

Pap Test as the First Step in Screening Genetic Stability in Cell-Based Therapy

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Abstract

The possibility of cell modifications compromises the safety of stem cell therapy under standardized conditions. The aim of this study was to analyze adipose tissue-derived mesenchymal stem cells (AT-MSCs) using the Pap test as a first screening step to evaluate genetic stability.

Methods: Human adipose tissue from six healthy female donors was obtained from elective liposuction procedures. The cells were isolated, cultivated at P2/P3, characterized by flow cytometric analysis, and differentiation induced. The AT-MSCs were stained by Papanicolaou (Pap) staining and analyzed according to the Bethesda classification, and viability-apoptosis relationships were evaluated.

Results: The Pap test for Sample I indicated high-grade alterations consistent with genetic instability; for Samples II-V, atypical cells of undetermined significance; and for Sample VI, normal cells.

Conclusions: These results demonstrate the potential of using the Pap test as an initial screening step to evaluate the genetic stability of cultured AT-MSCs as well for other adherent stem cells.

Keywords: Cultured; Mesenchymal stem cells; Therapy; Genetic stability; Human; Adipose-tissue; Papanicolaou; Safe

Introduction

The promise of stem cell-based therapy for advancement of research in regenerative medicine has stimulated a great number of clinical trials, particularly for previously untreatable diseases.

However, for new therapies, it is necessary to ensure the safety of processes and products in an efficient manner. Toward this aim, therapeutic use of biological products mandates the establishment and use of basic requirements known as Good Tissue Practices to prevent disease transmission, mix-ups, and cross-contamination, assuring safe cell therapy [1].

As with oncohematological diseases, cell-based regenerative therapy is expected to be useful in non hematological diseases with cells

of adult origin, such as mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs); both cell types are self-renewing

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populations and have pluripotent capacities to regenerate various tissues: cartilage, bone, muscle tendons, and the nervous system [2,3]. MSCs could also be used for transplantation in cell-based therapy, and for this purpose, must be able to expand *ex vivo* undercultivation. They should be able to grow in flasks based in their characteristic of adherence to plastic. However, the literature has demonstrated that transplantation is limited by the number of MSCs; thus, better results are dependent on producing a great number of cells independent of their source and consequently, large numbers of cells are usually required for clinical applications. Transplantation is also limited by mortality of the transplanted cells after transplantation by apoptosis or macrophage activity independent of the autologous or allogeneic procedures used. For the same reasons, iPSCs must also undergo expansion under *ex-vivo* conditions.

These considerations highlight the need for the *in vitro* expansion of stem cells prior to their commitment into tissue-specific applications. Before bioreactors can be used, several factors must be considered, including the need to increase the number of cells, the capacity to support high cell densities in relatively small volumes, the ability to scale up the design, and standardization of the bioprocess, including the safety of the products [4].

Difficulties have been reported during cultivation of MSCs for expansion. Spontaneous transformation of murine hematopoietic MSCs (mMSCs) has been described by many authors, although there are contradictory results [5,6]. However, another group observed spontaneous malignant transformation in a preclinical study in which the cells had modified characteristics after the third passage. Direct injection of cultured bone marrow-derived MSCs into immunocompetent mice has resulted in tumor formation, and a karyotype analysis showed that increased chromosome numbers and multiple Robertsonian translocations occurred at passage 3 coincident with the loss of contact inhibition [7, 8]. On the other hand, spontaneous transformation over the long-term in human MSCs (hMSCs) from bone marrow cultures has not been reported in other studies [9,10]. In humans, one group observed spontaneous malignant transformation of adipose tissue-derived (AT)-MSCs *in vitro* [11].

Ultimately, Røslund et al. [12] conclusively demonstrated spontaneous malignant transformations in long-term cultured bone marrow-derived human MSCs (5-106 weeks). The possibility of cell modifications compromises the reproducibility and safety of generation of MSCs under standardized conditions. The consequence of these results was increased requirements to maintain safe cell therapy beyond the basic requirements and regulations of government health organizations to ensure the identity, purity, potency, and clinical efficacy of MSCs.

