O R I G I N A L   A R T I C L E

Novel approach for quality assessment and improving diagnostic accuracy in cell-based infection imaging using 99mTc-HMPAO labeled leukocytes

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Summary. *Background and aim:* Labeled leukocytes with 99mTc-HMPAO are routinely used for infection imaging. Although cell labeling with 99mTc-HMPAO represents an imaging probe to detect infection sites, the diagnostic efficiency of the probe is largely influenced by cell manipulation, multidisciplinary interventions (i.e., biologist, technicians) and available technology (i.e., SPECT, SPECT/CT). The aim of the study was to assess in vitro and in vivo accuracy of a comprehensive approach for quality assessment (QA) of all steps of the procedure. *Methods:* Radiochemical purity (RCP), pH, labeling efficiency (LE) were measured in 320 procedures. White Cell Viability Factor (WVF) was determined in consecutive blood samples. Images (490 studies) were scored using a 5-point scale. Training program was evaluated using a Learning Questionnaire and a score system. *Results:* Pre/post-labelling WVF was 0.99% (max value 1%) in all blood samples. LE (mean value 72%) and RCP (>80% until 55 minutes) yielded considerably high values. The vast majority of images were scored as diagnostic by three independent observer (90% with score ≥4). *Conclusions:* This method appears highly reproducible and easy to use in clinical routine for leukocyte labeling, especially when standardized training and total QA system are implemented. (www.actabiomedica.it)

Key words: labeled leukocytes, 99mTc-HMPAO, radiopharmaceutical, biomedical imaging, quality assessment, leukocytes

List of abbreviations

ACD: Acid-citrate-dextrose
CT: Computed Tomography
EANM: European Association of Nuclear Medicine
HAES: Hydroxyethyl starch sodium chloride solution
HGB: Erythrocyte Hemoglobin
HMPAO: Hexamethylpropyleneamine Oxime
ITLC: Istant Thin Layer Chromatography
LE: Labeling efficiency
LQ: Learning Questionnaire
MR: Magnetic Resonance
PET: Positron Emission Tomography
PI: Propidium Iodide
QA: Quality assessment

QC: Quality Control
RBC: Red Blood Cells
RCP: Radiochemical Purity
SPECT: Single-photon Emission Computed Tomography
WVF: White Cell Viability Factor

Introduction

Leukocytes radiolabeling with 99mTc-HMPAO is the most common approach for infection imaging, thus allowing reliable detection of white blood cells (WBC) accumulation at the site of infection (1). This

process of cell migration can be visualized by planar and SPECT imaging, which enable differentiating sites of sterile inflammation from foci of pathogens infection . Infection imaging by means of labeled leukocytes also allows reliable monitoring of treatment efficacy (2-4).

Many cell types other than leukocytes can be labeled with ^{99m}Tc-HMPAO, thus including platelets (5-6), dendritic and endothelial cells (7-8), spermatozoa (9-10), but this approach can also be applied to labeling of liposomes (11), dendrimers (12) and nanoparticles (13).

Labelling of WBCs is an extemporaneous preparation of radiopharmaceuticals involving multiple steps in vitro (blood manipulation, dispensing) and in vivo (sampling, administering, etc.). This procedure is performed according to specific rules and recommendations, which require a classified environment and qualified personnel (14-16), since inadequate quality assurance of the compounding processes, involvement of inexpert personnel for carrying out compounding and inappropriate environmental conditions may all generate an unfavorable impact on the final product.

Some European countries have adopted specific guidelines and regulations for production of extemporaneous radiopharmaceuticals, especially for labeling of autologous cells, since these cannot be efficiently sterilized after the labeling procedure.

The European Association of Nuclear Medicine (EAMN) has performed a recent survey, concluding that WBC labeling is a well-established technique in Europe, which is mainly performed by trained personnel under sterile conditions in a laminar flow cabinet or cell isolator (class A), installed according to local regulations (17-18).

In 2005 the Italian standards of good preparation were approved by the National Healthcare System ("Roles of Good Preparation of the radiopharmaceuticals") .

According to this specific regulation, labeling procedure and staff training must be validated for guarantying patient safety as well as diagnostic efficacy and accuracy. Some critical issues during the labeling procedure may compromise imaging results and ultimately generate and adverse clinical impact, as clearly proven by the current scientific literature.

