BIOCELL
2008, 32(2): 175-183

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Behavior of mesenchymal stem cells stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) in osteogenic and non osteogenic cultures

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Keywords: bone marrow, cell cultures, rat, osteogenic differentiation

ABSTRACT: 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) is a DNA dye widely used to mark and trace stem cells in therapy. We here studied the effect of DAPI staining on the behavior of mesenchymal stem cells cultured in either a control, non-osteogenic medium or in an osteogenic differentiation medium. In the control medium, the number of stem cells/field, as well as the number of fluorescent cells/field increased up to the sixth day in both control and DAPI-treated cultures. Afterwards, both the number of fluorescent cells and their fluorescence intensity decreased. Control cells were fusiform and with some long extensions that apparently linked them to neighboring cells, while DAPI-treated cells were mostly round cells with fine and short extensions. The trypan-blue exclusion method showed 99% cell viability in both groups, however, both alkaline phosphatase activity and the thiazolyl blue formazan assay (indicative of mitochondrial metabolism) gave significantly lower values in DAPI-marked cells. The mitochondrial mass, as indicated by specific staining and flow cytometry, showed no differences between groups. Mesenchymal stem cells gave origin to mineralized nodules in the osteogenic differentiation medium and there were not DAPI-marked cells on the ninth day of culture. Alkaline phosphatase activity, viability assay and number of cells/field and of mineralized nodules/field were similar in both groups. So, DAPI treatment did not change cell viability and proliferation during osteogenic differentiation of mesenchymal stem cells. However, since these cells loose DAPI marking after 9 days in osteogenic cultures suggests that DAPI may not be an effective marker for mesenchymal stem cells implanted in bone tissue for long periods.

Introduction

The interaction of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) with DNA has been the subject of numerous studies (Kubista *et al*., 1987; Blaisdell and Wallace, 2007; Shin *et al*., 2007). This substance was developed as a diamidino compound, analogous to either diminazene aceturate (Berenyl) or stilbamidine, to be used as a trypanocidal agent. DAPI however showed a variety of biological effects, including antibiotic, anti-trypanosomal and antiviral activity, and it is now an important tool in molecular biology and citochemistry. As such, DAPI is a fluorescent substance which exhibits several binding modes to DNA (Wilson *et al*., 1990; Eriksson *et al*., 1993), being transferred to descendant cells during cell proliferation. Studies on

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Received on August 24, 2007. Accepted on May 15, 2008.

apoptosis utilize DAPI staining to verify the morphology of apoptotic bodies and the chromatin condensation (Kim *et al*., 2006).

Mesenchymal stem cells are pluripotent stem cells mostly residing within the adult bone marrow. In contrast to hematopoietic cells, these cells are adherent and can be expanded in culture. They may differentiate not only into osteoblasts, neurons, and skeletal muscle cells, but also into vascular endothelial cells and cardiomyocytes (Jiang *et al*., 2002).

With the rapid increase of reported uses of stem cells to treat cardiovascular (Li *et al*., 2006), retinal, neurological (Mayhall *et al*., 2004) and bone diseases (Kraus and Kirker-Head, 2006; Wang *et al*., 2006), it has become important to track stem cells *in vivo* during clinical trials. For such purposes, the labeling agent associated with a stem cell must be biocompatible, safe, non toxic and should allow the location and quantification of stem cells in the organism (Frangioni and Hajjar, 2004). DAPI apparently meets these criteria and is being widely used to mark and trace stem cells used in therapy (Thompson *et al*., 2004; Niagara *et al*., 2004; Richardson *et al*., 2005; Tang *et al*., 2006).

Studies in human leukemic marrow cells showed that DAPI doesn't modify their proliferation and it is not cytotoxic for these cells *in vitro* (Park *et al*., 1985; Tarnowski *et al*., 1993). Also, Niu *et al*. (2004) and Tang *et al*. (2006) have shown that *in vivo* DAPI marking does not show negative effects on mesenchymal stem cells used in the treatment of injured rat hearts. However, *in vitro* studies to verify the effect of DAPI on mesenchymal stem cells in osteogenic and non osteogenic cultures appeared necessary, and this was the aim of the present study.

Materials and Methods

Cell harvesting and culture

Adult, 130-140 g male rats were used. They were housed under a 12-hour- ligth/dark cycle and were fed ad libitum with a commercial rat chow containing 22% of crude protein, 1.4% of calcium and 0.6% of phosphorus. Water was also permanently available. To obtain bone marrow samples, rats were killed with an overdose of anesthesia (Tionembutal 2.5%), as approved by the Ethical Committee of the Federal University of Minas Gerais.

