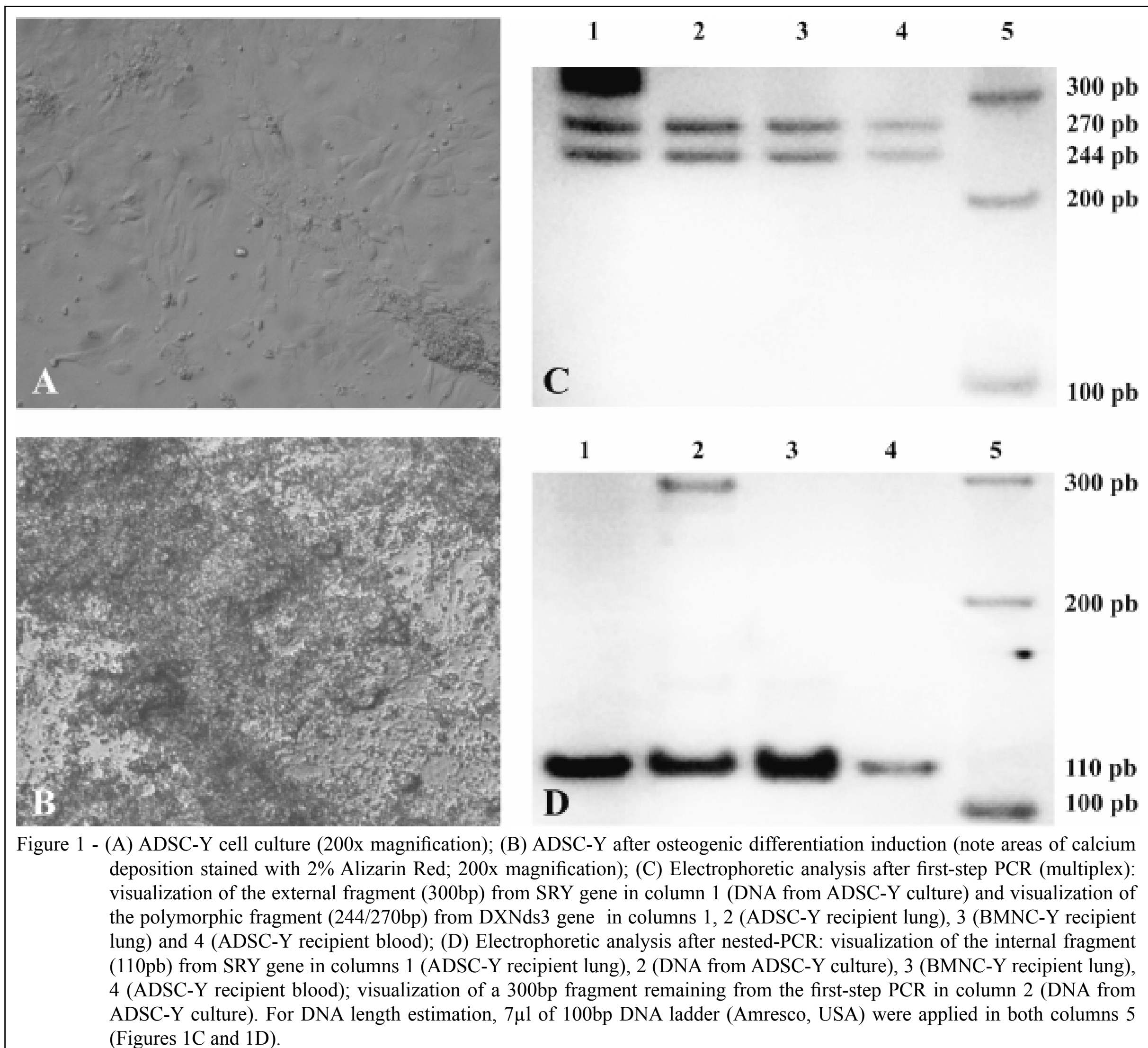

Figure 1 - (A) ADSC-Y cell culture (200x magnification); (B) ADSC-Y after osteogenic differentiation induction (note areas of calcium deposition stained with 2% Alizarin Red; 200x magnification); (C) Electrophoretic analysis after first-step PCR (multiplex): visualization of the external fragment (300bp) from SRY gene in column 1 (DNA from ADSC-Y culture) and visualization of the polymorphic fragment (244/270bp) from DXNds3 gene in columns 1, 2 (ADSC-Y recipient lung), 3 (BMNC-Y recipient lung) and 4 (ADSC-Y recipient blood); (D) Electrophoretic analysis after nested-PCR: visualization of the internal fragment (110pb) from SRY gene in columns 1 (ADSC-Y recipient lung), 2 (DNA from ADSC-Y culture), 3 (BMNC-Y recipient lung), 4 (ADSC-Y recipient blood); visualization of a 300bp fragment remaining from the first-step PCR in column 2 (DNA from ADSC-Y culture). For DNA length estimation, 7µl of 100bp DNA ladder (Amresco, USA) were applied in both columns 5 (Figures 1C and 1D).

al., 2007; LIU et al., 2014). Along with the increased sensitivity provided by the PCR second-step, the internal control of samples viability included in the PCR first-step (DXNds3 gene), improved the confidence of the diagnosis of the presence or absence of allogeneic cells DNA.

Other techniques may be used in order to increase the sensitivity and assuredness of molecular diagnostic, such as real-time qPCR, especially with specific probes (IMBERTI et al., 2011). However, expensive reagents and specific thermal cyclers are required for qPCR. Moreover, except for one pair of primers (internal), the nested-PCR does not require any type of reagent or equipment besides those used to single-step PCR. Furthermore, the second-step reaction is dispensable for the samples in which the gene of interest (SRY) is detected in the PCR first-step, diminishing the final cost of diagnosis. All these features accredit the nested-PCR as an extremely reliable technique, which is affordable to most research laboratories.

