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Original article

Validation of *in vitro* labeling method for human use of heat-damage red blood cells to detect splenic tissue and hemocateretic function

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Summary. *Background and aim:* Selective imaging of the splenic tissue is obtained with heat-damaged, or heat-denatured, red blood cells (RBCs) of the patient labeled with 99mTc in a variety of clinical scenarios. Aim of the study was to validate the process used for labelling heat-damaged red blood cells "totally *in vitro*", after blood sample collection, before re-inject labeled RBCs to the patient. Moreover, we assessed efficacy of the staff training programme in order to guarantee repeatibility and method standardization in the clinical routine. *Methods:* The validation process of the labeling procedure was performed in three different patients during three consecutive days. After collection of a blood sample using a heparinized syringe, we isolated erythrocytes from other blood components by centrifugation and washing steps. Then, we added the stannous pyrophosphate (PYP) to the erythrocytes pellet, after pH control. The 'pretinning' of RBCs was necessary to reduce Tc-99m once pertechnetate was entered them. After the labeling reaction with 130 MBq of 99mTc-pertechnetate, the erythrocytes were denatured in a water bath at a temperature of 49°-50°C, for 10 min. Radioactivity of blood aliquotes was measured with a dose calibrator and labelling efficiency (LE%) was determined. The labelling purity was measured using a gamma counter and calculated using the formula: counts of pellet/counts of pellet+(counts of surnatant)*100.Training program was evaluated using a Learning Questionnaire (LQ). with a grading score from 6 ("") to 1 ("nothing") for each operator (n=3). *Results:* We didn't observed the presence of macroaggregates during the entire process, until the final sample. The labelling efficiency resulted at very high values in the three consecutive measured aliquotes (mean value 73.67%) as well as the labelling purity (>95.22%). In our instituion, we use splenic imaging with labelled heat-damaged RBCs to detect ectopic spleen, splenosis, extramedullary hematopoiesis. We performed 3 procedures with heat-damaged labeled RBCs with a mean labelling efficiency 73.67%.Training and learning programmes were scored by key objective areas with a mean value of 5. *Conclusions:* Our *in vitro* labeling process of heat-damaged RBCs is simple and safe, providing a useful technique easy to implement in clinical routine for splenic imaging Learning outcome of the training programme was scored as effective by all the operators with evidence of high-efficiency-reproducible procedure mantained over time. (www.actabiomedica.it)

Key words: labeled red blood cells, biomedical imaging, quality assessment, splenic imaging

List of abbreviations:
ACD: Acid-citrate-dextrose
CT: Computed Tomography
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
PYP: Stannous pyrophosphate
PI: Propidium Iodide
QA: Quality assessment
QC: Quality Control
RBC: Red Blood Cells
RCP: Radiochemical Purity
SPECT: Single-photon Emission Computed Tomography

Introduction

Red blood cells are found in large numbers in the circulatory system, are easily harvested and are relatively resistant to physical and chemical stimuli that would damage other cells (1).

Red blood cell labelling techniques with Tc-99m evolved from ex vivo labelling methos to in vivo labelling methods aided by the availability of commercial kits (2).

Circulating senescent erythrocyte have an important role in the estimation of splenic reticuloendothelial function, as damaged red blood cells and cellular debris are sequestered by the spleen when phagocytized by the macrophages. Through the process of heating, RBCs undergo fragmentation and spherocytosis, leading to increased stiffness and, consequently, entrapment by the spleen.

This makes the heat-damaged RBC study a sensitive and specific method of identifying splenic tissue and investigating reticulo-endothelial function. One of the most frequent indications for the study is to assess for the presence and location of splenic tissue in patients who have undergone surgical removal of the spleen after trauma or suffering for idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura and hereditary spherocytosis.

Labelling of erythrocyte is one of the extemporaneous preparation of radiopharmaceuticals involving multiple steps in vitro (blood manipulation, labelling and pre-labelling procedures, 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 (2, 3), 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.

EAMN performed a survey demonstrating that cell labelling is a well-established technique in Europe and it is mainly performed by trained personnel under sterile conditions in a laminar flow cabinet or cell isolator (class A), installed according to local regulations (4-6).

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

According to the specific regulation, labeling procedure and staff training have to be validated in order to guarantee patient safety, diagnostic method efficacy and accuracy (7, 8).

The radiolabelling of blood cells may be occasionally problematical. Failure to label may be due to pharmaceutical factors, such as difficulties with collecting sufficient cells, sedimentation problems or instability of the cell chelator, or problems may be patient-related, such as patient medications or the presence of specific disease. In conclusion, labeled red blood cells require adequate technique and adequate training/experience (9, 10).

In our Nuclear Medicine Department RBCs are heat-damaged and labelled with Tc-99m (^{99m}Tc-RBCs) using a "totally *in vitro*" process after collection of the blood sample. Aim of this study was to validate the process used for labeling the damaged red blood cells with Tc-99m. Moreover, we assessed the staff training programme in order to guarantee repeatibility, high-efficiency results and method standardization in the clinical routine.

