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Riassunto

La marcatura radioattiva e la re-iniezione di leucociti autologhi è una tecnica di medicina nucleare utilizzata nella rilevazione scintigrafica di infezione nascoste.

Nel presente lavoro l'intera procedura è stata eseguita utilizzando un kit chiuso monouso che include la possibilità di una marcatura multi-paziente utilizzando un solo vial di HMPAO; i parametri per la ottimizzazione delle condizioni sperimentali di marcatura vengono riportati in dettaglio. La resa finale dei leucociti marcati con ^{99m}Tc-HMPAO è stata di circa l'80%, con un volume di sangue prelevato di 32 ml. I valori in vitro dei parametri ematologici ottenuti dopo la marcatura radioattiva sono stati sempre migliori dei valori minimi di accettabilità. Le cellule marcate hanno sempre mostrato una assenza di persistente attività polmonare, e una alta qualità generale in tutte le visualizzazioni scintigrafiche in vivo.

Parole chiave: Leucociti - Globuli bianchi - ^{99m}Tc-HMPAO - Marcatura radioattiva - Marcatura multidose

Abstract

The radiolabelling and re-injection of autologous leukocytes is a nuclear medicine technique used in the scintigraphic detection of hidden infection. In the present paper the whole procedure was performed with a closed, disposable kit, that allows a multi-patient leukocytes labelling by using a single HMPAO vial; experimental parameters for the optimization of the process are reported in detail. The final yield for ^{99m}Tc-HMPAO labelled leukocytes was around 80%, with a withdrawn blood volume of 32 ml. All in-vitro hematological parameters obtained after the radiolabelling were always better than the minimum acceptance threshold. The labelled cells always showed the absence of persistent pulmonar activity, and a general high quality of all in-vivo scintigraphic scans.

***Keywords**: Leukocytes - White blood cells - ^{99m}Tc-HMPAO – Radioactive labelling - Multidose labelling*

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Introduction

Radioactive labelling and reinjection of autologous leukocytes is currently used for the scintigraphic detection of soft tissue or musculoskeletal infection, due to the characteristic of leukocytes to accumulate in inflammatory foci. In order to efficiently radiolabel the leukocytes, various ^{111}In complexes have been proposed along the years (1-3), ^{111}In-oxime being the only one still currently used (4,5). As ^{99m}Tc is currently the preferred radioisotope in nuclear medicine, due to its ideal nuclear and chemical characteristics and its low cost, many ^{99m}Tc labeled molecules have been tested and used for leukocyte labelling (6-8). The molecule ^{99m}Tc-HMPAO (hexamethylpropyleneamine oxime - ^{99m}Tc(III)) is a complex developed for brain scintigraphy; due to its lipophilicity, the molecule has the ability to cross the blood-brain barrier. This complex has also the ability to penetrate through the leukocyte membrane by passive diffusion, is retained and converted into the cells in a hydrophilic complex (9), and is currently the most widely used molecule for white blood cells labelling in clinical practice. In normal individuals, after injection, about 60% of the radioactivity associated to labeled leucocytes is taken by the liver, spleen, bone marrow, followed by an exponential clearance from the blood, with a half-life between 5 and 10 h, resulting in a final uptake of about 20% in the liver, 25% in the spleen, 30% in the bone marrow and 25% in other organs (10). Clearance of labelled leucocytes from liver and spleen is rather slow, while the pulmonary activity is rapidly eliminated; an increasing lung leukocyte uptake extending for 30 min, or more, is generally considered a clear indication of cell damage or activation of leukocytes, and the clinical examination results can be questionable. By using a standard ^{99m}Tc- HMPAO labelling procedure, leukocyte recovery in different experiments was 55% ,while cell-bound activity in the final cell suspension was 60% (10). In the present paper we describe the optimization for the use of a single lyophilized HMPAO vial for multi-patient labelling with a disposable mono-patient closed kit for WBC labelling. This choice lowered the cost of the diagnostic

procedure per patient, for different reasons: (a) one single vial of cold HMPAO can be used for up to six patients; (b); the operator time is optimized, as basically the same time is required for the safe labelling of one or more patients, by using a single vial; (c) the yield of WBC labelling is, paradoxically, increased by using a multi-patient procedure; (d) the time scheduling can be easily optimized in order to increase the gamma camera throughput, insuring the same three scan protocol for all patients (before 1 h, from 2 to 4 h and about 24h p.i.).