In turn, *in situ* hybridization may be used for elucidating the structural location of allogeneic cells in tissue samples. However, this technique is laborious, requires histological preparation and may be less sensitive for low concentrations of allogeneic cells (TAKEHARA et al., 2013). On account of this, even when the location of allogeneic cells into tissues is desired, a preliminary mapping of their distribution in the recipient organism is recommended. In these cases, a screening by nested-PCR can be used to avoid unnecessary processing of samples from negative tissues by *in situ* hybridization.

The nested-PCR test can be used to monitor the fate of allogeneic transplanted cells into

recipient organism, without cell labeling by genetic manipulation. Therefore, nested-PCR test is adequate for experimental or clinical purposes. The presence/absence diagnosis shows the organs to which transplanted cells have spread (or not) and settled (or not) and can be done at several sampling points over time elapsed from transplantation. Although molecular detection of the SRY gene has been designed exclusively for male-female transplants, it is important to note that the incidence of chronic graft-versus-host disease is lower in transplants involving male donors (GRATWOHL et al., 2001; VERDIJK et al., 2004).

In this study, ADSC-Y were transplanted after four passages in culture and BMNC-Y were transplanted soon after collection, consisting of very different cell populations regarding their original niche and characteristics (LIAO et al., 2008; MIELCAREK et al., 2012; OTSURU et al., 2012; TAKEHARA et al., 2013). Since the nested-PCR multiplex described in this study was reliable for two different populations of allogeneic transplanted cells, this method may be used for other cell types, such as adult or embryonic stem cells.

CONCLUSION

Both internal control and PCR second-step increased the reliability of the molecular diagnosis for the presence or absence of different types of allogeneic cells in blood and tissues of recipients. The nested-PCR multiplex developed in this study provides a more sensitive evaluation of the organic distribution of male-derived cells in body fluids and tissues of female recipients in comparison to single-step PCR.

ETHICS COMMITTEE

Procedures involving animals were performed in accordance to CEUA-UFSM (registration number: 095/2011).

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