The femurs and tibias were dissected from attached muscle and connective tissue under aseptic conditions and the epiphyses were removed. Bone marrow was flushed out with Dulbecco's modified Eagle's medium-DMEM (Gibco, Grand Island, NY). The released cells were suspended in DMEM supplemented with 10% fetal bovine serum (Gibco) plus antibiotics (60 µg/L gentamicin, + 25 µg/L amphotericin B, 10.000 U/mL penicillin, and 10.000 µg/mL streptomycin, (Merck, Germany), and collected in a 75 cm^2 culture flask containing 10 mL culture medium. The cells were grown at 37ºC and 95% O_2 + 5% CO_2 for 3 days. The nonadherent cell population was removed and the adherent layer washed once with fresh media. Culture medium was changed twice weekly. After 3 weeks of culture, the adherent cells were detached by trypsin and seeded for experiments.

Phenotypic characterization (experiment 1 and 2)

Cells at passage fourth were harvested with trypsin/EDTA, centrifuged at 1400 rpm for 10 min, and resuspended at $1x10^6$ cell/well in phosphate buffered saline (PBS, 0.01 M phosphate buffer, pH=7.4, and 0.9% NaCl). Exposure of cell suspensions to a 1: 50 dilution of a single primary antibody (either against CD45, CD90, CD73 or CD54, BD Biosciences, San Jose, CA USA) were made for 30 min at 4ºC, while control cell suspensions with no antibody were simultaneously run. Afterwards, the cells were washed in PBS and incubated with fluorescent conjugated secondary antibody for 30 min at 4ºC. At least 20.000 events per sample were acquired into a FACScan cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) with the CELLQuest software (Becton Dickinson). Data were analyzed by WinMDI program through histogram graphics.

Experiment 1: Mesenchymal stem cells stained by DAPI in DMEM

DAPI staining: Cells were counted in a Neubauer camera and adjusted to a density of $1x10^5$ cells/well. Cells were incubated in DAPI (Sigma, St. Louis, MO) solution (50 nM) for 30 min in the dark, and were observed under a fluorescence microscope (Olympus, Japan). Cells were allocated to two experimental groups: untreated (control) and DAPI-treated cells, and were cultured for 0, 3, 6 or 9 days.

Cell counting: The number of cells was quantified in of material obtained from each well (3 microscope fields), under a light microscope (Olympus, Japan) with a 4x objective. Results are expressed as the mean and

standard error of triplicate determinations made for each group and for each experimental period. Also, the number of DAPI-marked cells was determined by counting marked cells in 7 microscope fields per well, under a fluorescence microscope with green filter (Olympus, Japan) and a 10x objective. Also, results were expressed as the mean and standard error of triplicate determinations for each experimental period.

Viability assay: Cytotoxicity was measured by 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and trypan blue exclusion assays. In brief, control and stained cells were incubated with MTT for 2 h at 37ºC. After incubation they were treated for 12 h with the solubilization solution (1% sodium dodecyl sulfate -SDS- and 10% HCl) and the absorbance at 595 nm of solubilized MTT formazan product was measured. For trypan blue exclusion assay, control and stained cells were exposed to trypan blue for 10 min. The number of viable cells (by dye exclusion) was counted under the light microscope using a Neubauer camera and expressed as a percentage of total cells.

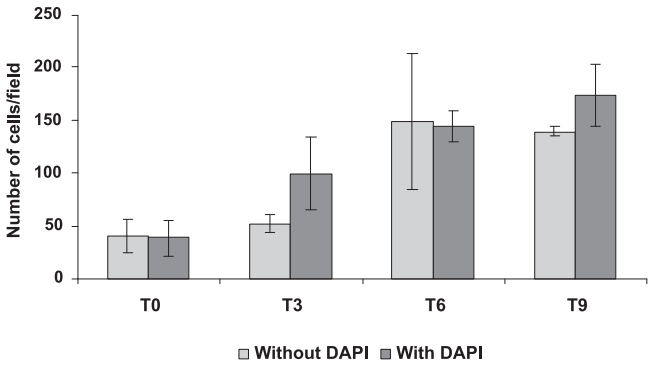

FIGURE 1. Number of cells per field (mean±SE) in groups without DAPI (control) and with DAPI treatment.