Detrimental effects of intracellular radiolabelling on leukocytes include viability problems related to DNA damage (19-27), maintenance and over time reproducibility of the method ,which can hence impair clinical efficacy, complexity and stability of the labeling technique (28). The recent introduction in clinical practice of hybrid diagnostic technology for producing images with different 3D modalities (CT/MR for morphology, SPECT/PET for metabolic and functional information) necessitates a highly efficient detection rate of the target biological process, so improving localization within fused images, along with characterization and over time monitoring of disease.

The current evidence hence suggests that accurate infection imaging with labeled leukocytes requires accurate techniques and adequate training/experience (29-30). Both these factors were main aspects of our study, based on a comprehensive approach to assess the quality of infection imaging, including all the different steps of the whole process (in vitro, in vivo, learning). The quality of acquired images was also assessed in the fused setting of SPECT/CT in our investigation.

Materials and methods

Infection imaging is a routine diagnostic tool performed in our institution (University Hospital of Parma). The local method is based on a standardized multi-step procedure entailing leukocytes isolation and labeling, quality control of prepared radiopharmaceutical, acquisition and post-processing of images. All phases of this process need appropriate learning and adequate training of the staff, both conducted with a standardized approach.

The purposes of this study were scoring and measuring single step data obtained throughout the entire procedure, without impacting routine activity.

In vitro quality assessment was performed by assessing the effect of ^{99m}Tc-HMPAO on blood cells and leukocyte viability, the number of cells efficaciously labeled using ^{99m}Tc-HMPAO at the lowest level of radioactivity and within the smallest possible volume, the maintenance of high radiolabeling yield and complex stability. The high over time reproducibility of labeling method obtained with standardized operator training

and high quality diagnostic images were defined as *in vivo* quality indicators of infection imaging. Written informed consent was obtained from all subjects.

Isolation of leukocytes

Peripheral venous blood (40 ml) was drawn from patients using a 19 gauge i.v. line into a sterile syringe containing 12 ml of acid-citrate-dextrose anticoagulant solution (ACD; formulation A according to the European Pharmacopoeia, consisting of 0,73 g of anhydrous citric, 2,2 g of sodium citrate dihydrate and 2,45 g of dextrose monohydrate in 100 ml of water for injection) and 6 ml of hydroxyethyl starch sodium chloride solution (HAES-sterile according to the European Pharmacopoeia, consisting of 100 g of Poly (0-2-hydroxyethyl) starch and 9 g of sodium chloride) (16).

The isolation of leukocytes from other blood components was performed by centrifugation at 1000x g at room temperature for 10 min, thus finally yielding a leukocyte pellet ready to be labelled.

Radiopharmaceuticals and cell labeling

Hexamethyl propylene amine oxime (exametazime) was supplied as a ready-for-labeling kit (Ceretec®; GE Healthcare). The lipophilic primary ^{99m}Tc-HMPAO complex was obtained after resuspension of Ceretec® with freshly eluated sodium ^{99m}Tc-pertechnetate, according with the manufacturer's instructions (2).

Quality control (QC) of the ^{99m}Tc-HMPAO preparation was performed in agreement with the procedure described by the manufacturer with the accompanying leaflet.

Freshly prepared ^{99m}Tc-HMPAO (750-1000 MBq) in 1 ml of saline was added to the mixed leukocytes suspension (or purified granulocytes) and incubated for 10 minutes at room temperature. The labeling process was stopped by adding 5 ml of NaCl 0,9% (w/v) in the solution. Labelled cells and unbound ^{99m}Tc-HMPAO were then separated by centrifugation. The radioactivity of both supernatant and cell pellet was measured in a dose calibrator, and the efficiency of the labeling method (labeling efficiency, LE) was estimated as the percentage of residual radioactivity in the cells.

The pellet containing the labelled mixed leukocytes was resuspended in 5 ml of NaCl 0.9%(w/v) and the dose (recommended dose 370-740 MBq) was administered to the patient.

In vitro quality measures

As regards the quality control of ^{99m}Tc-HMPAO-labelled WBC, several methods have been described (16), although only a few of them are used in routine clinical practice. In our QC laboratory the quality of each ^{99m}Tc-HMPAO preparation is regularly checked, as for manufacturer's guideline.

