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Preclinical assessment of Tc-99m labeled Nano colloid Liposomes as an Effective Blood Pool Radiopharmaceutical.

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ABSTRACT:

This study evaluated two Tc99m-liposome formulations as potential blood-pool agents in comparison with standard 99mTc-red cells and 99mTc-human serum albumin (HSA). 40 rabbits: Liposomes with no surface modification or coated with polyethylene glycol (PEG) were labeled with 99mTc using the lipophilic chelator, HMPAO. Autologous red cells were labeled with 99mTc using in vitro or in vivo techniques. Technetium-99m-HSA was supplied commercially. Rabbits were injected intravenously with 99mTc-liposomes, 99mTc-red cells or 99mTc-HSA. Static images were acquired and blood samples collected. Results: Technetium-99m-liposome images showed prominent blood-pool activity compared to lung and liver activities, which were similar to those acquired for 99mTc-red cells, but better than 99mTc-HSA. Heart-to-lung ratios were not significantly different between 99mTc-liposome as compared to 99mTc-red cells. The ratios were higher than for 99mTc-HSA. Heart-to-liver ratios were higher for PEG 99mTc-liposomes than they were for neutral 99mTc-liposomes and 99mTc-HSA, but were not significantly different than 99mTc-red cells. Bladder activities for both 99mTc-liposome was 3-6 times lower than for the other radiopharmaceuticals. PEG 99mTc-liposomes remained in circulation 1.6 times longer than any of the other agents.

Conclusions: Technetium-99m-liposomes, independent of surface modification, had excellent in vivo stability when compared to 99mTc-red cells and 99mTc-HSA. PEG 99mTc-liposomes are better than neutral 99mTc-liposomes due to lower liver background activity.
Advantages of PEG liposomes compared being excellent in vitro and in vivo stability and lack of drug interference so it can be used in blood pool imaging so it can be used in blood pool imaging studies.

**Key Words:** liposomes; technetium-99m-liposomes; blood-pool imaging; technetium-99m-HMPAO.

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**INTRODUCTION:**

The development of a safe, convenient and stable radiopharmaceutical for blood-pool imaging studies of cardiac function, venography and detection of gastrointestinal bleeding has so far remained an elusive goal (1). The most commonly used blood pool agent has relied on labeling autologous red cells with 99mTc (1). Recently, human serum albumin (HSA) labeled with 99mTc has been substituted for 99mTc-red cells because of its simple preparation and reduced likelihood of transmission of potential blood borne pathogens (1).

The use of 99mTc-HSA as a blood-pool agent, however, has been shown to be less than ideal due to its poor in vivo stability compared to 99mTc-red cells (1). Several strategies have been explored to develop blood-pool agents that are more effective than 99mTc-HSA and that do not rely on the use of autologous 99mTc-red cells. First, HSA has been modified by conjugation with 2,3-dimercaptopropionyl before 99mTc labeling and shown to have greater in vivo (2,3). A second potential cell-free blood-pool agent currently in preclinical testing consists of polylysine polymer conjugation of diethyleneetriacetic acid (DTPA) for chelation of 99mTc and containing a polyethylene glycol (PEG) for increased circulation times (PEG 99mTc-DTPA polylysine polymer) (4). Finally, a cell-free system comprised of polyethylene glycol (PEG) surface-modified liposomes has been studied as another blood-pool agent (5,6).

In these early studies, PEG-coated liposomes were labeled with 99mTc using a DTPA phospholipid-based surface-labeling technique or with 67Ga using method (5,6). In the present work, a newly developed technique was used to label liposomes with 9mTc using hexamethyl-propylene-amine-oxime (HMPAO).
This lipophilic chelator is thought to carry 99mTc inside preformed liposomes, where it is trapped following conversion of the lipophilic HMPAO to its hydrophilic form in the presence of glutathione (7).

**Aim of the work:** to study blood-pool imaging characteristics of two 99mTc-liposome radiopharmaceuticals in comparison to in vivo and in vitro labeled 99mTc-red cells and 99mTc-HSA in rabbits. The formulations consist of liposomes with no surface modification (neutral) or a PEG surface coating.

**MATERIALS AND METHODS:**

Liposome Preparation Two formulations were tested:

1. **PEG liposomes** comprised of distearoyl phosphatidylcholine (DSPC): cholesterol: distearoyl phosphoethanolamine-N-[Poly (ethylene glycol) 5000] (DSPE-PEG 5000):alpha-tocopherol (50:38:10:2 molar ratio). 2. Neutral liposomes comprised of DSPC: cholesterol: alpha-tocopherol (66:32:2 molar ratio). These liposomes were prepared as previously described (8,9), except after rehydration with 100 mA/ reduced glutathione in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, liposomes were extruded through a series of polycarbonate filters.

