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Original article

Quality control by Flow Cytometry and Media Fill Test of autologous leukocytes marked with ^{99m}Tc-HMPAO to be used for the "*In Vivo*" infection diagnosis: A pilot study

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Abstract. *Background and aim:* Infectious processes can be diagnosed using ^{99m}Tc-HMPAO labeled autologous leukocytes, that once separated and labeled should be neither activated nor apoptotic, so capable of reaching the infectious site. Commonly, only cell vitality by Trypan Blue test is assessed. This work aimed at checking the quality of the labeled leukocytes used for diagnosis, using an optimized cytofluorimetry analysis. *Methods:* Leukocytes from 11 patients were separated, labeled, and controlled by cytofluorimeter after labeling with Annexin V, CD 66, CD 64, CD 45, and 7AAD, markers of activation, apoptosis, and lysis, respectively. In addition, the sterility of the entire process was assessed using "Media Fill test". *Results:* In all the patients tested, about 80% of the cells remained inactivated and suitable for diagnostic use "in vivo." The small portion of activated leukocytes did not affect the preparation's diagnostic use. Only one patient showed a total apoptosis of 76%, and another one of 30% vs the mean of the other 9 patients of 1±0.8%. The Media fill test demonstrated sterility throughout all the process steps. *Conclusions*: Cytofluorimetry was demonstrated to be an accurate and useful method. This could be used both in the process validation phase and when the scintigraphy analysis provides ambiguous results. With a major number of cases, this methodology could provide a good correlation between leukocytes vitality and scintigraphy result, more accurate than Trypan blue test. The *Media fill* used in the clinical context can be run during the validation and re-validation phases of the entire process. (www.actabiomedica.it)

Key words: ^{99m}Tc-HMPAO-WBC, flow cytometry, *Media Fill*, CD64, 7AAD, Annexin V.

Introduction

Infection is the leading cause of mortality worldwide. Multi-resistant bacteria are increasing alarmingly, forcing research toward setting up new diagnostic and therapeutic controls (1). Imaging with labeled autologous leukocytes, a procedure based on functional imaging is a "first choice" technique for localizing infections and is widely applied in Nuclear Medicine (2–4). Imaging analysis of an infected site is influenced by several factors, including the types of radiopharmaceuticals, interference with medications, and the patient's general conditions (5). Imaging with labeled autologous leukocytes is considered a "gold standard" for diagnosing various infected lesions such as osteomyelitis, inflammatory bowel disease (IBD), diabetic foot (6–8) and remains today the only radiopharmaceutical that can distinguish infection from inflammation (4,5). An

important consideration to be made is that, if leukocytes become apoptotic during the process of separation and labeling, or if they have already been activated by other factors (for example drugs) theoretically this can lead to a decrease in the sensitivity of scintigraphy (5). To obtain optimal imaging, you need to use a simple and robust labeling procedure yielding a good quality of radiopharmaceutical with viable leukocytes as little as possible activated. Exametazime (RR, SS)-4,8-diazo-3,6,6,9-tetramethylylundecane-2,10 Dione bisoxime; HMPAO®) is a highly lipophilic compound capable of forming complexes with Technetium-99m (^{99m}Tc), a radioactive isotope gamma-ray emitting, largely used for medical imaging in Nuclear Medicine. The radionuclide ^{99m}Tc emits photons; once administered, it circulates throughout the body and binds its target, so it can be captured for imaging with a gamma camera performing a SPECT scan. This complex can be used for studies on brain perfusion (9), or for labeling leukocytes, due to its easy penetration of the cytoplasmic membranes. Autologous leukocyte labeling with 99mTc-HMPAO is based on the non-specific lipophilic solubility of HMPAO (10) Leukocytes must be isolated from whole peripheral blood before the labeling step (3), but procedures to confirm cell viability before and after the labeling process are lacking (11). The commonly used procedure to evaluate viability is the Trypan blue staining method (vital exclusion), which is characterized by low sensitivity and does not give indications regarding cellular activation (12). In Italy, the D.M. 30-3-2005 "Norms of good preparation of radiopharmaceuticals for nuclear medicine", Annex A4 (13) refers to a general "cellular viability" for the Quality Control of the patient's autologous material, without specifying the method to be used. Within the Nuclear Medicine Units, the "Media fill" test represents the final stage of the validation process related to the preparation of the drug/radiopharmaceutical under aseptic conditions and should be performed only at the end of the validation tests of all elements involved in the process (workers, rooms, equipment, materials). Also, in this case, DM 30-3-2005 (13) does not specify the procedure to follow for performing the "Media fill" test. In our Nuclear Medicine Unit, when scintigraphy tests give doubtful results, is important availability of a validated, standardized procedure more accurate than the common practice of Trypan Blue. The purpose of this work was to optimize the analysis of some cell viability parameters by flow cytometry, and to apply to autologous leukocytes before and after the labeling procedure with 99mTc-HMPAO, currently used (Figure 1). In particular, we chose to monitor a) Annexin V for apoptosis, b) CD 66, CD 64, CD 45 for cellular activation, and c) 7AAD for cell viability assessment. In addition, we have optimized the procedure to evaluate the sterility of the whole process utilizing a "Media fill" (13), so that it can be easily performed in a Nuclear Medicine Unit and used to validate and re-validate the whole process.