Materials and Methods

Our method is based on a standardized multistep procedure: blood sample collection, red blood cells isolation and labeling, quality control of prepared

radiopharmaceutical, re-injection of labeled RBCs to the patient, 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.

In this study we propose to score and measure c results of the critical steps of the entire procedure, without any intervention on the clinical routine. Quality assessment of the "*in vitro*" steps was performed measuring effect of Tc-99m on blood cells and erythrocyte viability, amounts of cells efficaciously labeled using 99mTc ate the lowest level of radioactivity and in a volume as small as possible, maintenance of a very high radiolabeling yield and stability of the complex. The validation process of the labeling procedure was performed in three different patients during three consecutive days.

"*In vivo*" quality indicators of the staff training efficacy were considered a) feasibility of a standardized operator training for the whole staff; b) high quality of the obtained diagnostic images; c) high reproducibility of the labeling method over time.

Isolation of erythrocyte

Peripheral venous blood (7 ml) is withdrawn from the patient using a 19 gauge i.v. line into a sterile heparinized syringe. Then, 1ml of acid-citrate-dextrose anticoagulant solution is added to the syringe (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). The whole sample of blood-ACD solution is dispensed into a falcon centrifugation tube and centrifuged at 2500x g at room for 5 minute, then we separete the cell-free plasma (CPF) from the pellet via a long lumbar needle or a butterfly needle of at least 20 gauge, by gently pushing the piston of the syringe up, without disturbing the erythrocyte.

The pellet is washed three times with 10 ml 0.9% aqueous solution of sodium chloride (saline), centrifuged at 2500x g for 5 minute; then, the supernatant is removed from the erythrocyte pellet, also removingmore than 99% of plasma proteins electrolytes and antibodies from the starting sample.

Reconstitution of the stannous pyrophosphate (PYP)

Stannous pyrophosphate (PYP) is commercially supplied as a ready-for-labeling kit (TechneScan® PYP; Covidien). The lyophilized drug is reconstituted by adding 10 ml 0.9% aqueous solution of sodium chloride (saline), and shake until complete dissolution of the lyophilized. The pH of the reconstituted drug is between 4.5 and 7.5.

Treatment of Red Blood Cells with PYP

Pretinning of RBCs is necessary to reduce Tc-99m once pertechnetate has entered red blood cells. Therefore, we add 0.3 ml of stannous pyrophosphate (PYP) to the erythrocytes pellet, and incubate for 35 min using a mechanical agitator to facilitate the process of reduction. Then, the pellet is washed two times with 10 ml 0.9% aqueous solution of sodium chloride (saline), centrifuged at 2500x g for 5 minute and the supernatant is removed from the erythrocyte pellet, to physically separate stannous-treated cells from the non-cellular associated stannous ion in the sample.

Labelling of RBCs with $^{99m}TcO_4Na$

Freshly prepared $^{99m}Tc\ O_4Na$ (70-110MBq) in saline solution (1 ml) is added to the erythrocyte suspension and incubated for 10 minutes at room temperature, using a mechanical agitator to facilitate the process of labelling.

The labeling process is stopped by adding at least 10ml NaCl 0,9% (w/v) into the solution; labelled cells and unbound $^{99m}TcO_4Na$ are separated by centrifugation. Then, the supernatant containing unbound is removed via a long lumbar needle or a butterfly needle of at least 20 G, by gently pushing the piston of the syringe up, without disturbing the pellet and the amount of radioactivityin the supernatant is measured to calculate the labelling efficiency (LE).

The pellet containing the labelled mixed erythrocyte is gently resuspended in 3-5 ml of NaCl 0,9%.

Denaturation of red blood cells

The whole sample of the labeling red blood cells solution is dispensed into a falcon, and incubate at a

temperature 49°-50°C for a maximum of 10 min, with gentle agitation in a water bath to allow the denaturation of RBCs.

Immediately after finishing the denaturation, we transfer the ^{99m}Tc-labeled RBCs dose in a syringe for administering to the patient (11).

The sample is visually inspected and reinjected into the patient as soon as possible, and not later than 1 h after completation of the labelling procedure. injection of the labelled RBCs is performed slowly, preferably using a needle of at least 22 g to prevent cell damage due to shear stress.

In vitro quality measures

Regarding the quality control (QC) of ^{99m}Tc-labelled RBCs, although only a few of them are used regularly in clinical practice. In our QC laboratory we check the quality of each $^{99m}TcO_4Na$ preparation, according to the manufacturer's guideline or the specific monograph.

Immediately after the reconstitution of TechneScan® PYP is necessary a visual inspection of the final product searching for aggregates, clumps or clots.