The diameters of neutral and PEG liposomes were determined to be 138 nm ±47 nm and 134 nm ±37 nm, respectively, by particle-size analysis. Phospholipid concentration for the neutral and PEG liposomes was 63 mM and 60 mM, respectively (10). Intravascular GSH concentration was estimated using a commercial assay kit to be 0.6 mM and 0.2 mM for the PEG and neutral liposomes, respectively.

Liposome Labeling Procedure Liposomes (3 ml) were mixed with 1.5 ml of HMPAO preincubated with 10 mCi sodium pertechnetate in 5 ml 0.9% saline. Reconstituted kits were checked for contamination using a threestep, thin-layer chromatography system outlined in the HMPAO kit package insert. The kits used for the liposome labeling studies contained >80% lipophilic HMPAO. After 30 min, the liposomes were separated from any free 99mTc by passage over a Sephadex G-25 column. Labeling efficiencies were checked by determining the activity before and after column separation of 99mTc-liposomes using a dose calibrator. Mean labeling efficiencies of neutral 99mTc-liposomes and PEG 99mTc-liposomes were 52% and 66%, respectively. Post-column preparations of 99mTc liposomes were used immediately for injection.
In Vitro Technetium-99m-Red Cell Labeling Procedure using autologous blood (3 ml) was withdrawn via an ear artery into a heparinized syringe and labeled with 99nTc using an Ultra-tag kit. After 20 min, labeled red cells were washed in normal saline and spun at 800 X g for 10 min. Supernatant and pellet fractions were checked for activity in a dose calibrator. Labeling efficiency was >97%. In Vivo Technetium-99m-Red Cell Labeling procedure was done using 99mTc using a Pyrolite kit. Each rabbit was injected in an ear vein with 0.5 ml of Pyrolite rehydrated with 10 ml of normal saline, which represented 48-90 /mg stannous chloride, 0.5 mg sodium pyrophosphate and 1.5 mg trimetaphosphate. After 20 min, 2.5 ml sodium pertechnetate (4 mCi) was injected via an ear vein. Technetium-99m-HSA Labeling Procedure Technetium-99m-HSA was purchased as a commercial kit and reconstituted. Imaging Studies was performed under the National Institutes of Health Animal Use and Care guidelines. **40 rabbits you should mention the number studied.**

Male New Zealand white rabbits (3.5-4 kg) were anesthetized intramuscularly with ketamine-xylazine (50mg/10mg) and placed in the supine position. PEG ⁹⁹ᵐTc-liposomes (2.0 ml, 2 mCi, n = 6), neutral ⁹⁹ᵐTc-liposomes (2.0 ml, 1.3 mCi, n = 5), in vitro ⁹⁹ᵐTc-red cells (2.0 ml, 2.8 mCi, n = 4), in vivo ⁹⁹ᵐTc-redcells (2.0 ml, 4 mCi, n = 4) or ⁹⁹ᵐTc-HSA (2.0 ml, 3.0 mCi, n = 4) were injected through an ear vein. Phospholipid dose was approximately 17mg phospholipid/kg body weight for both neutral and PEG ⁹⁹ᵐTc-liposomes. Whole body imaging’s with zoom of 2 magnifying the heart region were acquired using a gamma camera equipped with a high-resolution collimator. The camera was interfaced to a dedicated computer workstation and 1-min static images were acquired using a 64 X 64 matrix at 5, 22, 45 and 120 min post injection. Five-minute static images were acquired for all radiopharmaceuticals at 20 hr to measure isotope decay. Image Analysis Heart-to-liver and heart-to-lung ratios were determined from region of interest (ROI) analysis of zoomed static images. The count density of a 2 X 2-pixel box located over the heart, lung and liver in each image was recorded and these values were used to determine the heart-to-lung and heart-to-liver ratios. Bladder activity was determined by drawing a ROI around the bladder in each whole-body static image. A region of interest was drawn around the entire body in the image to determine total body counts. Blood sampling to measure the circulation kinetics of the radiopharmaceuticals were done via an ear artery.
Samples were collected immediately following the infusion of the radiopharmaceutical (approximately 3 min), at 5 min, every 15 min for the first 2 hr, at 20 hr and at 44 hr. The activity of each sample was measured in a scintillation well counter. The activity measured in the sample at the 3 min time point for each animal was taken as the maximal value (100%) and the activities at the other time points were related to this value.

Each radiopharmaceutical was also counted as a standard reference.

Statistical Analysis  Statistical analysis was performed using software for the Macintosh computer. Student's unpaired t-test was used to compare the heart-to-lung ratios, heart-to-liver ratios and bladder activity for each agent at a given time point. A p value <0.05 was considered statistically significant. Blood clearance curves were fitted to an exponential model to generate circulation half-lives using Scientist for Windows software with supplemental Pharmacokinetic Model Library.