A visual inspection of the final product is needed shortly after Ceretec® resuspension, thus enabling to identify aggregates, clumps or clots.

Radiochemical purity (RCP) of ^{99m}Tc-HMPAO has been assessed with istant thin layer chromatography (ITLC) on iTLC-SG glass microfiber chromatography paper impregnated with silica gel (2.5 cm* 2.0 cm, Agilent Technologies) as stationary phase and using two solvent systems as mobile phases. 0.9% NaCl produces a tiny little pick of unincorporated pertechnetate ^{99m}TcO$_4^-$ at the front (R$_f$=0.8-1.0), whilst methyl-ethyl ketone yields a little pick of ^{99m}TcO$_2$ at the origin (R$_f$=0-0.15). RCP, that is the proportion of a radionuclide present in the desired chemical form, must be ^{3}80% (2).

The pH of the ^{99m}Tc-HMPAO preparation was measured using pH test strips, and must be comprised within 9.0-9.8.

LE (%) was assessed after each production, by measuring the amount of radioactivity of both supernatant (soluble ^{99m}Tc-compounds) and pellet (cell-associated ^{99m}Tc) of the labeling solution obtained after centrifugation. LE was calculated using the following formula:

$$\left(\frac{\textbf{Activity of cell pellet}}{\text{Activity of cell pellett + Activity of supernatant}} \right) \times 100$$

A LE values comprised between 40-80% is advisable. When LE is <40%, further quality controls (e.g., microscopic inspection and trypane blue exclusion test for cell viability) should be performed.

In vitro assessment of blood cells and leukocytes viability

Leukocytes viability was assessed with a dye-exclusion assay on three different blood samples before and after labelling with ^{99m}Tc-HMPAO. In this assay, dead cells are stained with a small-molecule dye which can only permeate cells with compromised plasma membranes, whereas the die is not incorporated by live cells with intact membranes, which hence remain unstained. Propidium iodide (PI) is a membrane impermeant dye, which is generally excluded from viable cells, binds to double-stranded DNA by intercalating between base pairs and finally becomes fluorescent.

The fluorescence intensity obtained by staining the nuclear DNA with propidium iodide is expressed as the White Cell Viability Factor (WVF; max value, 1), which represents the fraction of viable leukocytes.

The effect of the labeling process on blood cells was assessed *in vitro* with the fluorescence flow cytometer CELL-DYN Sapphire, a multi-parameter, fully-automated hematologic analyzer.

Quality assessment of operator training

The training process of the local radiopharmacy is scheduled according to the guidelines for safe preparation of radiolabelled blood cells (16, 29-31). This consists of theoretical instructions (local rules and recommendations, available guidelines and pharmacopoeia, guidelines for working in aseptic conditions, including the use of a Class IIa safety cabinet, equipment maintenance), trainee observation (1 wk), supervised practice (2-3 wk) and proficiency assessment (at least three test sets) by personnel certified for cells labelling and performing in vitro quality controls.

Training scheduling and competency assessment were standardized, following the Quality Assurance Manual of the local radiopharmacy.

Before the personnel is qualified for routine activity without supervision, each trainee undergoes competency assessment.

Training program was evaluated using a Learning Questionnaire (LQ). The main objectives of the program were converted into a list of items aimed to capture information about the extent of being comfortable with each of the key objectives (rules, safety cabinet,

equipment maintenance). Learning was assessed using a score system from 6 ("a lot") to 1 ("nothing") for each operator (n=3).

In vivo quality assessment by imaging analysis

Lung uptake was evaluated at 5 and 30 min after labeled leukocytes reinjection on planar image of the thorax, to detect aspecific accumulation of activated neutrophils or cell clumps due to the labeling process. Three independent readers reviewed all planar, SPECT and SPECT/CT images obtained from January 2012 and November 2013 and between February and June 2017, and graded image quality using a 5-point scale (1=non-diagnostic; 2=poor, diagnostic confidence significantly reduced; 3=moderate, but sufficient for diagnosis; 4=good, diagnostic, and 5=excellent).

Statistical analysis

Data were reported as mean ± standard error of the mean (SEM). PCR values were compared using Students paired *t*-test. The statistical analysis was performed with SPSS software.