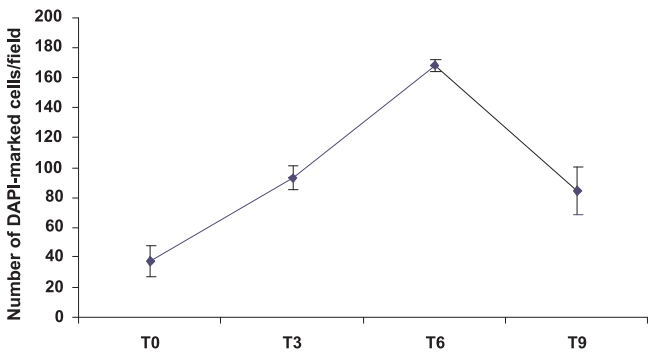

FIGURE 2. Number of DAPI-marked cells per field (mean±SE).

Alkaline phosphatase activity: Control and DAPI-stained cells were incubated with BCIP/NBT (5-bromo, 4-chloro, 3 indolylphosphate nitroblue tetrazolium) solution (Gibco) for 2 h at 37ºC. After incubation, they were treated for 12 h with the solubilization solution. The absorbance at 595 nm was measured using a microtiter plate reader.

MitoTracker Green (MTG) staining and flow cytometry analysis: MTG fluorescence has been used as a measure of mitochondrial mass (Pendergrass *et al.*, 2004; Lugli *et al.*, 2005). To study the mitochondrial mass, stem cells were analyzed after zero, three, six and nine days of culture with DMEM medium, with or without DAPI (1:50). The adhered stem cell was washed by trypsin action and collected by centrifugation (1400 rpm at 10 min). The pellet of cell was resuspended in DMEM medium and the cell concentration was adjusted to 3,0 x 10^5 cells/well and stained, in 96-well U-bottomed plates with MitoTracker Green (MTG) dye diluted 1:10 in PBS 0,15M, pH 7,0 and incubated by 30 min at 4ºC. Non-marked stem cells were used as controls. At least 20.000 events per sample were acquired into a FACScan cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) with the CELLQuest software (Becton Dickinson). Data were analyzed by WinMDI program through histogram graphics.

Experiment 2: Mesenchymal stem cell stained by DAPI in an osteogenic medium

The same cells were counted in a Neubauer camera and adjusted to a density of 1 x 10^5 cells/well were incubated in DAPI (Sigma, St. Louis, MO) solution (50 nM) for 30 min in the dark. Cells were allotted to two experimental groups: untreated (control) and treated with DAPI. After an attachment period of 24 h, the cells were cultured in an osteogenic medium consisting of DMEM supplemented with 10% fetal bovine serum (Gibco) plus antibiotics (60 µg/L gentamicin, 25 µg/L amphotericin B, 10.000 U/mL penicillin, and 10.000 µg/mL streptomycin, 10 mM ß-glycerophosphate, 50 µg/mL ascorbic acid, (Merck, Germany), 10 nM dexamethasone (Sigma). The cells were grown at 37ºC and 95% O_2 + 5% CO_2 for 21 days. The cells were observed under a fluorescence microscope along the culture period (Olympus, Japan). Cell proliferation and viability were determined as described above. At the end of the culture period, osteoblastic differentiation and mineral-

ization were assessed by determining alkaline phosphatase activity (as describe above) and by counting the number of mineralized nodules formed. For the latter purpose, cultures were rinsed in PBS, fixed in 70% ethanol and rinsed with deionized water. After the addition of 5% silver nitrate solution, the wells were exposed to light for 2 h. The plates were rinsed with deionized water, and the residual silver nitrate was neutralized by 5% sodium thiosulfate. The number of nodules was quantified in 35 microscope fields with a 10x objective, and the mean and standard error of triplicate determinations was calculated for each group.

Cellular morphology and cell counting: Control and stained DAPI cells were cultured in 24-well plates with coverslips. The cells were fixed in 70% ethanol solution for 30 min and hydrated in deionized water for 5 min, and then stained with hematoxylin and eosin. The number of cells was estimated in both groups by cell counting in 35 microscope fields with a 10x objective. Mean and standard error were determined in triplicate for each group.

Statistical analysis: Delineation was entirely at random in both experiments with a 2 x 3 factorial (two groups x three periods) in experiment 1. The data were subjected to analysis of variance (ANOVA), and means were compared by Student Newman Keuls Test (SNK) (Instat, version 3.00, 32 Win 95/NT; GraphPad Software San Diego, CA, USA). Differences were considered significant if $p < 0.05$.

