Effects of Methylene Blue Stabilizer on In Vitro Viability and Chemotaxis of ^{99m}Tc-Exametazime–Labeled Leukocytes

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This study was conducted to evaluate the effects of methylene blue/phosphate buffer stabilizer on the labeling efficiency, cell membrane integrity, chemotaxis, and in vitro stability of leukocytes labeled with ^{99m}Tc-exametazime. **Methods:** Whole blood (480 mL) was obtained from each of 3 healthy donors and divided equally into eight 60-mL aliquots. Two aliquots from each of the 3 subjects were used as a control (i.e., leukocytes were not labeled with either nonstabilized or stabilized ^{99m}Tc-exametazime) in this study. The remaining 6 aliquots from each of the 3 subjects were divided equally into the following formulation groups: (a) leukocytes labeled with nonstabilized ^{99m}Tc-exametazime, (b) leukocytes labeled with stabilized ^{99m}Tc-exametazime containing 250 μg methylene blue, and (c) leukocytes labeled with stabilized ^{99m}Tc-exametazime containing 500 μg methylene blue. Duplicate samples were evaluated to confirm the repeatability of the study. Six samples were studied under each experimental condition (i.e., control, a, b, and c groups). The cell membrane integrity and chemotatic capacity of leukocytes were measured at 1 and 3 h after preparation, whereas a complete blood count was obtained at 3 h after preparation. The labeling efficiency was calculated immediately on conclusion of cell labeling, whereas the in vitro stability of the radiolabeled leukocytes was evaluated at 3 h after radiolabeling. **Results:** None of the samples showed trypan blue–stained cells. For the chemotactic index, no statistically significant differences were noted between the 3 labeling formulations at 1 h ($P = 0.43$) or 3 h ($P = 0.10$) after preparation. None of the labeling formulations differed significantly from the control values of the chemotactic index (all $P > 0.25$). For the complete blood count, no significant differences were observed between any of the groups, with the exception of platelet number in the control group ($P = 0.004$). The labeling efficiency ($P = 0.10$) and in vitro stability ($P = 0.33$) were also similar in our comparison of the 3 labeling formulations. **Conclusion:** With no major statistically significant difference noted either between the 3 labeling formulations or between the control and the 3 labeling formulations, we concluded that the methylene blue/phosphate buffer stabilizer (250 or 500 μg methylene blue) did not affect the cell membrane integrity, chemotactic capacity, labeling efficiency, in vitro stability, or complete blood count of leukocytes labeled with stabilized ^{99m}Tc-exametazime.

Key Words: ^{99m}Tc-exametazime; radiopharmaceutical stabilization; radiolabeled leukocytes; methylene blue; chemotaxis

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Our laboratory recently published a study of the biodistribution and radiation dosimetry of leukocytes labeled with stabilized ^{99m}Tc-exametazime in 10 healthy subjects (*1*). The cell labeling efficiency and in vivo stability appeared to improve compared with the results of leukocyte labeling with nonstabilized ^{99m}Tc-exametazime. In addition, there are advantages in a more cost-effective preparation of the stabilized ^{99m}Tc-exametazime as well as an extended window for clinical usage. Our previous study showed that stabilized ^{99m}Tc-exametazime maintained an acceptable value of radiochemical purity (RCP) up to 6 h, even after the reagent kit was reconstituted with 4 or 5 times the recommended ^{99m}Tc activity (i.e., 7.4–8.0 GBq [200–215 mCi] vs. 2.0 GBq [54 mCi]) (*2*,*3*). No significant increase was observed in either specific organ or whole-body dosimetry estimates on comparison with our previous estimates using nonstabilized ^{99m}Tc-exametazime–labeled leukocytes (*1*,*4*).

However, we noticed that the stabilized ^{99m}Tc-exametazime–labeled leukocytes used in our study had a lower peak recovery and prolonged lung half-clearance time (*1*), which may represent a mild-to-moderate form of cell activation (*5*). Grüning and Franke (*5*) have suggested that the addition of methylene blue during labeling could contribute to cell activation.

Although the labeling efficiency, cell membrane integrity, chemotaxis, in vitro stability, and complete blood count are not synonymous with cell activation, they do indicate preserved viability and function. Thus, the purpose of this study was to evaluate the effects of methylene blue/phosphate buffer stabilizer on the labeling efficiency, cell membrane integrity, chemotactic behavior, and in vitro stability of leukocytes labeled with stabilized ^{99m}Tc-exametazime.