Results

Preparation and stability of ^{99m}Tc-HMPAO labeled at high specific activity

Ceretec® was supplied in amounts of 0.5 mg (1.85 µmol/L) of exametazime per vial, reacting upon reconstitution with NaTcO$_4$ (3.4-3.7 GBq) to a final volume of 3 ml, in a one to one molar ratio to form the ^{99m}Tc-HMPAO complex. According to manufacturer's guidelines, pertechnetate may be added to Ceretec® in amounts not exceeding 1100 MBq, and the preparation must be used within 30 min after resuspension. Larger amounts of pertechnetate (3-5 GBq) may be added with decreased tenability of preparation (2, 32).

Radiochemical purity control assays of ^{99m}Tc-HMPAO on ITLC-SG strips in 0.9% NaCl displayed showed a modest pick of unincorporated pertechnetate ^{99m}TcO$_4^-$ at the front (R$_f$=0.8-1.0), which significantly increased after 60 min. The radiochemical purity con-
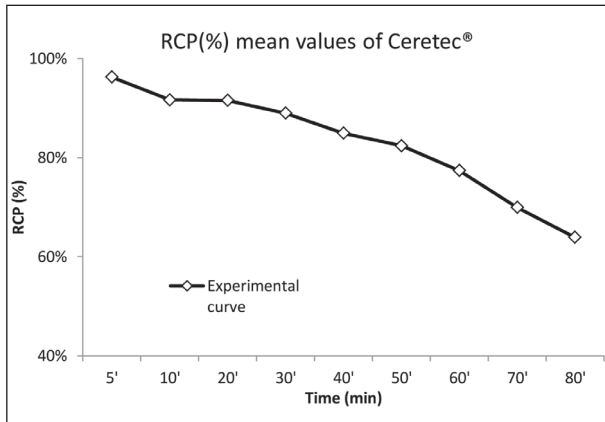
Figure 1. Radiochemical purity of ^{99m}Tc-HMPAO. Mean values over time (3 measures for each time interval from the labeling until 80 minutes)

trol assays on ITLC-SG strips in methyl ethyl ketone solvent showed displayed a modest pick of ^{99m}TcO$_2$ at the origin (R$_f$=0-0.15), which significantly increased after 50 minutes.

Final radiochemical purity was maintained as hisg as 80% up to 55 min (Fig. 1).

Effect of the labeling process on blood cells

Effect of the labeling process on blood cells is summarized in Fig. 2.

WVF was 0.99% in all blood samples, both before and after the labelling process.

The comparison of pre- and post-labeling data (Fig 3 - a,b) shows that the mononuclear component (lymphocytes and monocytes) exhibits a more compact cluster, probably attributable to coarctation of cytoplasmatic membrane, which then generates a reduction of cell volume for adhesion to nuclear membrane.

This morphological alteration is attributable to the method used for cell treatment (i.e., centrifugation) and not to the labeling reaction, as shown by the initial absence of morphological alarms.

After treatment, the appearance of these reflects the presence of immature granulocytes (7.2%) with high intensity (0.74%).

The comparison between pre- and post-centrifugation data (Fig. 3 - c,d,e,f) is suggestive for displace-

(a)

(b)