Results

The phenotypic characterization of bone marrow MSCs demonstrated absence of expression of CD45 in 97% of the obtained cells. But there was expression of

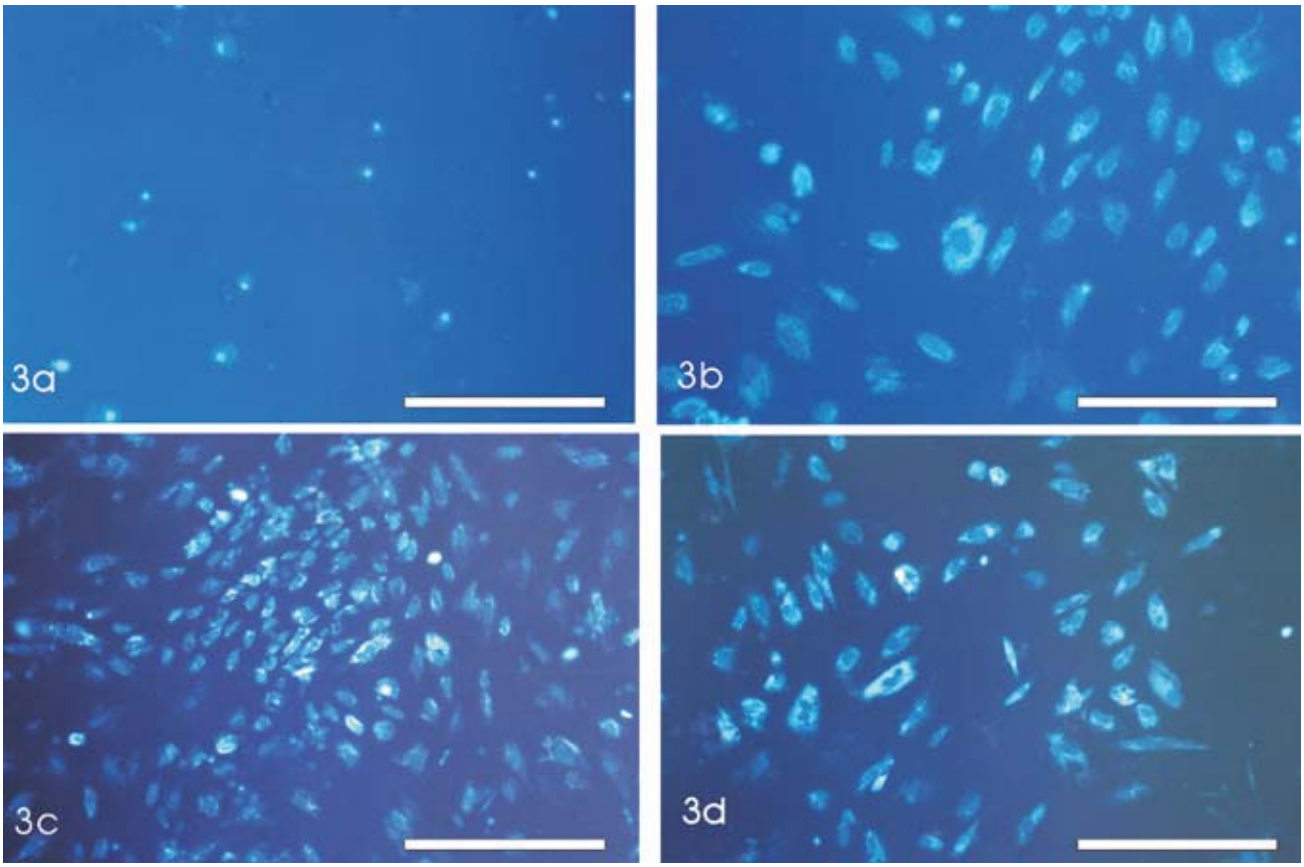

FIGURE 3. Mesenchymal stem cells stained with DAPI. Bar = 187 µm. a) Number of cells with DAPI-marked nucleus on day 0. b) on day 3, c) on day 6, and d) on day 9. The number of cells with DAPI-marked nucleus increased significantly up to day 6. On day 9, both the number of fluorescent cells and the fluorescence intensity were reduced.

CD73, CD54 and CD90 in 94%, 95.10% and 87% of the obtained cells, respectively.

Experiment 1

Proliferation and viability of mesenchymal stem cells after DAPI treatment.