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MATERIALS AND METHODS

Samples

Each control and each of the 3 labeling formulations (6 samples each) were evaluated in our study: (Control) Leukocytes were not labeled with either nonstabilized or stabilized ^{99m}Tc-exametazime; (labeling formulation a) leukocytes were labeled with nonstabilized ^{99m}Tc-exametazime; (labeling formulation b) leukocytes were labeled with stabilized ^{99m}Tc-exametazime containing 250 μg methylene blue; and (labeling formulation c) leukocytes were labeled with stabilized ^{99m}Tc-exametazime containing 500 μg methylene blue.

Separation of Leukocytes

Using aseptic technique in a vertical laminar flow hood, whole blood (480 mL) from each of 3 healthy donors (obtained with approval of the Mayo Institutional Review Board) containing 60 mL anticoagulant citrate dextrose (ACD) was divided into eight 60-mL portions. Duplicate samples for each of the 4 groups (i.e., control, a, b, and c groups) were evaluated to verify the reproducibility of the evaluation. The following steps were performed on the control and the 3 labeling formulations unless indicated otherwise.

Eight milliliters of hydroxyethyl starch (6% hetastarch in 0.9% NaCl solution [Volex; American Critical Care, McGraw Park, IL; and Hespan; DuPont Pharmaceuticals, Wilmington, DE]) were added to each sample and inverted gently to mix. Samples were then set at a 45° angle and allowed to sediment for 30 min. After sedimentation, the supernatant of leukocyte-rich, platelet-rich plasma was removed and centrifuged at 160*g* for 10 min to create platelet-rich plasma (PRP) and a button of mixed cells including leukocytes. PRP was removed and centrifuged at 3,200*g* for 10 min to create 9–12 mL platelet-poor plasma (PPP). Four milliliters of PPP were saved for final resuspension of the separated leukocytes, and the remaining PPP was discarded. The button of mixed cells was mixed with 8 mL 12.6% ACD/saline and centrifuged for 5 min at 100*g* to remove platelets that remained in the supernatant. The supernatant was removed and discarded to leave the white cell button, which was then mixed with 8 mL 12.6% ACD/saline (control sample preparation stopped at this point). For groups a, b, and c, the white cell button mixed with 8 mL 12.6% ACD/saline was centrifuged for 5 min at 100*g* to remove additional platelets. The supernatant was removed, leaving the leukocyte button.

Preparation of Nonstabilized and Stabilized ^{99m}Tc-Exametazime

Using a fresh ^{99m}Tc eluate from a ^{99m}Tc generator eluted previously within 24 h, the Ceretec kit (Medi-Physics, Inc., Amersham Healthcare, Arlington Heights, IL) was reconstituted with 2 mL 8.3–8.7 GBq (225–236 mCi) ^{99m}Tc activity. One milliliter of nonstabilized ^{99m}Tc-exametazime was removed and mixed with 1 mL physiologic saline to be incubated with samples taken from labeling formulation a (~925 MBq/0.5 mL [~25 mCi/0.5 mL] per sample). The other 1 mL nonstabilized ^{99m}Tc-exametazime was divided into 2 vials: vial 1 (~2.2 GBq/0.5 mL [~60 mCi/0.5 mL]) was mixed with 0.5 mL methylene blue stabilizing solution (0.5 mL 1% methylene blue injection U.S. Pharmacopeia [USP] and 4.5 mL phosphate buffer), and vial 2 (~2.2 GBq/0.5 mL [~60 mCi/0.5 mL]) was mixed with 0.5 mL methylene blue stabilizing solution (1 mL 1% methylene blue injection USP and 4 mL phosphate buffer). The stabilized ^{99m}Tc-exametazime solution in vial 1 was used to label leukocytes as identified in labeling formulation b (~925 MBq/0.5 mL [~25 mCi/0.5 mL] containing 250 μg methylene blue per sample). Stabilized ^{99m}Tc-exametazime from vial 2 was withdrawn to label leukocytes taken from labeling formulation c (~925 MBq/0.5 mL [~25 mCi/0.5 mL] containing 500 μg methylene blue per sample).