The process showed that a routinely yield of cells-bound activity ranging 74-89 % in multi-patient labelling can be easily reached, while retaining high viability of the labeled cells and excellent scintigraphic visualization in-vivo.

Leukocyte collection and labelling

White cell concentrates were obtained starting from the blood of patients, according to the following procedure: a 60 ml disposable syringe containing 8 ml of ACD-A (Acid Citrate-Dextrose Anticoagulant) solution was filled with 32 ml of whole blood. To this syringe 10 ml of 10% 2-hydroxyethyl starch (HES) pharmaceutical grade, molecular weight 200 kDa, were added, the content was gently mixed, and the syringe was kept in vertical upside down position to allow the erythrocytes sedimentation.

After 2 hours, the leukocyte rich supernatant fraction was collected, and centrifuged at 150g for 5 min. After removal of the plasma, the leukocyte pellet was resuspended and incubated with a solution of freshly prepared ^{99m}Tc-HMPAO in saline, with an HMPAO concentration of 125 μg/ml.

After 15-20 min, 5 ml of saline were added, the suspension was centrifuged at 150g for 5 min, and the sedimented erythrocytes and leukocyte depleted plasma fractions were discarded. All the separation and labelling steps were always performed by means of a disposable, ready-to-use closed system kit, containing all necessary sterile materials and reagents (WBC Marker Kit, Celltech, Turin, Italy).

The sterility of the process was tested by using, in the separation procedure, sterile and pyrogen free water (Acilia, PBI International); the final volume of water was incubated in ready-to-use plates, according to the manufacturer instructions, for the chromogenic revelation of bacteria, and of yeasts/molds (PBI International, Milan). The same procedure was also repeatedly used to test the possible presence of pyrogens, by using an immunogenic agglutination Limulus Amebocyte Lysate test (LAL test kit, PBI International, Milan).

Equal volumes of labelled leucocytes suspension and of a 0.4% trypan blue solution in saline were gently mixed, and the resulting solution visualized under microscopy (500x) for counting of blue-stained dead cells; the viable leukocytes were visualized by neutral red or acridine staining of cells.

In the separation process, the presence of any contamination, both from bacteria and yeasts/molds, was always found negative, and the pyrogens concentration was always (n=40) found below the revelation threshold of the LAL test (0.125 EU/ml).

Typical hematological parameters obtained after the ^{99m}Tc-HMPAO labelling process are reported in Table 1.

	RBC/WBC (n=650)	**PLT/WBC (n=40)**	**Total WBC before sedimentation (n=40)**	**Labelling yield (n=820)**	**Non vital leukocytes (%) (n=650)**	**Sedimentation time (n=820)**
Recommended values (11)	<3	<1	Typically $2 \cdot 10^8$	>40	<4	n.d
Experimental values (present paper)	1.62 ± 0.5	0.72 ± 0.2	$2 \cdot 10^8 \pm 0.3 \cdot 10^8$	82 ± 6	1.2 ± 0.3	112 min $\pm$15

Table 1

Results and discussion

It has been demonstrated (12) that during the blood sedimentation the percentage of white blood cells activity in the leukocyte-rich-plasma steadily increases its value from a mean value of 44% after one hour, up to a mean value of 74% after two hours. For this reason, a standard sedimentation time of two hours has been chosen in our Hospital as it significantly increases the total recovery yield of radiolabelled leukocytes, allowing the withdrawal of less blood from the patients, which can be an important clinical advantage, as an example, in pediatric or anemic patients.