In both groups: The number of stem cells/field increased progressively and significantly up to the ninth day of culture. There were no significant differences between groups (Fig. 1).

However, the number of fluorescent cells/field, i.e., cells with DAPI-marked nucleus, increased significantly up to the sixth day only. Then the number of fluorescent cells (Fig. 2) and fluorescence intensity decreased (Fig. 3). Cells in mitotic division and with two or more nuclei, were clearly visualized by DAPI-staining. DAPI was present in nucleus and cytoplasm.

Although the number of DAPI-treated cells increased progressively up to the ninth day, MTT assay showed a significant reduction of the spectrophotometric absorption of formazan in this group from day 3 (Fig. 4). However, trypan blue assay showed 99% of cell viability in both the control and in the DAPI-treated groups. The trypan blue assay showed no significant differences between groups, at none of the studied periods.

Alkaline phosphatase activity: In the control group, alkaline phosphatase activity increased significantly on the ninth day as compared to the previous days. DAPI significantly reduced mesenchymal stem cells alkaline phosphatase activity both at the sixth and ninth days (Fig. 5).

MTG staining and flow cytometry analysis: In all of the studied times, i.e., zero, three, six and nine days in culture, we found a similar mitochondrial mass in stem cells cultured with or without DAPI (Fig. 6). Nevertheless, with MTG staining, mesenchymal stem cell morphology differed between groups. On the sixth and ninth day of culture, DAPI-treated group most of the times, presented round cells with fine and short extensions. Control cells were mainly fusiform with several long extensions linked to neighboring cells. On the 0 day and third day, there was not significant difference between groups (Fig. 7).

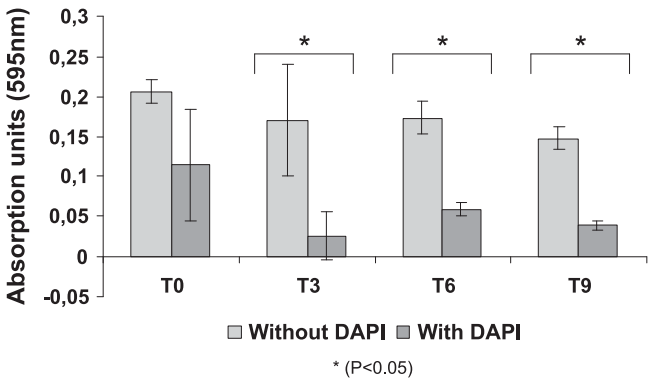

FIGURE 4. Cell viability (mean±SE) in groups without DAPI (control) and with DAPI treatment.

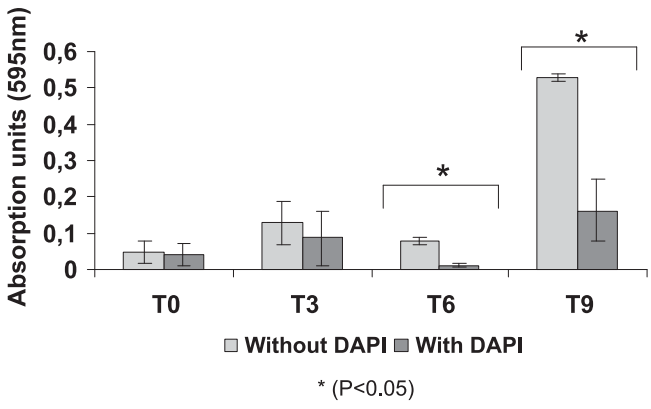

FIGURE 5. Alkaline phosphatase activity (mean±SE) in groups without DAPI (control) and with DAPI treatment.

TABLE 1.

Proliferation, viability, mineralization and alkaline phosphatase activity of mesenchymal stem cells grown during 21 days in an osteogenic medium, with and without DAPI treatment.

	Control	DAPI-treated cells
MTT assay (absorption units)	0.31±0.08	0.29±0.05
Alkaline phosphatase activity (absorption units)	0.41±0.12	0.38±0.07
Number of cells/field	57.89±13.15	66.56±9.69
Number of mineralized nodules/field	2.99± 0.78	2.61±1.17

*No significant differences were observed.

Experiment 2

The number of cells/field did not differed statistically between the control and the DAPI- marked groups. Fluorescence intensity was reduced along the experimental period in the DAPI-treated group. In the ninth day there were not DAPI-marked cells left. As opposed to experiment 1 the MTT assay showed no significant difference between groups, i.e., the number of cells/field, the viability assay, the activity of alkaline phosphatase and the number of mineralized nodules/field were similar in both groups (Table 1).