Figure 2. Effects of the labeling process on blood cells. Data acquired by CELL-DYN Sapphire. (a) Data analysis of the blood sample before labeling with ^{99m}Tc-HMPAO. (b) Data analysis of the blood sample after labeling with ^{99m}Tc-HMPAO
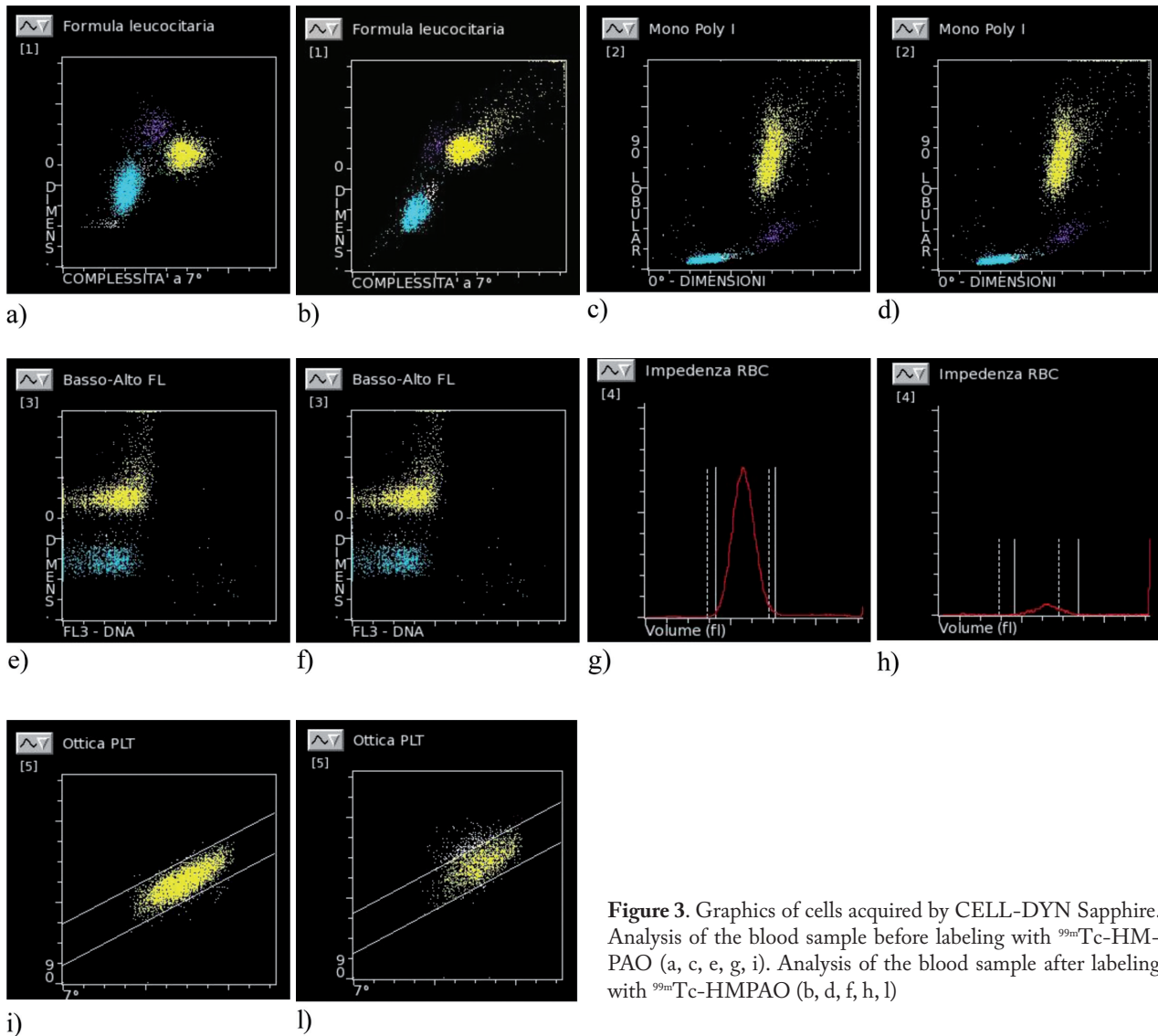
a)   b)   c)   d)

e)   f)   g)   h)

i)   l)

Figure 3. Graphics of cells acquired by CELL-DYN Sapphire. Analysis of the blood sample before labeling with ^{99m}Tc-HM-PAO (a, c, e, g, i). Analysis of the blood sample after labeling with ^{99m}Tc-HMPAO (b, d, f, h, l)

ment of cell clusters due to decrease of lymphocytes/monocytes volume and dispersion of neutrophils and eosinophils for plasma swelling.

Sample hemolysis (4.47×10^{-6}/µl vs 0.045×10^{-6}/µl; p<0.001) and hemoglobin content (152 g/l vs 1.75 g/l; p<0.001) were decreased after labeling (Fig. 3 - g,h).

The red blood cell distribution width (RDW) did not differ before and after the labeling process (11.0% vs 11.2%).

Platelet count assessed with the optical method was significantly reduced after labeling (26.5×10^{-9}/L vs 278×10^{-9}/L; p<0.001) (Fig. 3 - i,l).