The disadvantage of an increased sedimentation time, is compensated by the possibility, with the use of a closed-system disposable kit, of a safe processing of many blood samples in the same session (4 to 6 patients per session are routinely examined in our Nuclear Medicine Division).

Simultaneous radiolabelling of WBC from multiple patients is usually discouraged in clinical practice, in order to prevent possible cross-contamination, and the labelling of WBC from different patients is usually carried out at physically separated locations, unless closed labelling systems are used.

Since ^{99m}Tc-HMPAO-labelled WBC have to be reinjected into the patient, strict aseptic conditions are required for the radiolabelling procedure(11). The kit used in the present work supplies sterile, disposable, pyrogen-free reagents vials and valves, that are used in every separation and manipulation step, strongly simplifying the preparation process, and virtually eliminating the possibility of an accidental contamination of the sample and of the operator, also in multiple labelling sessions.

The whole process was evaluated as optimal because of the in-vitro hematological obtained parameters, the constant high radiolabelling yield, the absence of pulmonar activity, and from the general in-vivo high quality of all the scintigraphic scans. In all examined patients (n=820) the lung activity always showed a complete clearance well

before 60 minutes, proving that no significant leukocyte activation was induced from the separation and labelling process.

Due to the cost of HMPAO cold kit and to the need to prolong the shelf life of 99mTc-HMPAO, various attempts have been carried out to increase labelling yield and stability of the product. Inconsistent reproducibility and declining stability with time have been reported in these attempts (13-16), due to radiolysis problems, low stability of labeled HMPAO, low amount of reducing Sn(II) present in the commercial vial kit. In one paper (17) the freezing of reconstituted HMPAO at various temperature (up to -70 °C) has been proposed to overcome these problems, but also this optimized fractionation procedure can be subjected to manipulation errors or accidental contaminations. For all these reasons we have chosen to always use the HMPAO immediately after reconstitution. In the study of our procedure, an accurate process of optimization of labelling parameters has been carried out, in order to investigate the possibility of using a single vial for multi-patient WBC labelling. Activities of 0.37, 0.74 or 1.11 GBq of 99mTc per patient has been used in the labelling step of HMPAO; an increase in mean labelling yield has been obtained, up to the mean value of 82% for the 1.11 GBq activity. By using this activity per patient, a single vial has been used for the labelling of 1 to 6 patients per preparation session. By decreasing the HMPAO amount per labelling, a paradoxical increase in labelling yield has been found, from a mean value of 66 % for the use of a vial per single patient up to a mean of 82% when the same vial was used to label four to six patients. Some papers (17) have evidenced that the reducing tin(II) present in the HMPAO kit can be a limiting factor for cell labelling, due to the oxygen present in the vials and test tubes, that can cause a partial oxidation of tin. In the kit used in the present work, a total absence of air during all the labelling, washing and centrifugation step is always ensured, and this factor can explain the constantly high yields obtained in our multi-dose labelling procedure. The scintigraphic quality has always been accurately monitored, in order to ascertain if any modification in

diagnostic quality (scintigraphic accuracy and sensitivity, lung activity, liver/spleen radioactivity ratio, vitality of leukocyte preparations) could arise from this procedure modification. No detrimental effect has never been demonstrated, and the process has been officially validated and approved in our Nuclear Medicine Division Procedure Protocol for routinary clinical use. Our data confirm some of the data published in the past from some research groups; in a paper dealing with optimization of HMPAO labelling to improve the shelf-life of reconstituted exametazime (18), an aliquot of a freshly prepared stannous solution was added to to exametazime solution followed by 400-500 MBq pertechnetate; the median labelling efficiency was 72%, range 30-96%. Due to the small volumes of the exametazime solution used, up to 15 doses for leukocyte labelling could be prepared from one single vial. In two publications dealing with efficiency labelling optimization (19,20) a mean labelling efficiency of 64 +/- 7% (n = 29) was achieved when the whole HMPAO vial was used for white blood cell labelling of a single patient. The labelling efficiencies increased to 78 +/- 5% (n = 43), 83 +/- 3% (n = 37) and 85 +/- 5% (n = 18) when one-half, one-third and one-fifth of the lyophilized kit content was used, respectively.