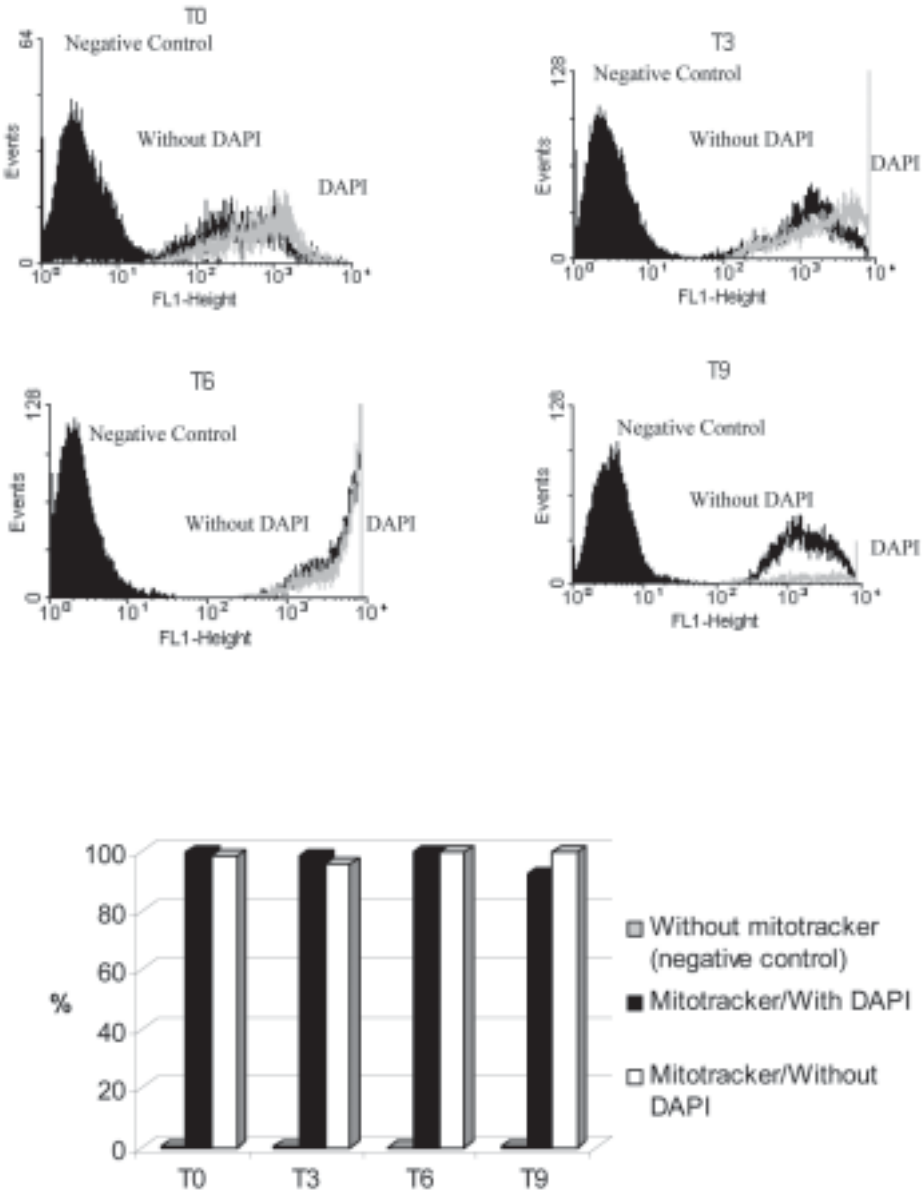

FIGURE 6. Mitochondrial mass evaluation in stem cells from rats by MitoTracker Green (MTG) staining and flow cytometry. The histograms show the frequency of mitochondria in stem cells cultured for 0, 3, 6, or 9 days, with or without DAPI treatment. The fluorescence scale (X axis) is considerably positive when the peak of cells is above 10^1. The full black peak refers to unmarked stem cells. The empty green peak refers to stem cells cultured with DAPI and the empty black peak refers to stem cells cultured without DAPI treatment. The same results are presented in bars in the bottom graph.