Each of the 3 Ceretec kits was reconstituted with 2 mL 8.3–8.7 GBq (225–236 mCi) ^{99m}Tc activity, and the RCP of ^{99m}Tc-exametazime was determined using a single-strip chromatography method, which was developed initially by Jurisson et al. (*6*). This RCP testing method involves using Gelman Solvent Saturation Pads (1 × 8.5 cm) (Pall Life Sciences, Ann Arbor, MI) as the stationary phase with ether used as the mobile phase (*6*).

Radiolabeling of Leukocytes

Approximately 925 MBq (~25 mCi) nonstabilized or stabilized ^{99m}Tc-exametazime (with 250 or 500 μg methylene blue) was added to the leukocyte button (~90 min after the blood collection step), mixed gently, and incubated for 20 min. After incubation, 3 mL 12.6% ACD/saline were added to the solution and centrifuged for 5 min at 150*g* (first dilution process) (*2*). The supernatant was removed, and 9 mL 12.6% ACD/saline were added to the leukocyte button. The mixture was then centrifuged for 5 min at 150*g* (second dilution process) (*2*). The supernatant was removed and the leukocyte button was suspended in 4 mL PPP.

Evaluation of Leukocytes

Labeling Efficiency. The labeling efficiency of the radiolabeled leukocytes with nonstabilized or stabilized ^{99m}Tc-exametazime was calculated by the equation:

$$\text{Labeling efficiency (\%)} = \frac{\text{Final radiolabeled activity}}{\text{Total activity after 20-min incubation}} \times 100.$$

Cell Membrane Integrity. All leukocyte samples in our study were stained with 0.4% trypan blue solution (Allied Chemicals, Morristown, NJ) to evaluate the integrity of the cell membrane. Twenty-five microliters of trypan blue were added to a test tube, and an equal volume of mixed leukocyte suspension was added to the tube. This combination was mixed gently, and a drop of this solution was placed on a hemocytometer, which was then placed under a light microscope for observation. Any uptake of trypan blue by the leukocytes was an indication of nonviability of the cells. Cell membrane integrity was measured at 1 and 3 h after preparation.

Chemotaxis Assay. The ability of neutrophils to migrate toward a chemoattractant was evaluated with a modified under-agarose technique originally described by Nelson et al. (*7*). This assay was performed at 1 and 3 h after preparation.

Six sets of 3 wells were cut into a layer of agarose on a plastic Petri dish. The middle well of each of the 6 sets contained leukocyte samples from 1 of the labeling formulation groups. The leukocytes settled to the bottom of the well and attached to the plastic plate. With regard to the 2 surrounding wells (180° apart), the inner wells contained a neutral buffer solution (i.e., Gelatin Hanks' balanced salt solution [Life Technologies Inc., Rockville, MD]), whereas the outer wells contained a chemoattractant (i.e., *Escherichia coli*), in which the attractant diffused into the agar to establish a concentration gradient.

The plates were allowed to incubate in a 37°C, 5% CO_2 incubator for 3 h (original method of Nelson et al. (*7*) requires a 2-h incubation time). During the incubation, the leukocytes adhered to

the plastic surface and migrated under the agarose in random fashion (e.g., toward either the buffer well or the attractant well). The migration distances were used as indicators of random and directed chemotaxis. After incubation, migration of leukocytes was halted by the addition of 5 mL 2.5% gluteraldehyde and 0.01 mol/L phosphate buffer applied to the center of each plate, allowing the solution to remain on the plate for 30 min (stoppage of leukocyte migration, as stated by Nelson et al. (*7*), involves the application of 3 mL absolute methanol for 30 min and 3 mL 47% buffered formalin for 30 min) and staining with Wright's stain (Sigma, St. Louis, MO). The leading edge of the leukocyte population migrating on the plastic out of the well was measured. The distance of migration toward the chemoattractant was designated as directed migration, whereas the distance of migration toward the neutral buffer was designated as random migration. The chemotactic capacity (i.e., chemotactic index) was calculated by taking the ratio of directed migration to random migration:

$$\text{Chemotactic index} = \frac{\text{Directed migration}}{\text{Random migration}}.$$

In Vitro Stability. The in vitro stability of ^{99m}Tc-exametazime–labeled leukocytes was determined by measuring the amount of unbound ^{99m}Tc activity eluted from the radiolabeled white cells. The suspension of the mixed leukocytes labeled with stabilized ^{99m}Tc-exametazime and 4 mL PPP was stored at room temperature for 3 h. The radioactivity in the supernatant and the radiolabeled leukocyte button was measured separately to determine the amount of ^{99m}Tc activity released from the labeled mixed leukocytes. This evaluation process was conducted at 3 h after leukocyte radiolabeling.