Material and Methods

Materials

The accepted practice to evaluate cell viability is to use trypan blue, as indicated in the ISO9001-certified quality manual. For cell vitality assessment, the monoclonal antibodies CD45 V500, CD64 PE, CD66 V450, ANNEXIN V FITC, and 7AAD percp-Cy5,5 were used (BD S.p.A. Buccinasco, Milan, Italy). HMPAO (Exametascan) is manufactured and marketed by Radiopharmacy Laboratory Ltd (2040 Budaörs, Gyár st. 2 Budaörs Industrial and Technology Park, Gutenberg st. 125, Hungary).

Biological samples

The peripheral blood of 11 patients arriving at the Nuclear Medicine Unit for suspected infection, to be diagnosed by scintigraphy with labeled leukocytes, was used for this Quality Control study.

Ethical consideration

All patients signed the notice and gave their informed consent for the routine daily scintigraphy, which routinely requires the peripheral blood sample collection for labeling with 99mTc-HMPAO. Those who refused to take part in the study were excluded. Our Italian legislation (DM 30-3-2005) prescribes at the end of the process for autologous granulocytes

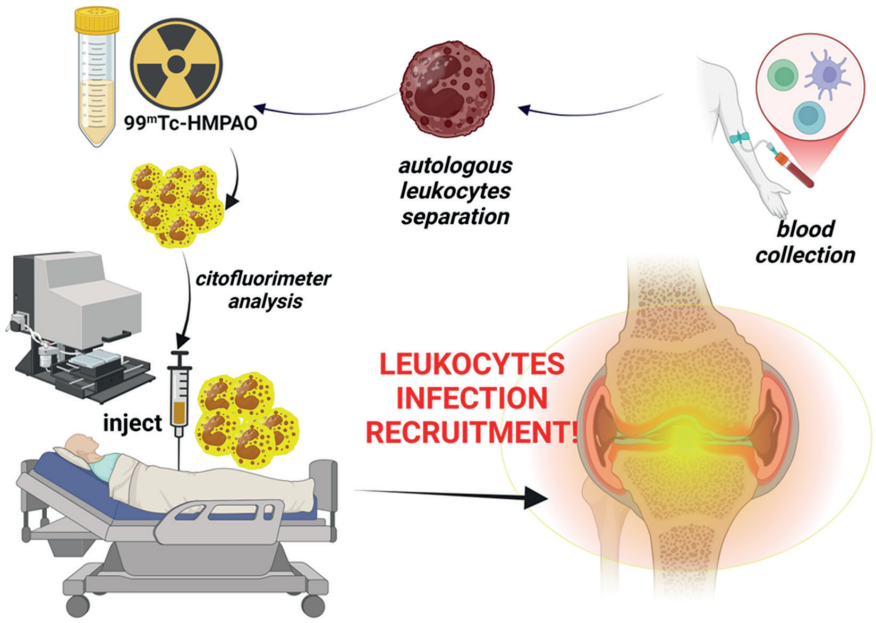

Figure 1. The whole process proposed to validate labelled leukocyte preparation for use in Nuclear Medicine diagnostics.

labeling, and before re-injection into the patient, a quality check on the viability of the labeled leukocytes, without, however, specifying the method. Here we report a more refined quality control at molecular level than the commonly used Trypan Blu assay (14). This study was performed in line with the principles of the Helsinki Declaration and was approved by the Research Commission of the Health Sciences Department, University of Milan, on 19th April 2023 (project No. F_DOTAZIONE_2023_DIP_013).