The pH of the stannous pyrophosphate (PYP) preparation is measured by pH test strips and it has to be 4.5-7.5. After each production, LE (%) is determined with a dose calibrator (Capintec CRC-15 R) by measuring the amount of radioactivity in the supernatant (soluble ^{99m}Tc-compounds) and the pellet (cell-associated ^{99m}Tc) of the labeling solution after centrifugation. LE is calculated using the formula:

LE between 50% and 80% is expected.

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

To determine the purity of the labeling, we withdraw a 10 ul aliquot of the pellet, put it in a test tube A containing 990 ul of NaCl and centrifuge at 2500x g for 5 minutes. Then, we withdraw 500uL of supernatant and put it in a test tube B.

The purity of labeling is expected ≥95 and, it is determined measuring the radioactivity of blood aliquots with a gamma counter (Videogamma-1250 ACN) and calculated using the formula: counts of pellet/counts of pellet+(counts of surnatant)*100.

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 (12-15). 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 are 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).

Results

Preparation and stability of PYP

TechneScan® PYP was supplied in amounts of 20 mg of sodium pyrophosphate decahydrate for vial. According to manufacturer›s guidelines, the kit was reconstituted whit 10 ml of sodium chloride 0.9%, and the preparation used within 4 hours after resuspension. The pH of the reconstituted drug was 6 in all the samples.

Efficiency and purity of RBC labeling

Labeling efficiency of the samples used to validate the process resulted 73.56%, 72.56% and 73.88%. Mean value of LE (%) during three consecutive days

also indicate that our labeling method lead to high erytrocyte labeling efficiency over time, with an average value of yield around 73.47%.

The purity of labeling was ≥95% in all the samples, confirming the efficiency of the labelling method.

Assessment of training

Training and learning programmes were scored by key objective areas with a mean value of 5 (4.8, 5.8, 4.8 for each area respectively) also in the context of high operator turn-ver.

The education programme was well received by the operators and made it possible to achieve rapidly the main goals of the labelling technique.

In all the performed exams two indipendent nuclear physicians assessed images as high-quality ones also allowing hybrid imaging (SPECT/CT) to increase accuracy in localizing labeled RBCs accumulation, expecially to detect splenosis foci (16) or extramedullary hematopoiesis.

Discussion

As in the case of other nuclear medicine examinations, the final result of a a scan obtained with ^{99m}Tc-RBCs is dependent on the labeling efficiency (Hunter and Pezim, 1990; Kuehne, 1999; Wieseler et al., 1994) in order that the impurities do not interfere with the quality of the image or result in an unacceptably high radiation dose to the patient. Therefore, it is important that high quality images be acquired starting from an excellent radiopharmaceutical preparation (Marson et al., 1998; Poter. 1983). Our "totally *in vitr*" labeling procedure showed high LE with a mean value of 73.67% as well as a labeling purity >95.22% in all the measured samples, ensuring the absence of macro-aggregates until the final product.

The results emerged from our study also indicate that a specific standardized training modality associated to our labeling method lead to highly efficient and reproducible procedure over time, easy to implement in clinical routine.

Personnel involved in preparation and release of radiopharmaceuticals has to be appropriately trained in quality systems, current good radiopharmacy practice (cGRPP) and the specific regulatory requirements (17).

Increasing staff turn-over related to the progressive reduction of financial resources in the National Healthcare System, makes it mandatory to implement rigorous competency based Radiopharmacy Training assuring to operate safely and effectively in the "hot" laboratory, to reduce the risk to patients as many of the radiopharmaceuticals come in the form of injections and/or formulations containing radioisotopes. In our Department growing attention is focused on a standardized delivery of learning and training, customized for the specific competences of the multidisciplinary team. Training modality, learning outcome and staff validation goals are defined in the Validation Master Planof the Department (8). Running 3 consecutive batches for each procedure used in the Radiopharmacy is performed for each new operator prior to initiation of clinical activityand after the standardized training. Periodic assessments of the effectiveness of training programme is taken (every 6 months) and after any significant change in the methods or reagents.

Conclusions

Our results show that the "totally in vitro" labeling of heat-damaged red blood cells with Tc-99m can be easily implemented in routine clinical practice despite a "multistep" complex procedure requiring cell manipulation if a standardized operator training is regularly performed and assessed, encouraging continuous update of core competencies in hot laboratory staff, also in the context of high operator turn-over. Our labeling method appears highly reproducible, efficient, and stable over time, due to implementation of a comprehensive QA system targeting all the critical steps of the process, including staff skills.

Finally, high labeling efficiency and purity of this procedure, allows to obtaining high-quality hybrid (SPECT/CT) images.

Authors' contributions:
SM and AS carried out the labeling procedures, participated in the data analysis and drafted the manuscript. AS carried out

the labeling procedure and collaborated in collecting data, GB, MS and 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), nuclear medicine (GB, MS, LR).

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

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