The protocol governs quality control of cells by the FDA, excluding genetically modified cells, and primarily addresses safety concerns with cell differentiation to undesired cell types and potential uncontrolled cell proliferation or tumorigenicity [13]. Others concerns about the safety of this type of therapy are related to the stability of the product and/or the cell line, specifically, the number of passages/doublings over time, and maintaining desired differentiation properties without karyotypic alterations [14,15].

All of those proceedings impose great costs in translating these therapies to humans and barriers to democratization of advanced therapy approaches. If cell-based therapies could be made more efficient, more people could access their benefits; ideally, the cost of

each step would be reduced, without prejudicing the safety of the therapy.

In our laboratory, over the last eight years, we have made morphological observations of undifferentiated MSCs from human bone marrow and adipose tissue up to P5 (not for transplantation use), reviewing and recording a diary of cultured cells using an inverted microscope for the purposes of research academics. We have observed some issues that would be of concern regarding the health of cultured MSCs for potential use in transplantation cell-based therapy. In developing countries, particularly in Brazil where our group is established, we have limited financial resources and at times, we need to answer certain questions without great cost. Why not “do simple things in a simple way” [16]?

With that concept in mind, we chose the Papanicolaou test, known as the Pap smear, a cytological analysis of exfoliated cells from the cervix originally described by George Papanicolaou in 1928 [17]. This test is efficient in diagnosing early abnormal changes in the cervical epithelium that could indicate the presence of cervical cancer, and is universally used for screening for cervical cancer in woman [18].

The possibility of analyzing cultured MSCs at low cost using the Pap test appears very attractive to avoid additional steps, as a first step why it is a simple and short procedure requiring laboratory equipment available to everyone, in contrast to the more cumbersome trial procedures required by the FDA, if, similar abnormalities as for squamous cells of the cervix can be demonstrated. The aim of this study was to analyze AT-MSCs using the Pap test.

Materials and Methods

Isolation of adipose tissue-derived MSCs

Human abdominal AT from six healthy female donors was obtained and designated Samples I-VI (ages 47, 38, 24, 45, 35, and 41 years with body-mass index 22.3, 29.4, 24.7, 29.4, 22.8, and 23.6, respectively). Samples were obtained during elective liposuction procedures for plastic and cosmetic purposes under local anesthesia after informed consent, following the guidelines of the Brazilian Ethics Committee on Use of Human Subjects and as approved by the Pequeno Príncipe Hospital Complex Ethics Committee, number 0617-08.

The AT-MSCs were isolated in accordance with Zuk et al. (2001) [19]. The lipoaspirates were washed extensively with sterile PBS to remove contaminating debris and red blood cells. The washed aspirates were treated with 0.075% collagenase (type I, Sigma, USA) in PBS for 30 minutes at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of DMEM/10% FBS (Gibco BRL, Life Technologies, Inc Grand Island, NY) and the pellet was centrifuged for 10 minutes at low speed before being resuspended in DMEM/F12/10% FBS with 1% antibiotic (streptomycin- penicilin; Gibco BRL, Life Technologies, Inc, Grand Island, NY, USA), and filtered through a 100- μ m mesh filter to remove debris. The filtrate was centrifuged and plated onto conventional tissue culture plates and the samples were then characterized by flow cytometric analysis.

Cell characterization and viability-apoptosis relationships

Flow cytometric analysis (FACScalibur; Becton Dickinson) was performed to validate the MSCs from adipose tissue. The harvested cells from the culture dishes underwent flow cytometric analysis to

characterize the immunophenotypic profile of cellular subsets using the surface markers CD45, CD34, CD49d, CD73, CD90, and CD105.

Viability-apoptosis relationships were characterized using conjugated 7AD-annexin. The procedure used was “stain then lyse;” lysis was performed after staining with monoclonal antibodies (MAb). The cells were incubated with the specific MAb panel for 15 minutes at room temperature in the dark, lysed (FACS lysing solution, Becton Dickinson), washed with 0.1% PBS, resuspended, and analyzed on a FACS calibur using Cell Quest software (Becton Dickinson).