Separation and radiolabelling of leukocytes

The separation of leukocytes and their labeling were performed in a class A isolator with a class B pre-chamber in a class D room, as per DM 30-3-2005 (13). Every precaution has been taken to protect the preparation from possible bacterial contamination, trying to minimize the passages and centrifugations. In addition, the preparation has been protected from undue radiation doses. All the reagents used were sterile, pyrogen-free, and intended for in vivo use. The procedure was performed at room temperature (22-24 °C) and completely traced by a dedicated working program. The volume of peripheral blood collected, and the radioactivity added to the leukocytes were in proportion to the body weight of the patients, the radioactivity was within the limits of the Low Dose Radiation (LDR) (370 MBq). Peripheral blood (35 ml/75 Kg BW) was taken with a 19G needle on a syringe using 5 ml ACD-A (Baxter SpA, Roma, Italy) as an anticoagulant and 10 ml Gelofusine® (Succinylated gelatin solution 4% for intravenous infusion) (B. Braun Melsungen AG, Werk Pfieffewiesen, Germany) as a precipitating agent (15). The syringe then remained in a static position to sediment the erythrocytes for 30 to 60 minutes inside the isolator. The supernatant containing leukocytes and platelets was transferred to a sterile tube and centrifuged at 150 g for 10 minutes and after the centrifugation, the supernatant containing platelets was discharged. Approximately 1 ml of fresh preparation of the 99mTc-HMPAO radiopharmaceutical (740 MBq) was then added to the leukocyte pellets. After 10 minutes of incubation, the preparation was centrifuged at 150 g for 5 minutes, the supernatant containing the cell-free radiopharmaceutical was removed and the pellet containing the autologous labeled leukocytes was suspended in 2 ml

of saline and then promptly infused into the patient. Radioactivity was measured in the waisted supernatant and in the leukocyte suspension to assess the labeling efficiency using the formula:

Labeling efficiency % =
Radioactivity of labelled cells (MBq)/Total radioactivity added to the cells (MBq) × 100
A labelling efficiency of ≥ 30% was considered acceptable.

Biomarkers of cell vitality for Flow cytometry

CD66 antigens belong to the family of embryonic carcinogen antigen (CEA) molecules closely related to the superfamily of immunoglobulin glycoproteins. Studies of CD66 molecules suggest a potential adhesion function in vivo. Members of the CEA family may be involved in transmembrane signaling and activation of neutrophils. CD66 is therefore a marker for the regulation of neutrophil and eosinophil granulocytes (16,17) CD64 antigen is expressed on monocytes, macrophages, and at low levels on polymorphonuclear neutrophils (18). CD64 antigen is a marker of infection and sepsis (19) CD45 is a family of tyrosine phosphatase transmembrane proteins, involved in the regulation of activation signals. Expression of CD45 is found in all hemopoietic cells, such as granulocytes, monocytes, macrophages, and lymphocytes (20) In flow cytometry, annexin V is a cellular protein used to detect apoptotic cells for its ability to bind to phosphatidylserine, a marker of apoptosis when found on the outside of the plasma membrane (21) 7-amino-actinomycin D (7-AAD) is a fluorescent dye that intercalates into double-stranded DNA (regions rich in GC). It is excluded from viable cells but can penetrate dead or dying cell membranes. Therefore, it can be used for the evaluation of cell death and late apoptosis (22).