Complete Blood Count. Aliquots from all leukocyte preparations (i.e., control, a, b, and c groups) were analyzed using the Coulter STKS cytometer (Beckman Coulter, Inc., Miami, FL). The STKS is a hematology flow cytometer that yields a full blood count report, which includes erythrocytes, platelets, leukocytes, and a 5-part leukocyte differential (i.e., neutrophils, lymphocytes, monocytes, eosinophils, and basophils). This instrument also measures volume, conductivity, and light scatter simultaneously for each cell.

Statistical Analyses

Continuous variables are summarized as a mean ± 1 SD. To compare the effects of the labeling formulation on chemotaxis, labeling efficiency, and complete blood count, a linear mixed-model ANOVA approach was used to account for the repeated experimentation on the same subject. To compare the labeling formulation with the control, a paired *t* test was used, and this was done separately for each of the 3 labeling formulations.

RESULTS

Radiochemical Purity of ^{99m}Tc-Exametazime Preparations

The average RCP of the 3 preparations, before the addition of methylene blue/phosphate buffer stabilizer, was 91.8% ± 1.0%. According to the package insert for Ceretec, an RCP of >80% is necessary for ^{99m}Tc-exametazime used for leukocyte labeling (*3*).

TABLE 1
Effects of Methylene Blue Stabilizer on Performance of Leukocytes

Labeling formulation	Chemotactic index		Labeling efficiency at 0 h (%)	In vitro stability at 3 h (%)
	1 h	3 h		
Control	2.4 ± 0.1	2.4 ± 0.2	NA	NA
a	2.5 ± 0.3	2.2 ± 0.2	85.8 ± 6.3	95.7 ± 1.0
b	2.9 ± 0.9	2.5 ± 0.1	83.8 ± 6.1	94.6 ± 3.0
c	2.5 ± 0.3	2.6 ± 0.5	87.0 ± 6.0	94.7 ± 1.3

Control = leukocytes not labeled with nonstabilized or stabilized ^{99m}Tc-exametazime; NA = not applicable; a = leukocytes labeled with nonstabilized ^{99m}Tc-exametazime; b = leukocytes labeled with stabilized ^{99m}Tc-exametazime containing 250 μg methylene blue; c = leukocytes labeled with stabilized ^{99m}Tc-exametazime containing 500 μg methylene blue.

Data are expressed as mean ± SD (n = 6).

Evaluation of Leukocytes

Labeling Efficiency. Table 1 shows that the labeling efficiency results of the radiolabeled leukocytes using nonstabilized (i.e., labeling formulation a) or stabilized ^{99m}Tc-exametazime with 2 different methylene blue formulations (i.e., labeling formulations b and c) were 85.8% ± 6.3%, 83.8% ± 6.1%, and 87.0% ± 6.0%, respectively. The labeling efficiency (P = 0.10) was similar between the 3 labeling formulations (Table 1).

Cell Membrane Integrity. Using visual assessment, none of the samples (i.e., control or labeling formulations a, b, and c) showed trypan blue–stained cells, indicating a viable mixed leukocyte population in each of the 4 groups.

Chemotaxis Assay. For chemotaxis, there was no statistically significant difference between the 3 labeling formulations either at 1 h after preparation (P = 0.43) or at 3 h after preparation (P = 0.10) (Table 1). Also, no statistically significant difference was found between the labeling formulations with regard to the change in chemotactic capacity between the 1- and 3-h measurements (Table 1). None of the labeling formulations differed significantly from the control study either after initial preparation or at 3 h after preparation (all $P > 0.25$).

In Vitro Stability. The in vitro stability was similar between the labeling formulations (P = 0.33) (Table 1).