For the 4-color staining immunophenotyping analyses, the following rat antibody conjugates, coupled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), CyChrome (PE-Cy5), or allophycocyanin (APC) were used: CD45-PE-Cy5 (clone HI30), CD34-FITC (clone 581), CD49d-PE (clone 9F10), CD105-FITC (clone 266), CD73-PE (clone AD2), and CD90-FITC (clone 5E10). All antibodies were used at the concentrations recommended by the manufacturer. The respective isotype-matched control was PE MoAb.

Induced differentiation

Differentiated AT-MSCs were processed as described in Zuk et al. [20]. Briefly, the AT-MSCs were washed extensively with sterile PBS to remove contaminating debris and were cultured in induction medium

DMEM/F12 and 10% FBS supplemented with the growth factors for each cell type: for adipogenic, 0.01 Mm 1,25-dihydroxyvitamin D3, 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 1% antibiotic (streptomycin-penicilin); and for osteogenic, 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, and 1% antibiotic (streptomycin-penicilin).

Cytology and immunocytochemistry

Induced AT-MSCs were stained for the assays with Alizarin Red for osteogenesis and Sudan Red for adipogenesis.

Papanicolaou staining

The Pap test is based on specific changes in the cytoplasm and/or nucleus of the epithelial cells of the cervix, which can be identified through light microscopy. A combination of two coloring agents is used: a nuclear dye, Harris hematoxylin; and a mixture of two cytoplasmic dyes, orange G and polychrome mixture (EA51) highlights the cellular aspects that are fundamental in differentiating normal squamous cells of the cervix from low- and high-grade squamous intraepithelial lesions (LSILs and HSILs, respectively) [21,22]. These aspects include nuclear pigmentation, position, volume, and morphology; membrane regularity and integrity; and the chromatin arrangement. Other important parameters are the cellular size and shape, and the ratio of the nucleus to the cytoplasm, which is increased in pre-cancerous cells

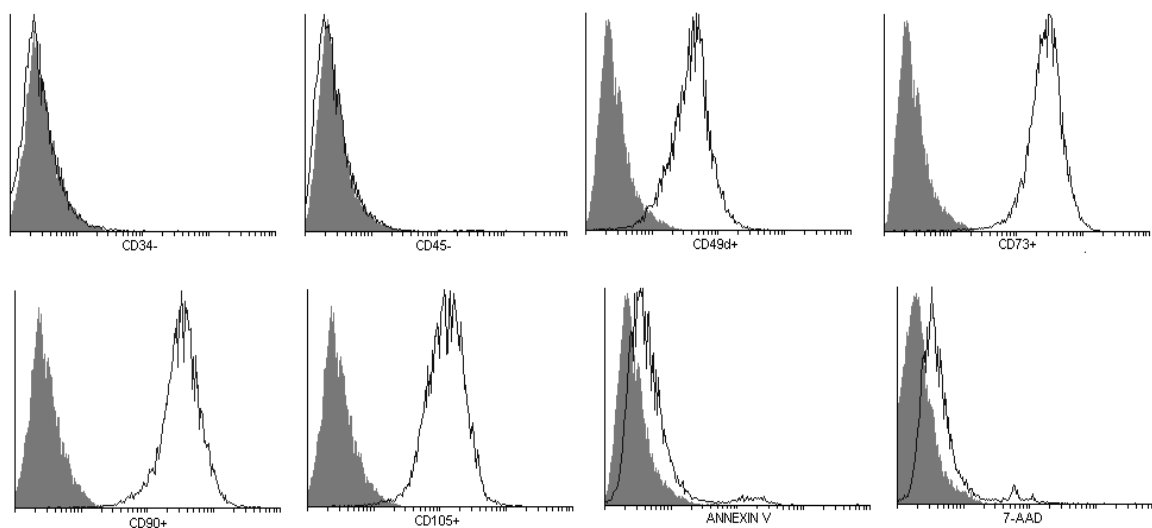


Figure 1: Four-color staining immunophenotyping analyses of Sample I. The following rat antibody conjugates, coupled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), CyChrome (PE-Cy5), or allophycocyanin (APC) were used: CD45-PE-Cy5 (clone HI30), CD34-FITC (clone 581), CD49d-PE (clone 9F10), CD105-FITC (clone 266), CD73-PE (clone AD2), CD90-FITC (clone 5E10), and conjugated 7AD-annexin (Becton Dickinson). The respective isotype matched control was PE MoAb.