Flow cytometry

Cytometric analysis of leukocyte samples was performed in 11 patients using a FACS Lyric cytometer (BD). Immediately after the blood collection in ACD/Gelofusine for the leukocyte separation, an aliquot of peripheral blood was collected from each patient in EDTA to be used as the baseline or pre-treatment sample. Approximately 0.3 ml of the preparation used for diagnostic purposes, consisting essentially of saline and autologous labeled leukocytes, was analyzed as the post-treatment sample.

Samples were prepared as follows:

- Washing, and resuspension following the indications of Annexin V Staining Protocol (BD) using Annexin Binding Buffer, buffer solution at pH 7.4
- Labeling with CD45 V500, CD64 PE, CD66 V450, Annexin V FITC (Fluorescein isothiocyanate), Via Probe (7-ADD) PerCP-Cy5,5 BD
- Incubation in the dark for 15 minutes
- Red Blood Cell Lysis with Pharm Lyse (BD)
- Washing and resuspension of the post-lysis sample with Annexin Binding Buffer at pH 7.4 (BD)
- Cytometric analysis by acquisition of 50,000 total events
- Exclusion of debris with forward scatter (FSC) and side scatter (SSC) parameters
- Gate on granulocyte population using CD45/SSC parameters
- Exclusion from granulocyte analysis of NON-VIABLE events defined as Annexin V-/7AAD+
- Evaluation of the following parameters on vital granulocytes:
  - EARLY APOPTOSIS: evaluated as Annexin V+/7AAD-expression
  - LATE APOPTOSIS: evaluated as Annexin V+/7AAD+ expression
  - ACTIVATION: evaluated as co-expression of CD66 and CD64

The results were expressed as % positive signal cells to the total neutrophil granulocytes. The pretreated not labeled samples were analyzed to assess the inherent fluorescence in the baseline sample.

In addition, as a positive activation control in 5 samples, before and after 1 and 3 hours from labeling,

the autologous leukocytes were treated with TNFα at 10 nmol/L for 10 minutes at 37 °C, before measuring the expression of CD64, Annexin V, and 7AAD.

"Media Fill" Test

The microbiological growth medium used for optional aerobic and aerobic bacteria was BACT/ALERT FA PLUS while BACT/ALERT FN PLUS was used for optional anaerobic and anaerobic bacteria. Instead of peripheral blood, BACT/ALERT FA PLUS soil was used throughout the process, and saline instead of ^{99m}Tc-HMPAO. The whole preparation process was then faithfully reproduced as described above in the "Separation and radiolabeling of leukocytes" paragraph. The validation process involved repeating the process 3 times for both aerobic and anaerobic bacteria. All the samples collected were sent to the microbiology laboratory where they were incubated for 5 days at 35 °C to assess possible aerobic and anaerobic bacterial growth. Figure 2 shows the scheme of the whole procedure.

Statistical analysis

The results were expressed as mean and standard deviation. The comparison of the results in the various periods was performed through non-parametric tests for paired data using the T-test to compare the results before and after 1 hour from the marking and the Friedman test to compare the results of the three

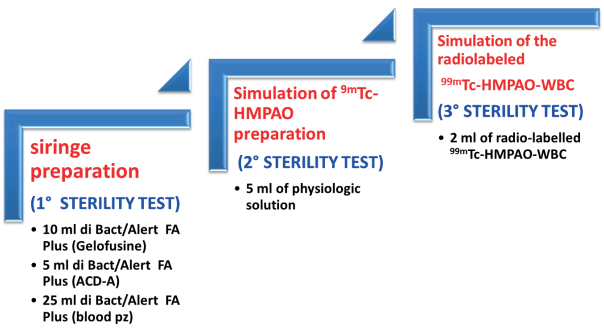

Figure 2. Scheme of the Media Fill test procedure.

time points (0, 1 and 3 hours after marking). The significance has been set at $P<0.05$.

Results

In Table 1 the anthropometric and clinical data of the patients used for this quality control study on leukocytes vitality are reported.

The mean results of the Trypan blue test and of the expression of the CD64, CD66, Annexin V, and 7AAD before and after the labeling procedure are reported in Table 2. By Trypan blue test, cells were vital at 99.0%. As an example, in Figure 3 is reported Flow Cytometry analysis of all the assessed parameters before (on the left), and after (on the right), the labeling of autologous leukocytes with ^{99m}Tc-HMPAO. In Figure 4 the trend of the single leukocyte preparations used for infection diagnosis in Nuclear Medicine are reported in detail.