Imaging results and assessment of training

A mean number of 160 studies with labeled leukocytes are performed each year in the local facility (1203 from January 2009 to June 2017) for diagnosing infection and identifying the. Overall, bone infection or orthopedic implant infection (27% hip, 23% knee) was the reason for ordering the test in 94% of cases, whilst the test was performed for other causes in the remaining 6% of patients (i.e., for prosthetic vascular graft infection, endocarditis, cardiac device infection, abdominal abscess or fistula, fever of unknown origin).

Planar images were recorded from the segments involved in all patients; SPECT imaging and SPECT/CT was performed in 26% and 10% of them, respectively (Fig. 4).

For the purpose of this investigation, we reviewed 490 studies with labeled leukocytes (planar, SPECT and SPECT/CT images) obtained from January 2012 and November 2013, and those review between January and March 2017.

Lung uptake of labeled leukocytes could not be detected in any patient at the end of operator training.

In two cases lung uptake was identified during the phase of new operator validation (LE of 68% and 66% respectively; RCP of ^{99m}Tc-HMPAO 96.54%).

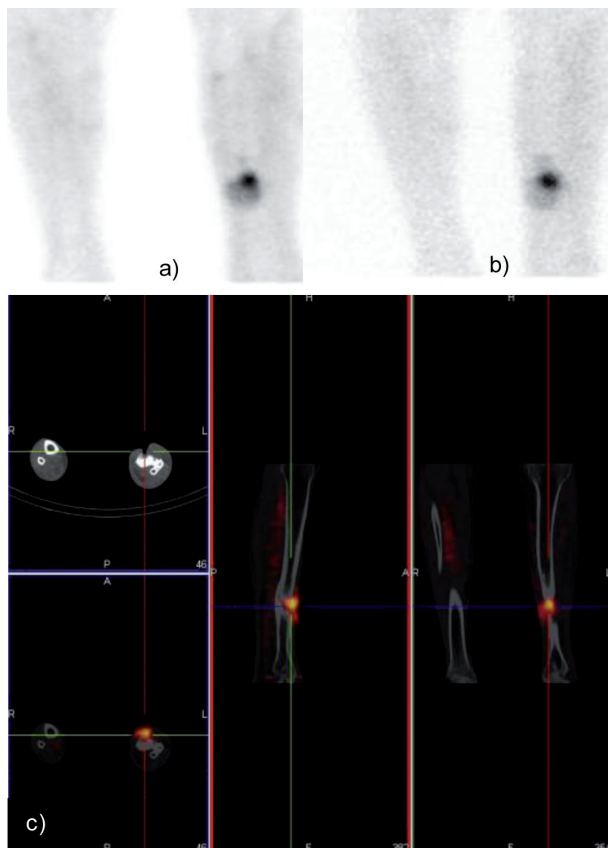An optimal absolute agreement in image quality was found among the three independent observers and all the images were score as diagnostic (90% of observation with score ≥4).

SPECT post-processing was performed with iterative algorithm (8 iterations, 4 subsets) and in all cases the reconstructed images were scored as diagnostic (i.e., score comprised between 3-5), thus allowing localization of the leukocyte accumulation foci in the fused images. The training and learning programs were scored by key objective areas, with a mean value of 5 (4.8, 5.8 and 4.8 for each area, respectively), a good result also considering operator turnover (5 operators were changed from 2012).

Efficiency of leukocytes labeling

The comparison of mean LE (%) values during six months of two consecutive years (i.e., averaging 188 measures) also indicated that the labeling method used in this study was effective to produce a high leukocytes labeling efficiency over time, with an average yield of approximately 72% (Fig. 5).

Moreover, no statistical significance was found (p=0.95) comparing data distribution of LE (%) mean values during the different semesters of the two years, thus confirming the reliability of the labelling technique.

Discussion

The radiolabeling of white blood cells has been introduced in 1976 as an imaging procedure, and has

Figure 4. Imaging of bone infection with labeled leukocytes. M, 49 yrs, tibia/fibula fracture: from 1990 recurrent infections. ^{99m}Tc-HMPAO labeled leukocytes scintigraphy detecting leukocytes accumulation in the left tibial plateau.
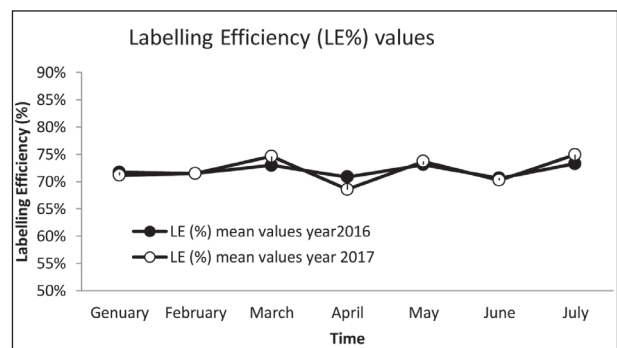Planar images at 3 (a) and 24 hrs (b) and SPECT/CT images (c).