AT-MSC	CD34-	CD45-	CD49d+	CD73+	CD90+	CD105+	ANNEXIN+	7-AAD+
I (a)	99.64	99.43	84.07	99.58	99.67	98.55	4.41	4.98
II (b)	99.64	99.83	96.38	99.84	99.95	99.41	6.48	8.43
III (c)	98.81	99.99	94.09	99.12	99.52	99.84	13.69	12.98
IV (d)	96.68	99.85	93.74	99.66	99.86	99.80	4.78	7.35
V (e)	99.19	99.88	88.77	99.88	99.87	99.91	3.8	5.78
VI (f)	97.04	99.91	81.24	99.45	99.86	99.20	9.6	4.83

AT-MSC: adipose-tissue mesenchymal stem cells; CD34: CD34 FITC (clone 581); CD45: CD45 PE-Cy5 (Clone HI30); CD49d: CD49d PE (clone 9F10); CD73: CD73 PE (clone AD2); CD90: CD90 FITC (clone 5E10); CD105: CD105 FITC (clone 266); 7-Aminoactinomycin D (7-AAD) - dead cells. Annexin V - Apoptosis as well as other forms of cell death

Table 1: Immunophenotypic expression by flow cytometric analysis (%).

[23,24].

In this study, the AT-MSCs were seeded in 25-cm² flasks at 5 × 10⁴/mL, then cultured for 7-14 days to P2 and P3 until 70% confluence was reached. The cells were then cultured for three days on double-chamber slides, after which the cultures were ended and the stain applied directly to the slides. Undifferentiated AT-MSCs were stained by Papanicolaou staining. Each slide was immersed in vats containing the staining solutions in sequence for the appropriate immersion times in accordance with the manufacturer's instructions (Newprov, Pinhais, Brazil). The slides were then mounted and observed under a light microscope. The evaluation of PAP-stained cells have been done by three independent pathologists and the result was determined in accordance with the Bethesda classification as (a) normal cells, (b) atypical cells of undetermined significance, or (c) high-grade alterations consistent with genetic instability [22,24].

Results

Figure 1 shows the results of the flow cytometric analysis for Samples I, as well the viability/apoptosis relationships (Table 1). The obtained cells were demonstrated to be MSCs from adipose tissue why the cells were marked with antibodies that are in consensus of adipose origin as well the cells were integrity preservation demonstrates by great viability and small apoptosis fraction; all samples have demonstrated the normal profile of undifferentiated adipose derived stem cells, the cells were adherents and all samples were capable of differentiation in lipid cells demonstrated by oil Sudan red and bone by alizarin osseous matrix identification, respectively (Figure 2). The Pap test for Sample I showed high-grade alterations consistent with genetic instability (Figure 3), Samples II-V contained atypical cells of undetermined significance (Figure 4a, Figure 4b) and Sample VI comprised normal cells (Figure 4c, Figure 4d).

Discussion

These results demonstrate the potential for using the Pap test as the initial step of a screening methodology to ensure the genetic stability of cultured MSCs. As described by various authors, the difficulty in assuring accuracy in the Pap test is more in the preparation of cervical smears than in the analysis, because it is possible to use rescreening methods for negative results [23,25,26].