The quote of NON-vital neutrophil granulocytes (annexin V-/7AAD+) was very low at the baseline (mean 0.77%) and only one patient showed an increase from 0.56 to 8.2% after the labeling procedure. In the other preparations, NON-vital cells remained always well below ≤3%, with a mean of NON-vital cells of 1.64% not significantly different from the beginning (p=0.12). This confirmed that the vitality of the cells was well conserved before and after the labeling process which proved to be safe and unable to significantly damage the cells. The labeling process resulted, instead, in a statistically significant activation of neutrophil granulocytes (CD64+ and CD66+) that increased from 13.5% in the pre-treatment population to 18.5% after labeling (p=0.0069) (Table 2). Two samples were more activated than the others (+16, +13% *vs* a mean activation of +5%). The "in vivo" scintigraphy images were always completely adequate. As for early apoptosis concerns (annexin V+/7AAD-), there were no statistically significant differences before and after the labeling procedure (p=0.15), and the same was observed for late apoptosis (annexin V+/7AAD+) (p=0.16). An average of 10.45% of the cells resulted positive to total apoptosis markers, not reaching, however, the statistical significance (p=0.10). The positive

Table 1. Patients' characteristics

PATIENTS	N	AGE (yrs)	WEIGHT (Kg)	HIGHT (cm)	^{99m}Tc-HMPAO -WBC (MBq)	DIAGNOSTIC QUESTION	POSITIVE SCINTIGRAPHY REPORT No. (%)	NEGATIVE SCINTIGRAPHY REPORT No. (%)
MALES	**4**	**53±19**	**72±19**	**174±8***	**405±74**	1 suspected lower limb osteomyelitis	**3 (75%)**	**1 (25%)**
						1 suspected distal tibia infection		
						1 suspected infection hip prosthesis		
						1 suspected osteomyelitis toe foot		
FEMALES	**7**	**66±13**	**74±18**	**162±4**	**383±73**	4 suspected infections knee prosthesis	**1 (14%)**	**6 (86%)**
						2 suspected distal tibia infection		
						1 suspected septic hip arthritis		

*p<0.001 vs females.

Table 2. Cytofluorimetry analysis of neutrophil granulocytes before and after the labeling process with ^{99m}Tc-HMPAO

Biomarkers of cell vitality	BEFORE mean ± SD (%)	AFTER mean ± SD (%)	(Δ%)	p
Trypan Blue test	-	**99.0±1.0**	-	-
NON-vital (annexin V-/7AAD+)	**0.77±0.47**	**1.64±2.38**	**+0.87**	**0.12**
Early apoptosis (annexin V+/7AAD-)	**0.53±0.77**	**7.48±21.46**	**+6.95**	**0.15**
Late apoptosis (annexin V+/7AAD+)	**0.91±1.18**	**2.98±7.25**	**+2.07**	**0.16**
Total apoptosis	**1.44±1.88**	**10.45±23.43**	**+9.01**	**0.10**
Activated (CD64+ CD66+)	**13.5±7.41**	**18.6±9.50**	**+5.0**	**0.0069***
NON-Activated	**86.4±7.41**	**81.4±9.50**	**-5.0**	**0.0069***

*Significance p<0.05. Δ% represents the increase of neutrophil granulocytes damaged after the process or the decrease of the intact ones.

control, leukocytes treated with TNF-α, showed an increase in CD64 expression (from 2.3 to 43%) of annexin V (from 0.8 to 47%) and an increase in cell death (from 0.9 to 49%). The "media fill" test, carried out in triplicate on all the samples, evidenced sterility in all the steps of the process, for both aerobic and anaerobic bacteria.