Complete Blood Count. For the number of leukocytes, no statistically significant differences were found between the 4 groups (P = 0.71). The average number of leukocytes isolated from the initial 60 mL whole blood was 138.2 ± 34.7 × 10^6 with 65.8% ± 3.3% neutrophils. The measured counts for the other cell components (with the exception of platelets) were comparable between the 4 groups (P = 0.82, 0.90, 0.55, and 0.88 for lymphocytes, neutrophils, monocytes, and erythrocytes, respectively). The average platelet number for the control group (i.e., 202.7 ± 69.2 × 10^6) was considerably larger than that for the other 3 groups (i.e.,

group a, 73.0 ± 16.9 × 10^6; group b, 70.0 ± 16.5 × 10^6; group c, 50.3 ± 7.9 × 10^6; $P = 0.004$).

DISCUSSION

A recent study by Roca et al. (*8*) suggests that methylene blue causes a decrease in the cell chemotactic capacity of radiolabeled leukocytes, even though the data show that the indicator that they used to evaluate the chemotactic capacity of the labeled leukocytes (i.e., directed migration or the chemotactic index, as designated by Roca et al., and the chemotactic index or the ratio of the chemotactic index and the random index) shows a "large magnitude" of within-sample (i.e., replicated measurements under the same condition) and between-sample (i.e., measurements from different condition) variation. Although most of the difference can be accounted for by the directed migration and chemotactic index data, the random migration data appear to be similar between the 2 groups (i.e., leukocytes radiolabeled with either nonstabilized or stabilized formulation). The source of within-sample and between-sample variation of chemotactic capacity observed by Roca et al. is unclear. Nevertheless, the authors conclude that methylene blue–stabilized ^{99m}Tc-exametazime should be avoided in leukocyte radiolabeling (*8*).

Some major noticeable differences between the studies performed by Roca et al. (*8*) and our laboratory (*2*) may directly affect the outcome of both studies:

- The stabilizer used in their study is an unmixed methylene blue solution (i.e., 0.6 mg/mL in saline) (*8*) rather than the recommended mixture of 10% methylene blue injection USP (10 mg/mL) and 0.003 mol/L monobasic sodium phosphate USP and dibasic sodium phosphate (buffer solution specified in the Ceretec package insert (*3*).
- The amount of methylene blue solution applied to each test sample of the study performed by Roca et al. (*8*) was ~20 μg, which is significantly less than the amount that we used in our previous study (i.e., 250–500 μg) (*2*) and in this study (i.e., 250 and 500 μg).
- Roca et al. (*8*) incubated the mixed leukocytes with much less ^{99m}Tc-exametazime activity than we used in our previous study (*2*) and in this study (i.e., ~333 MBq vs. ~925 MBq [~9 mCi vs. ~25 mCi]).
- The chemotaxis evaluation method used by Roca et al. (*8*) was a modified Boyden chamber system that separates 2 solutions with 2 porous membranes (*9,10*). The cells are placed in solution on 1 side of the membranes, and a chemoattractant is placed in the solution on the other side. The number of radiolabeled cells that have migrated into the membrane nearest the attractant solution (i.e., directed migration) is quantitated by measuring the radioactivity of the membrane. This finding is then compared with the counts from a membrane in a chamber that had no attractant, just buffer (i.e., random migration).
- The Boyden method involves the use of a radioassay to determine migration values (*9,10*), whereas the chemotaxis assay system that we used in our study, which was a modified version of the technique of Nelson et al. (*7*), simply measured leukocyte migration distances (i.e., directed and random) on agarose to determine the chemotactic index values.

There is no established gold standard for the evaluation of leukocyte chemotaxis. The agarose system (i.e., modified method of Nelson et al. (*7*)) used in this study has a drawback: It is difficult to know whether all cells being examined on the agar plate have been radiolabeled. Even though one may assume that the labeled cells are homogeneously distributed in the isolated leukocyte preparation, as such, the measured chemotactic index using the agarose system can be considered to be a representative value. The chemotactic radioassay used by Roca et al. (*8*) attempted to overcome this problem by measuring the radioactivity retained on the membrane. However, the results obtained using the Boyden chamber system (*9,10*) may be affected by cell adhesion to the filter material and by the tortuosity and size of the pore channels of the filter membranes. It is conceivable that the methylene blue/phosphate buffer could affect the chemotactic capability of the overall leukocyte population, not just the labeled cells. Consequently, the issue of whether the chemotactic index is truly representative of the radiolabeled leukocytes may be irrelevant.