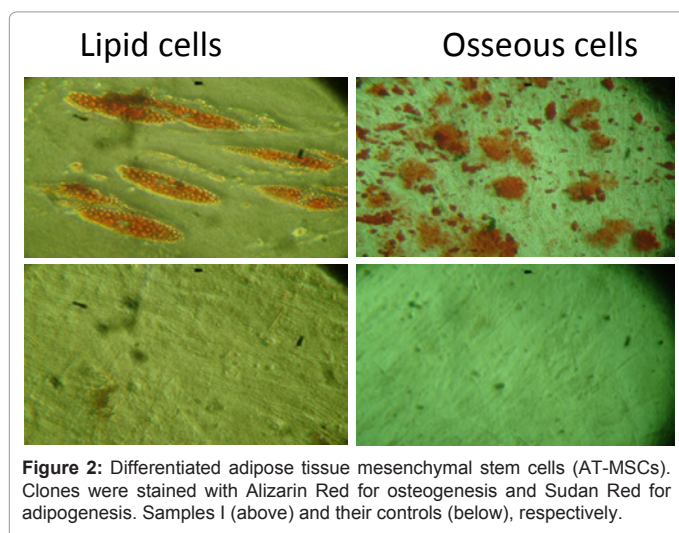


Figure 2: Differentiated adipose tissue mesenchymal stem cells (AT-MSCs). Clones were stained with Alizarin Red for osteogenesis and Sudan Red for adipogenesis. Samples I (above) and their controls (below), respectively.

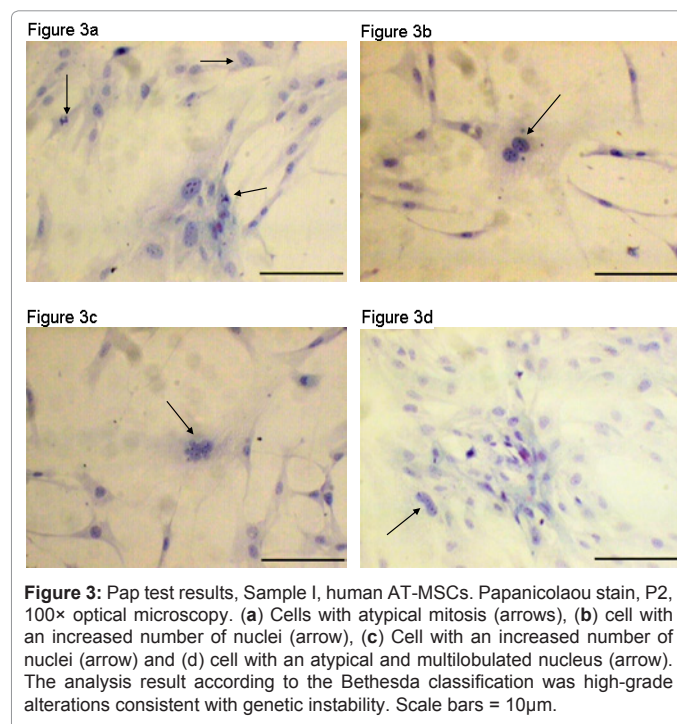


Figure 3: Pap test results, Sample I, human AT-MSCs. Papanicolaou stain, P2, 100× optical microscopy. (a) Cells with atypical mitosis (arrows), (b) cell with an increased number of nuclei (arrow), (c) Cell with an increased number of nuclei (arrow) and (d) cell with an atypical and multilobulated nucleus (arrow). The analysis result according to the Bethesda classification was high-grade alterations consistent with genetic instability. Scale bars = 10µm.