Discussion

The labeling of autologous leukocytes with 99mTc-HMPAO remains a "gold standard" for the "in vivo" infection search. In patients treated with drugs and with active infections ongoing, the activation of 99mTc-HMPAO-labelled autologous granulocytes could lead to a potential deficit in the chemotaxis process and, ultimately, to a final scintigraphic imaging of poor quality and/or false negative results. Trypan Blue is a dye that can selectively distinguish, by a rapid microscopic analysis, dead cells from live cells, having the membrane intact and not allowing the penetration of this dye into the cytoplasm. Despite the Trypan Blue exclusion assay being simple, inexpensive, rapid, and recommended by the guidelines (23), it has limitations and remains a basic test. The dye can be incorporated

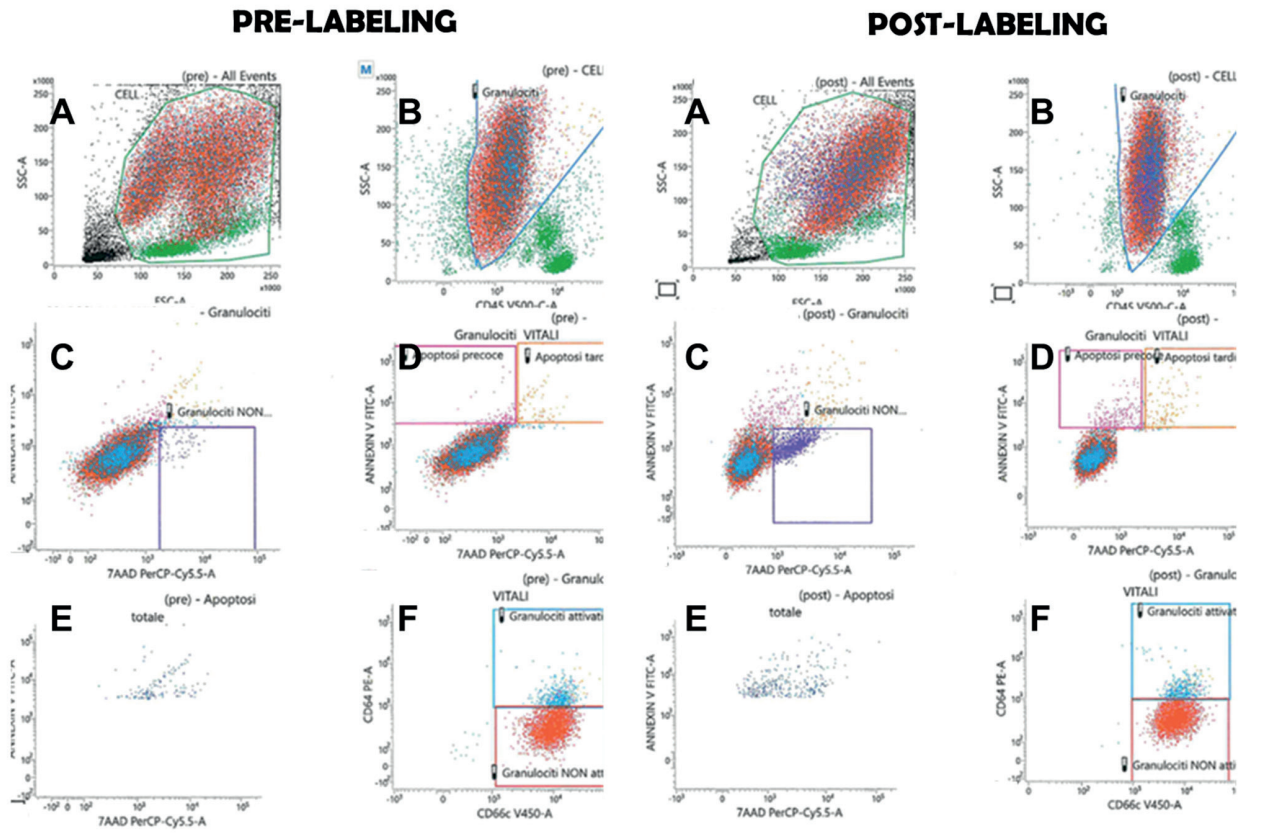

Figure 3. Examples of flow cytofluorimetry of autologous labeled leukocytes with ^{99m}Tc-HMPAO, carried out pre- (on the left) and post- (on the right) labeling procedures. A) Physical parameters of all the blood cells. The Region of Interest (ROI) evidences the Leukocytes. B) All leukocytes. ROI evidence all granulocytes. C)Vital granulocytes. ROI evidence non-vital granulocytes. D) Vital granulocytes ROI showing the quote of early and late apoptosis. E) Granulocytes with total apoptosis. F) Vital granulocytes. ROI evidences the activated (up) and non-activated (down).