Figure 5. Mean values of radiolabelling yields.
Mean values of LE (%) during six months of two consecutive years 2016-2017 (188 measures)

been used as routine technique in nuclear medicine for detecting infection and inflammation (2-4).

Since the time when Peters et al. described the possibility of labeling granulocytes with ^{99m}Tc-HMPAO, this compound has been considered the preferred labeling agent and has then been commercially available for nearly 30 years (33). Nevertheless, several studies described that radiotoxic effects can be expected with intracellular labeling of leukocytes due to Auger electrons originating from decay of ^{99m}Tc. Detrimental effects of intracellular radiolabeling on leukocytes have also been described, such as response to blastogenic stimulation, chromosomal aberrations, structural changes and DNA damage (19-27).

Taken together, the results of our study provide clear evidence that ^{99m}Tc-HMPAO-labelling do not alter leukocytes viability. Our labeling procedure hence allows maintaining all leukocytes vital. The White Cell Viability Factor, which aimed to assess leukocyte viability (max value, 1), was 0.995% before labeling and remained virtually identical (i.e., 0.994%) afterwards. Our results also demonstrate that the observed morphological alterations were exclusively due to the centrifugation process and not to the labeling reaction.

The centrifugal treatment of the cellular elements caused a substantial reduction of red blood cells (over 99.9%) and erythrocyte hemoglobin (approximately 99%). Although the platelet number was also contextually decreased by approximately 90%, this is an expected outcome during separation of cellular elements by centrifugation. Regardless of these results, WVF did not vary from the baseline, thus confirming the effectiveness and efficiency of the whole labeling process.

The results emerged from our study also indicate that a specific standardized training modality combined with our labeling technique ensure high reproducibility over time, facility of implementation in routine clinical practice despite operator turn-over. We could also document preservation of high leukocytes labeling efficiency (average yield 72%) and high stability (55 minutes) because the agent (Ceretec®) remains bound to labeled leucocytes without decreasing their vitality.

The education program was well received by the operators and made it possibile to achieve the main goals of the labelling technique.

Finally, such a reproducible and stable method allows to obtain high-quality imaging of infection sites also with tomographic (SPECT) and hybrid (SPECT/CT) technology (score 3-5), ultimately enabling an accurate localization of leukocytes accumulation foci in routine clinical practice.

Conclusions

In conclusion, our results shows that cell-based infection imaging with ^{99m}Tc-HMPAO-labeled leukocytes can be easy implemented in routine clinical practice using a standardized approach for training and learning. This can hence allow high reproducibility and establishment of a quality assessment system for reducing vulnerability in lab activity or images acquisition, especially in the challenging context of an increasing turnover of the staff.

This technique also enable to maintain labeled leukocytes vital, is reproducible and stable over time. Finally, this technique allows obtaining high-quality imaging of infection sites also using SPECT and SPECT/CT technology in the daily practice. ^{99m}Tc-HMPAO (Ceretec®) may be used as an efficient and safe tool to study leukocytes turnover and activity in inflammation/infection diseases.

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Authors' contributions

SM and AS carried out the labeling procedures, participated in the data analysis and drafted the manuscript. CC, GB, AS and MS carried out the imaging studies and participated in the images scoring. CG and GS participated carried out technology assessment and performed the statistical analysis. SP and GL performed the cell viability assessment, AS carried out the labeling procedure and collaborated in collecting data, LR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

Authors of this paper are competent in many different disciplines from basic science to clinic. Cell. based procedures are complex and require a multidisciplinary approach and different skills related to radiochemistry (SM, AS), medical physics (CG and GS), biochemistry (GL and SP), nuclear medicine (CC, GB, MS, AS, LR).

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