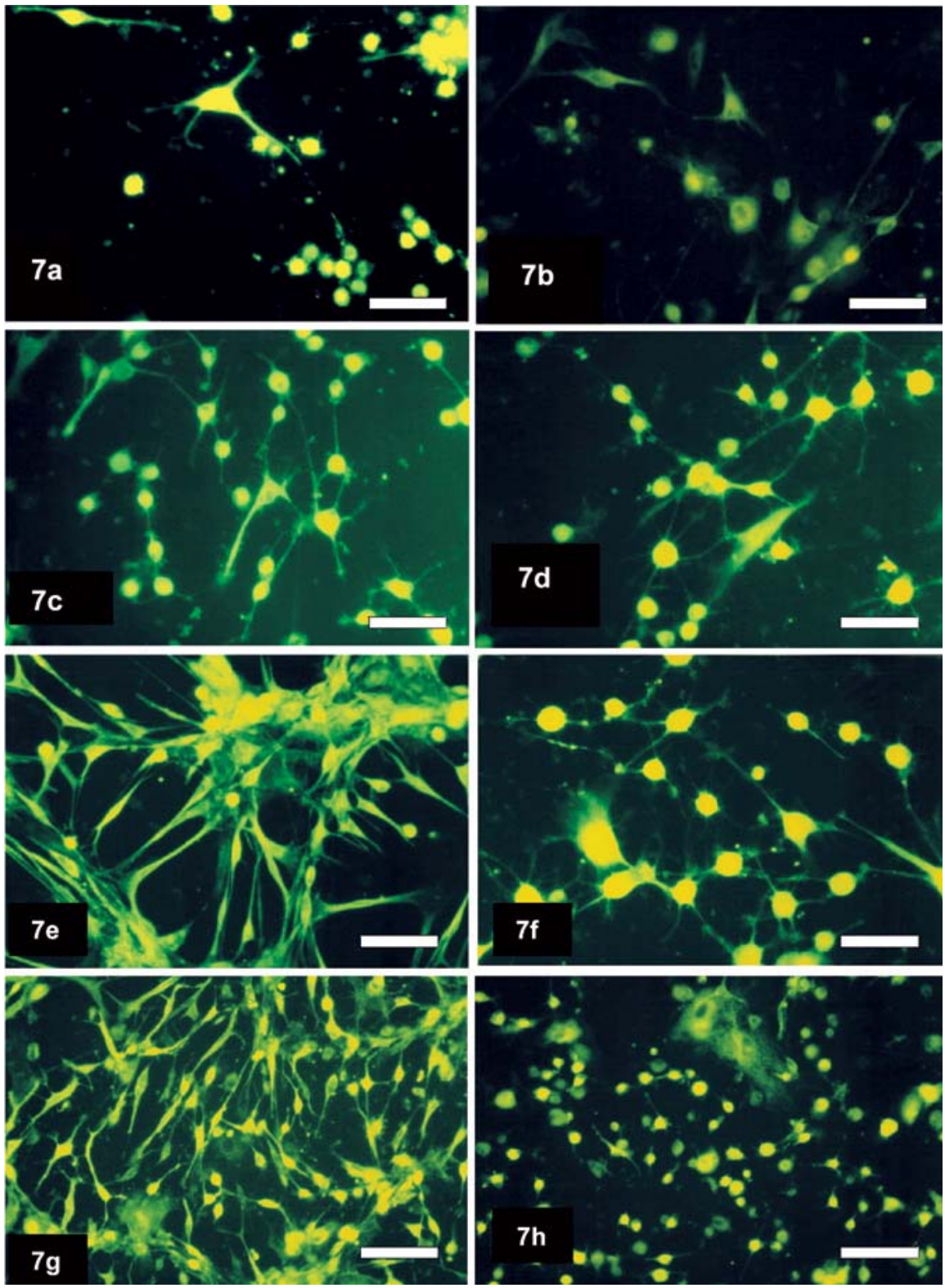

FIGURE 7. Mesenchymal stem cells stained with MitoTracker Green. Bar = 67 μm. Panels a and c: control groups on days 0 and 3, respectively; panels b and d: DAPI-treated groups on days 0 and 3, respectively, showing mostly round cells, with fine and short extensions, and with no differences between groups. Panels e and g: control group on day 6 and 9, respectively, showing fusiform cells with many long extensions, apparently linking to neighboring cells. Panels f and h: DAPI-treated groups on days 6 and 9, respectively, showing mostly round cells with fine and short extensions.