In general, scintigraphy with labeled leukocytes requires the acquisition of images at different times. An early acquisition , not later than 60 minutes, allows to carry out a quality control in vivo (disappearance of the lung within 60 minutes, activity ratio hepatic / splenic < 1 are indicative of a good quality labelling), but also shows the shape of blood vessel in the body area under investigation, which is useful for the interpretation of the subsequent scans and can prove hyperemia in the district of interest. The second acquisition is typically performed between 2 and 4 hours after intravenous injection of leukocytes : this interval time is suitable in most clinical studies, except in bowel inflammatory disease, vascular prostheses and when abdominal pathologies are suspected; in all these cases bowel activity, due to biliary excretion of HMPAO, can seriously interfere with a proper assessment of the images. In these occurrences, according to current literature, and in our clinical experience, it

is appropriate to perform a second acquisition after about 90 minutes, and a third within 150 minutes after injection. The last acquisition is carried on after 18 -24 hours, and can be essential to establish an affordable diagnostic, particularly if the clinical question concerns skeletal sites. Both segmental acquisitions and total body scans can be performed: in our experience, by using a low energy high resolution collimator, 100-250 Kcounts in 2-5 minutes (depending on the body area and the characteristics of the patient) are acquired for static images in the first day, and in 16-40 minutes on the second day. We acquire total body scans in the first (12cm/min) and in the second day (8 cm/min); these can be useful to verify the kinetics of disappearance from the bloodstream, and to prove satellite nodal stations. In some cases , in relation to the clinical question and the body area under investigation, a tomographic acquisition is considered necessary; this acquisition, in our experience, generally lasts about 20 minutes if acquired during the first day, about 40 minutes on the second day.

The majority of patients undergoing WBC scintigraphy in our Nuclear Medicine Division are examined for suspected septic infections of joint replacement. In these cases a standard acquisition protocol includes planar segmental acquisition approximately 20-30 minutes after injection in the joint study, a second planar segmental acquisition 2-4 hours p.i., associated with a total body scan and, finally, an acquisition in the second day of the planar static, associated with a second total body. In our experience, based on more than 800 patients in the last five years, with a dual head gamma camera and adequately trained technical and nursing staff, four patients per day can be easily examined for WBC scintigraphy; with two gamma cameras this number can easily be raised to six per day.

Conclusions

HMPAO has originally been developed as a lipophilic tracer for cerebral scintigraphy, but its characteristics have been in a second time exploited for the in vitro labelling of white blood cells, in mild conditions. The amount of exametazime that is present in a single vial has been designed for the optimization of a cerebral scintigraphy after injection of a single vial in patients; this amount is, however, clearly in excess respect to the number of white blood cells present in about 30 ml of whole blood. It is interesting the fact that by lowering the total amount of exametazime, the labelling yield of white blood cells, paradoxically, increases. A possible phenomenon of site saturation on leukocyte seems to be present, that deserve an extensive and deeper investigation, in order to fully clarify the kinetics and chemical equilibrium parameters of ^{99m}Tc-HMPAO cell labelling .

Indipendently from underlying mechanism, the described procedure has clearly demonstrated the practical clinical feasibility, and after approvation it has been used in our Division in more than 800 patients. This new experimental protocol, together with the use of a single-use closed-system labelling kit, not only allowed us a more widespread use of ^{99m}Tc-HMPAO in WBC labelling, thanks to cost reduction of radiopharamaceutical kit, to the optimization of the working time of the operators and to full exploitation of gamma camera work time, but also demonstrated a higher cell labelling efficiency respect to the standard procedure, with uncompromised high quality of scintigraphic in-vivo visualization.

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