by live cells after a short exposure time, the faint blue staining of the viable cells after longer incubations can be misleading, and there is binding of small amounts of trypan blue by proteins. So, personal reliability, related to the expertise of the analyst, can affect the results as the subjective differentiation between dead cells and stained cell debris is difficult (i.e., difficult to compare data between different studies). Thus, the disadvantage of this method is that it tests only cells' membrane integrity. Whether the cells are truly nonviable or just damaged remains unknown, and trypan blue is unable to distinguish between viable cells and cells that are losing cell functions (i.e., detection of necrotic cells only) (24). Additionally, it was suggested that trypan blue might be toxic for the cells over longer incubation times (i.e., ≥10 min). The trypan blue assay thus serves as a good indicator of cell membrane integrity, but it is not an optimal indicator of cell viability. Indeed, the cell membrane damage indicated by trypan blue might recover, and conversely, it might arise from the intracellular compartment and therefore, not be detected by trypan blue at the early stages. Thus, it is very important to select the optimal time for the trypan blue assay to obtain truly relevant data (25). DM30-3-2005 (13), active in Italy, does not suggest or specify the technique to be used for cell viability assessment. The study presented here was mainly focused on the development and validation of a methodological protocol of high accuracy and definition in comparison with the current routine method (Trypan Blue) which is limited

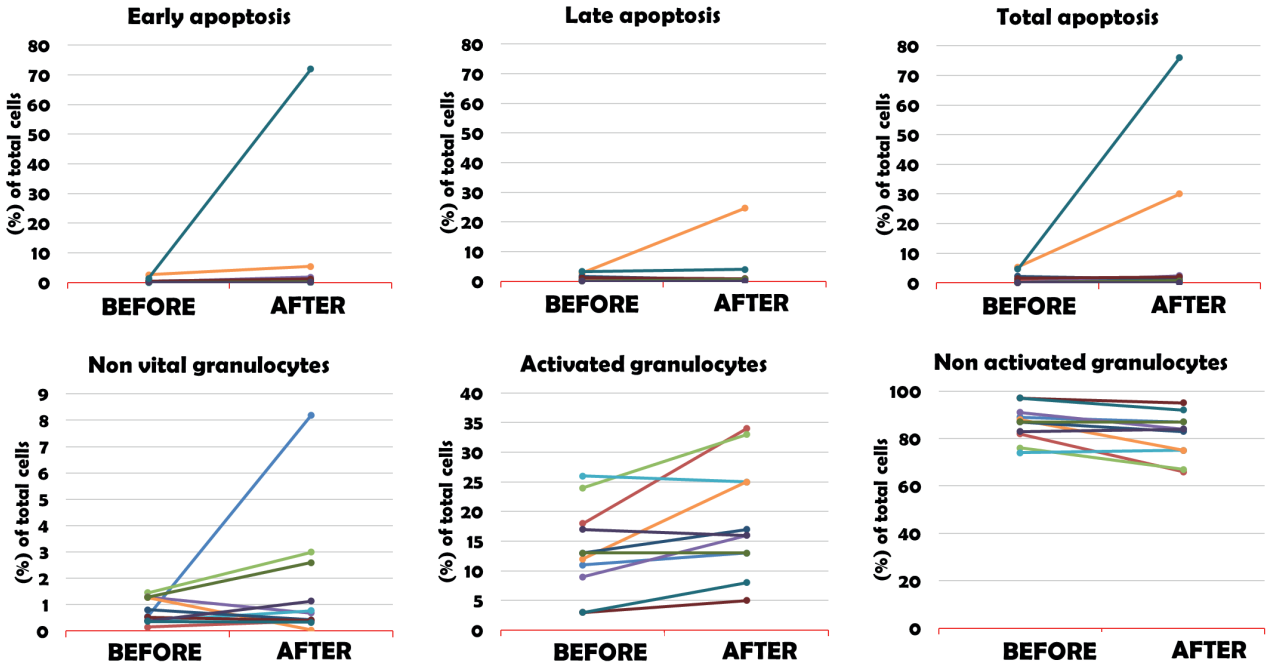

Figure 4. Movement of the tested cell vitality biomarkers in the 11 patients' preparations after the ^{99m}Tc-HMPAO labeling treatment.