Discussion

The bone marrow is known to contain mesenchymal stem cells, hematopoietic cells (Bianco *et al*., 2001; Bobis *et al*., 2006) and fibroblasts (Ishii *et al*., 2005a). The cells used in the current study have been characterized as being positive for CD73, CD54 and CD90, and negative for CD45. The latter antigen is expressed by hematopoietic cells (Pittenger *et al*., 1999; Bobis *et al*., 2006) and some fibroblasts (Ishii *et al*., 2005b), while CD54 and CD90 are typical of mesenchymal stem cells (Covas *et al*., 2003; Bobis *et al*., 2006). On its part, CD73 may be expressed by both fibroblasts and mesenchymal stem cells (Ishii *et al*., 2005a; Bobis *et al*., 2006). It is concluded, therefore, that the bone marrow cells used in this study are indeed mesenchymal stem cells.

A potentially interesting finding of the current study is the diminishing effect of DAPI staining on formazan formation after MTT treatment from the third day on, even though cell viability (as detected by the trypan blue method remained high) and the proliferation ability of DAPI stained cells seemed not impaired. The MTT test is a sensitive and quantitative colorimetric method, which measures the ability of mitochondrial succinate dehydrogenase enzymes of viable cells, to convert MTT into formazan crystals (Altman, 1976; Holt *et al*., 1987). At least two hypotheses may explain DAPI interference with the MTT assay: (1) that DAPI may reduce the mitochondrial mass present in cells; and (2) that DAPI may affect the mitochondrial reduction of MTT. The first hypothesis was rejected since a similar mitochondrial mass was determined by MTG staining and flow cytometry in cells that were cultured either with or without DAPI during 3-9 days.

As expected, DAPI treatment did not affect the increase in the number of both DAPI-marked cells and mitosis up to the sixth day in culture. Ideally, a stem cell marker must be transferred to descending cells with minimal or no dilution during subsequent cell divisions (Frangioni and Hajjar, 2004). DAPI was transferred to descendant cells in the current study; however, both the number of DAPI-marked cells and the intensity of DAPI labeling on the day nine of culture were lower than on day 6 in non-osteogenic cultures. Dissapearing of DAPI marking occurred even faster in osteogenic cultures. This is in contrast to *in vivo* studies in which mesenchymal stem cells stained by DAPI were observed up to 10 weeks after inoculation and differentiation in cardiomyocytes (Niu *et al*., 2004).

Cells with more than two nuclei were observed in the DAPI-treated group, which may suggest that they were undergoing nuclear division with no cytokinesis. This apparent failure in cell division, together with the observed morphological changes of stem cells after DAPI treatment, may suggest that this dye may be affecting the cytoskeleton.

The activity of alkaline phosphatase by cells may be estimated through BCIP-NBT solution (5-bromo,4-chloro,3-indolylphosphate-nitroblue tetrazolium). BCIP-NBT is used to as substrate for alkaline phosphatase enzyme (De Jong *et al*., 1985). Alkaline phosphatase cleaves phosphate group of BCIP producing bluish staining at the same time that NBT is reduced and produces a dark red insoluble deposit. DAPI-treated cells present a low activity of alkaline phosphatase in non osteogenic cultures. But DAPI treatment did not alter either the activity of alkaline phosphatase or the synthesis of mineralized nodules in those osteogenic cultures, up to day 21.

X-Ray-based methods, optical imaging, ultrasound, single-photon emission computed tomography, positron emission tomography, and magnetic resonance imaging are contrast methods for *in vivo* stem cell tracking. At present no imaging technology presents all the appropriate characteristics for clinical stem cell trials. DAPI is being used thoroughly to mark and trace stem cells route which will be used in the therapy of diseases (Thompson *et al*., 2004; Niagara *et al*., 2004; Richardson *et al*., 2005; Tang *et al*., 2006). Although stem cells are being used to treat bone diseases (Kraus and Kirker-Head, 2006; Wang *et al*., 2006), this is the first study in which effects of DAPI on mesenchymal stem cells were shown in osteogenic cultures.

It is concluded that in non osteogenic cultures, DAPI changes the morphology of mesenchymal stem cells and reduces the production of alkaline phosphatase and formazan crystals, probably by changing mitochondrial enzymatic activity and not by changing the mitochondrial mass. But, DAPI did not affect cell viability, proliferation and the process of osteogenic differentiation. The fact that the cells loose their mark after day 9 in culture in osteogenic cultures suggests that DAPI may not be a good marker for long term studies of mesenchymal stem cells implanted in bone tissue.

Acknowledgements

This work was supported by grants from Fundação de Amparo a Pesquisa de Minas Gerais (Fapemig) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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