RESULTS:

Whole-body gamma camera images of rabbits acquired 45 min post injection of each blood-pool agent are shown in Figure 1. For cardiac function studies, the relationship of the distribution of the agent in the heart compared to the background activity associated with the lungs and liver is important, especially for accurate quantitation of ejection fraction. Comparison of these regions in the images showed that the activity associated with the blood pool was prominent for all agents except 99mTc-HSA. In these images, blood-pool activity was greater than lung activity for all agents tested. This observation was further substantiated by determining that the heart-to-lung ratios for each agent were >1, as outlined in Table 1. Comparison of the 45 min heart-to-lung ratios, which correspond to the images, showed that the ratio for PEG 99mTc-liposomes was greater but not significantly different than the neutral 99mTc-liposome ratio. Also the ratio for PEG 99mTc-liposomes was lower than the value determined for in vitro Tc-red blood cells, whenever the mean value determined for neutral 99mTc liposomes, which was significantly less mean in vitro Tc- Red blood cells (p < 0.01). Ratios determined for both liposome-based agents were slightly greater than the in vivo 99mTc-red blood cell value, but with no statistical difference. Both liposome formulations, were significantly has higher mean value than the ratio for 99mTc-HSA (p < 0.05).
Table 1: Heart lung ratio of Tcliposomes, Tc-RBCs and Tc HAS.

| Time (min) | PEG liposomes (n = 6) | Neutral liposomes (n = 5) | In vitro RBCs (n = 4) | In vivo RBCs (n = 4) | HSA (n = 4) |
|------------|-----------------------|--------------------------|-----------------------|---------------------|------------|
| 22         | 2.09 ± 0.21           | 2.09 ± 0.11*             | 2.76 ± 0.16*          | 1.92 ± 0.05*        | 1.66 ± 0.02|
| 45         | 2.40 ± 0.25*          | 2.20 ± 0.09tt            | 2.71 ± 0.11*          | 2.02 ± 0.12*        | 1.58 ± 0.05|
| 120        | 2.30 ± 0.21           | 2.31 ± 0.21*             | 2.75 ± 0.07*          | 2.09 ± 0.16*        | 1.70 ± 0.05|

Values represent the mean ± s.e.m.
*<p < 0.05 versus HSA.
**p < 0.01 versus in vitro RBCs.
***p < 0.01 versus HSA.
****p < 0.001 versus HSA.

Also, Heart-to-lung ratios for both 99mTc-red cell preparations were significantly higher mean value than for 99mTc-HSA (p < 0.001 for in vitro 99mTc-red cells; p < 0.05 for in vivo 99mTc-red cells). During the initial 120 min, there were no statistically significant differences in the heart-to-lung ratios as a function of time for any of the agents.

The images also showed that blood-pool activity was greater than liver activity for all agents except 99mTc-HSA, which was the only agent with heart-to-liver ratios < 1, as outlined in Table 1. Blood-pool activity was similar for both 99mTc-liposome formulations, but there was less liver activity associated with PEG 99mTc-liposomes than neutral 99mTc-liposomes, which lead to a higher heart-to-liver ratio for PEG 9mTc-liposomes compared to neutral 99mTc-liposomes.

This ratio for PEG 99mTc-liposomes was not significantly different from the in vitro 99mTc-red cell ratio, whereas the ratio for neutral 99mTc-liposomes was significantly lower. Heart-to-liver ratios for both liposome formulations were significantly greater than 99mTc HSA at all-time points studied. Likewise, the ratios for both in vitro and in vivo 99mTc-red cells were significantly greater than the ratios for 99mTc-HSA at all-time points.

A comparison between 99mTc-red cell labeling methods showed that the ratio for in vitro 99mTc-red cells was greater than the ratio for in vivo 99mTc-red cells (p < 0.05) at 45 min. As with the heart-to-lung ratios, there were no significant effects of time on the heart-to-liver ratios for the blood-pool agents, with the exception of the ratio for 99mTc-HSA, which decreased significantly.
At 45 min post injection, there were major differences in the organ distribution of the blood-pool agents in the abdominal region where blood-pool agents are used to detect sites of gastrointestinal bleeding (Fig. 1). There was very little spleen activity seen with either in vitro or in vivo 99mTc-red cells, indicating that the red cells were not damaged during the labeling process. The 99mTc-liposome formulations showed greater activity in the spleen than either 99mTc-red cells or 9mTc-HSA.