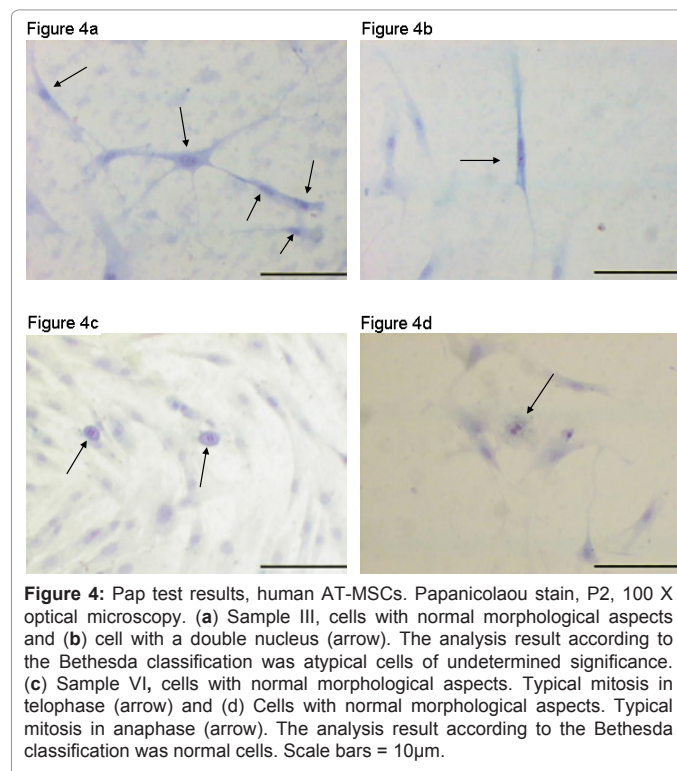


Figure 4: Pap test results, human AT-MSCs. Papanicolaou stain, P2, 100 X optical microscopy. (a) Sample III, cells with normal morphological aspects and (b) cell with a double nucleus (arrow). The analysis result according to the Bethesda classification was atypical cells of undetermined significance. (c) Sample VI, cells with normal morphological aspects. Typical mitosis in telophase (arrow) and (d) Cells with normal morphological aspects. Typical mitosis in anaphase (arrow). The analysis result according to the Bethesda classification was normal cells. Scale bars = 10µm.

It is much easier to obtain a thin and clean smear starting from a cell culture than from the cervical samples normally used in a conventional Pap test. The Pap test is used to differentiate cells in many types of specimens: cerebrospinal, abdominal, or pleural fluids; synovial tissues; tumor samples; or other materials containing cells [23,25,26]. The cytopathological findings for the AT-MSC samples in this