to simply assessing the percentage of dead leukocytes. This is a pilot study conducted on a limited number of subjects (n=11) to assess the cell safety along the labeling process, which leaves in a second phase the clinical application study on a more extensive case history and a follow-up in the long term to relate cell viability to the imaging diagnosis of scintigraphy. Here we have presented a battery of cell viability tests by cytofluorimeter using CD45, CD64, CD66, Annexin V, and 7AAD markers that could be applied in the validation and revalidation process and after the labeling process with ^{99m}Tc-HMPAO, in case of ambiguous scintigraphic images. After having optimized the analytical conditions for cytofluorimeter analysis, the quality test was applied to eleven patients referred to Nuclear Medicine for suspected infection. All patients tested had a suspicion of infection and positive inflammation markers. Our data suggests that the simple centrifugation leads to a natural cell activation, due to contact with the flat bottom of the glass test tube used (about 13.5±7.41 of activation in the leukocytes preparation before the process of labeling with 99mTc-HMPAO). After the labeling process, activation of the labeled autologous leukocytes reaches a mean value of 18.6±9.50. As regards apoptosis, only one patient revealed total apoptosis of 76% *vs* a mean value of 10±23% and a second one a total apoptosis of 30%. We observed in the patient with total apoptosis of 76% that the scintigraphy result was negative. This could have affected the negative scintigraphy result because the cells were mostly apoptotic. Of course, this observation must be confirmed and validated with a larger number of patients' cases. The patient that showed a 30% total apoptosis, on the contrary, resulted positive fto the scintigraphy. This result may suggest the need to identify a % apoptosis cut-off that can be correlated with the scintigraphy result. In these patients, we can also envisage a "pharmacological" interference due to the anticoagulant (ACD-A), the plasma expander (GELOSUFINE), or the radiopharmaceutical (^{99m}Tc-HMPAO). However, this occurrence did not affect the result of the scintigraphy images, which were considered of acceptable quality, showing the expected total body distribution of the labeled leukocytes in all the 11 patients studied. All patients regularly receive a medical report, as well as those with impaired apoptosis values. The procedure is cost-effective (as it's an average of 5€/test), it is available in almost every center, and the time required for

cytofluorimetric tests (about half an hour) is compatible with the patient's timing (separation, labeling, and re-injection process). The "Media Fill Test" results in the simulation of the radiolabeling of autologous leukocytes have shown both the absence of clots and fibrin aggregates and the lack of microbial growth, validating the operating procedure described as an aseptic process. However, this conclusion is not permanent but must be reconfirmed annually through a re-validation process as indicated by DM 30-3-2005 (13).

Study limitations

The low number of leukocyte preparations investigated.

Conclusions

We suggest using the proposed comprehensive procedure instead of Thypan Blue to perform this programmed Quality Control, especially during the method validation phase. In addition, the panel of vitality analyses here presented can be useful in evaluating and comparing the available commercial methods for the separation and labeling of autologous leukocytes.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: CM Conceptualization, investigation, methodology, Writing – Original Draft Preparation, GC investigation, GC investigation, GV investigation, methodology, MN methodology, AS methodology, IG methodology, AP Supervision, AC Review & Editing, RP Writing – Review & Editing, Project Administration, Funding Acquisition. All authors revised the manuscript content critically, edited it for intellectual content, and contributed to the discussion. All authors revised the final version and approved the submitted version.

Declaration on the Use of AI: "None".

Ethic Approval: This study was performed in line with the principles of the Helsinki Declaration.

Consent to Participate: The participants signed an informed consent form and approved publishing the scientific results. Those who refused to take part in the study were excluded.

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