According to the Ceretec package insert (*3*), the recommended amount of methylene blue to be used for stabilization is ~2,000 μg per vial of stabilized ^{99m}Tc-exametazime. Our study tested the effects of methylene blue, using either 250 or 500 μg per sample. If a stabilized ^{99m}Tc-exametazime vial contains ~8.1 GBq (~220 mCi) ^{99m}Tc-pertechnetate, one should be able to dispense an average of 4–6 doses (925 MBq [25 mCi] each) of ^{99m}Tc-exametazime from this vial. Consequently, the amount of methylene blue in each dose should be in the range of 333–500 μg. We have taken the lower end of the aforementioned average usage of stabilized ^{99m}Tc-exametazime vials (i.e., 4 doses per vial) as a worst-case scenario, in which each dose of a 925-MBq (25 mCi) stabilized ^{99m}Tc-exametazime dose contained 500 μg methylene blue. On the other hand, the 250 μg methylene blue per 925-MBq (25 mCi) dose was taken from the best-case scenario of dispensing an ~8.1-GBq (~220 mCi) stabilized ^{99m}Tc-exametazime vial (i.e., 2,000 μg methylene blue per vial ÷ 8 doses per vial = 250 μg methylene blue per dose).

The labeling efficiencies (i.e., 83.8% ± 6.1% and 87.0% ± 6.0%; $n = 6$ each) for the stabilized ^{99m}Tc-exametazime-labeled leukocytes in this study are similar to the labeling efficiency (i.e., 89.1% ± 2.8%; $n = 6$) for the ^{99m}Tc-exametazime–labeled leukocytes with 0-h-old stabilized ^{99m}Tc-exametazime from our previous study (*2*). The overall elution of unbound ^{99m}Tc activity from each of the 2 labeling formulations of the stabilized ^{99m}Tc-exametazime–

labeled leukocytes (i.e., labeling formulations b and c) was similar to the results obtained in our previous study with radiolabeled leukocytes using 0-h-old stabilized ^{99m}Tc-exametazime (*2*).

Our study design included a control group in which the mixed leukocytes were not labeled with either nonstabilized or stabilized ^{99m}Tc-exametazime. This group provided a true baseline of the chemotactic index with regard to unlabeled leukocytes. Overall, the chemotactic index values for all tested groups (i.e., control, a, b, and c groups) of both evaluation time periods (i.e., 1 and 3 h after preparation) in our study were similar, and the SD for each average chemotactic index value was small (Table 1). The chemotactic index values obtained in our study were within the normal range (i.e., 1.5–2.9; *E. coli* as chemoattractant) established in our institution with a modified technique of Nelson et al. (*7*). The chemotactic index for leukocytes and *E. coli* combination as measured in the original study by Nelson et al. (*7*) is 3.8 ± 0.9 (range, 2.8–5.7).

The significantly higher number of platelets in the control group was most likely due to the fact that the mixed leukocytes in the control group did not undergo a second washing and centrifugation process (i.e., mixing with 8 mL 12.6% ACD/saline and centrifugation for 5 min at 100*g*) to remove additional platelets. This outcome clearly illustrates the importance of the second washing and centrifugation in the isolation process of leukocytes, which would yield a leukocyte preparation with much less platelet contamination.

Because no major statistically significant difference was noted either between the 3 labeling formulations or between the labeling formulations and control groups, we concluded that the methylene blue/phosphate buffer stabilizer had no effect on the labeling efficiency, cell membrane integrity, chemotactic behavior, in vitro stability, or complete blood count of leukocytes labeled with stabilized ^{99m}Tc-exametazime. Although these 5 parameters that we evaluated in our study do not necessarily have any direct connection with cell activation, they do suggest that the cell preparations used in formulations b and c (i.e., incubated with different amounts of methylene blue) still maintained their viability and chemotactic function.

CONCLUSION

With no statistically significant difference noted between the 3 labeling formulations (i.e., a, b, and c) or between the control group and the 3 labeling formulations (Table 1), we concluded that the methylene blue/phosphate buffer stabilizer (250 or 500 μg methylene blue) did not affect cell membrane integrity, chemotactic capacity, labeling efficiency, in vitro stability, or complete blood count of leukocytes labeled with stabilized ^{99m}Tc-exametazime.

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