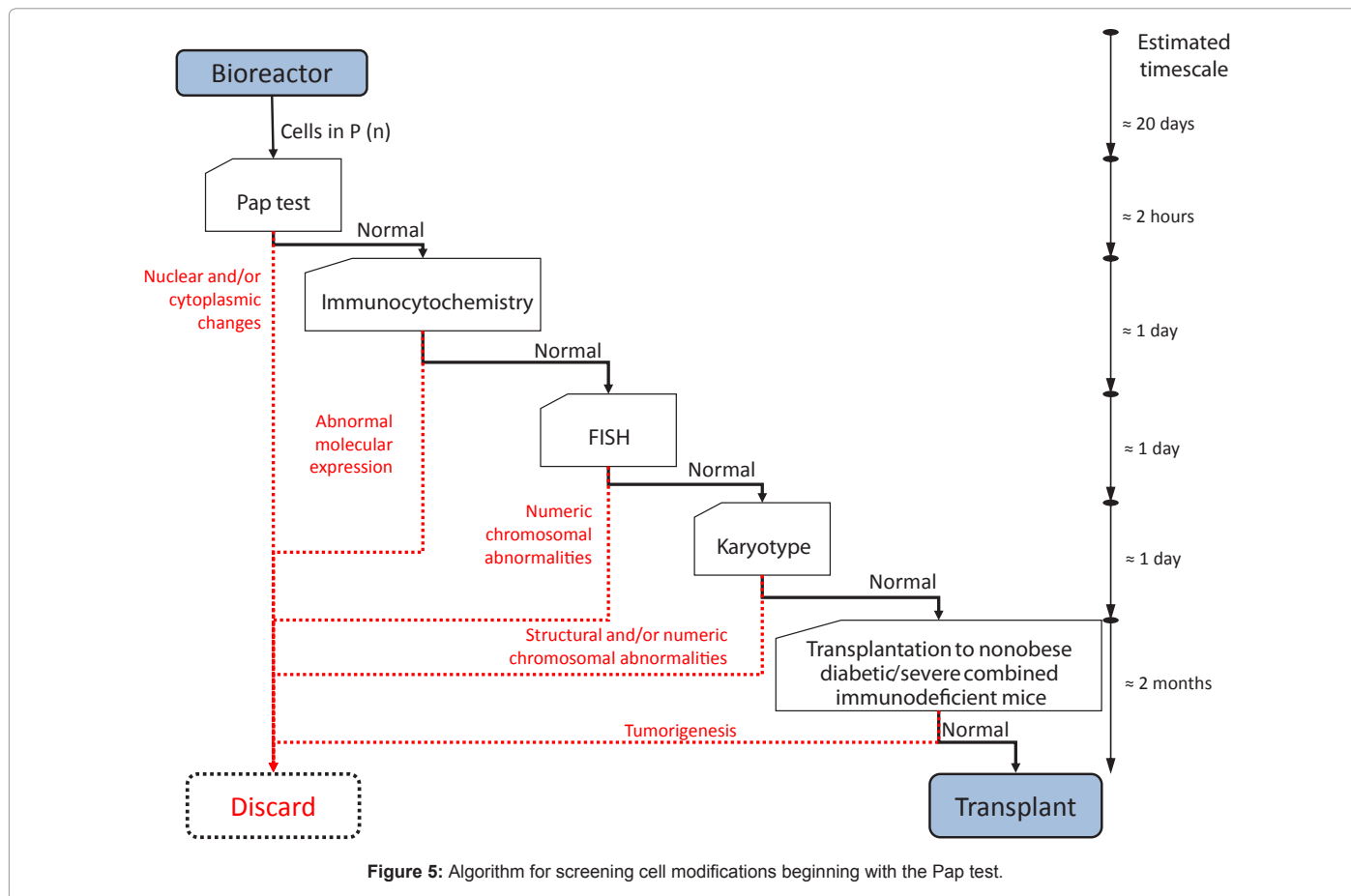


Figure 5: Algorithm for screening cell modifications beginning with the Pap test.

study may be explained by suppression of RNA polymerase II serine-2 phosphorylation. This suggests a conserved mechanism of transcriptional quiescence in various stem cells and, in the absence of telomerase expression, why AT has a slow turnover and AT-MSCs participate in tissue homeostasis and respond to tissue injury, but stay in a quiescent form prior to injury longer than others [27,28].

The AT is “aged”-the differentiated adipocytes as well their quiescent AT-MSCs and these cells are likely at increased risk of genetic instability. The adult MSCs were demonstrated to differentiate in varying concentrations in both solid and hematopoietic tissues in blood suspension; however, while the pluripotentiality of AT-MSCs may be similar to those of hematopoietic origin, their genetic stability may not be [29,30]. The abnormality findings in a previous study of AT-MSCs suggest that inclusion of the Pap test for both allogeneic and autologous donors would be valuable, because abnormalities may develop in cultured cells independent of the type of donor [31].

These finding suggests that the Pap test could be used not only for adult sources, but also for all adherent cells from embryos, fetuses, and adult tissue such as iPSCs or MSCs. The results of our study demonstrate the usefulness of the Pap test as the initial quality control step for cultured stem cell therapy, independent of the origin, source, or type of transplantation. This approach seems reasonable if identification of modified cells in this first step could avoid additional costs. If modified cells were present at this first step, they would not be considered safe for use in transplantation. The atypical cells and cells of unknown significance are not safe; they

may not be pathogenic, but they may transform to cancer stem cells. Caution is required and the risk is not considered acceptable. If there were no modified cells according to the Pap test results, the next step of the screening procedure involving the karyotype analysis would be followed.

Tsuji et al. [32] has suggested with respect to iPSCs transplantation that to await the results of injecting cells into mice and subsequent tumor development to exclude cells for transplantation after two months will increase the delay in deciding whether to use the cells. The reprogramming process and subsequent culture of iPSCs in vitro can induce genetic and epigenetic abnormalities as well the human embryonic stem cells that could acquire an abnormal number of chromosomes. They justify the caution with cultured stem cell therapy [33].

On the other hand, we discuss the impact of concentration of FBS could be responsible for “fibroblastic” transformation of AT-MSC (making them potentially more tumorigenic) as the concentration of the 10% Fetal Bovine Serum-containing media used to expand cells and whether this Lower FBS media might reduce the cellular abnormalities seen during expansion as the cells would divide less rapidly [34]. We concur, and further suggest that our results support the proposition to introduce the Pap test into the algorithm for screening cell modification as an initial step (Figure 5). To contribute for the usefulness of this test as a screening step is the possibilities to exclude in the start of screening the cells that have definite and indubitable high-grade alterations.

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