**DISCUSSION:**

Technetium-99m-labeled liposomes were evaluated in this study in order to determine if they meet the criteria of being a safe, convenient and stable radiopharmaceutical for blood-pool imaging studies. Both 99mTc-liposome formulations were compared with 99mTc-red cells labeled using standard in vivo and in vitro methods. The labeling efficiencies, blood circulation times and bladder activities for both red cell labeling methods were similar to those seen in other studies comparing the in vivo and in vitro methods (1).
Also, as demonstrated by other researchers and confirmed in the present study, heart-to-lung and heart-to-liver ratios were better for the in vitro 99mTc-red cells than in vivo 99mTc-red cells due to lower background 99mTc activity in the lungs and liver for in vitro 99mTc-red cells \(^1\). 99mTc-liposomes could potentially be substituted for 99mTc-red cells as blood-pool agents. At 45 min, heart-to-lung and heart-to-liver ratios for PEG 99mTc-liposomes were not significantly different from 99mTc-red cell ratios. On the other hand, heart-to-lung and heart-to-liver ratios for neutral 99mTc-liposomes did not significantly differ for in vivo 99mTc-red cells, but were significantly less than in vitro 99mTc-red cells. Therefore, PEG 99mTc-liposomes would make a better blood-pool agent than neutral 99mTc-liposomes. Although heart-to-lung and heart-to-liver ratios for the 99mTc-liposomes were lower than in vitro 99mTc-red cells in this rabbit model, both 99mTc-liposomal agents remained in the vasculature longer than 99mTc-red cells. The half-life of 8.8 hr for in vitro 99mTc-red cells determined in rabbits in this study is less than the biological half-life of 29 hr reported in humans, but agrees with other studies in rabbits by the manufacturer. Also, during the first 120 min, bladder activities for both 99mTc-liposome formulations were significantly lower than both 99mTc-red cell preparations. This increased blood retention and low bladder activity for both 99mTc-liposome formulations show better in vivo stability of 99mTc label associated with the liposomes compared to Tc-red cells labeled by either method. Labeled liposomes may be better radiopharmaceuticals with increased safety, because the technologist does not have to handle potentially contaminated blood during the red cell labeling procedure. Technetium-99m-liposomes are also more convenient to use than 99mTc-red cells because they require only one venipuncture and can be labeled before the patient arrives.

In addition, 99mTc liposomes have been found to be very stable following reconstitution and labeling with little decomposition of the 99mTc label (>6 hr) in previous studies \(^7\). Also, it is recommended that in vitro 99mTc-red cells be re-injected into the patient within 30 min. Therefore, unlike both in vivo and in vitro 99mTc-red cells, 99mTc liposomes could be prepared ahead of time for emergency situations. Technetium-99m-liposomes would be more reliable than 99mTc-red cells for gastrointestinal bleeding studies because of their excellent in vivo stability, as shown by the lack of significant bladder activity.
Furthermore, 99mTc-liposomes are unlikely to be affected by medications prescribed for the patient that can interfere with the labeling and stability of 99mTc-red cells \(^{(1,12)}\). Both 99mTc-liposomes and 99mTc-HSA are convenient radio pharmaceuticals because they require few steps, use cheap and widely available sodium pertechnetate and can be prepared prior to the patient’s arrival. Technetium-99m-liposomes would be safer to produce and administer than \(^{"^{18}Tc}\) HSA, because HSA is derived from human blood, which is a source for the transmission of blood borne pathogens in some patients. However, has this safety issue been overcome by producing HSA using recombinant DNA Technology. Another difference between the two agents is the improved in vivo stability of 99mTc-liposomes over 99mTc-HSA. The 99mTc HSA is cleared rapidly from the blood pool compared to both 99mTc-liposome formulations. By 120 min, there was 8 times (34.4% for 99mTc-HSA versus 4.5% for PEG 99mTc-liposomes) more renal excretion of the 99mTc label. This lack of in vivo stability produces heart-to-lung and heart-to-liver ratios for 99mTc-HSA that are significantly less than the ratios for both 99mTc-liposome formulations. Verbeke et al. \(^{(2,3)}\) developed a dimercapto-propionyl-modified HSA and showed that it had superior in vivo stability when compared to a conventional kit of 99mTc-HSA in human volunteers. This in vivo stability was similar to that shown for 99mTc-liposomes in that there was good retention of the modified HSA in the bloodstream and low bladder activity. Heart-to-lung and heart to-liver ratios for the modified HSA were also shown to be comparable to ratios determined for in vitro 99mTc-red cells. Despite these positive features. Long-term storage of modified HSA may not be as reliable as 99mTc-liposomes due to oxidation of the sulfhydryl groups \(^{(2-4)}\).

**CONCLUSIONS:**

99mTc-liposomes would both be safe because the agents are derived independent of potentially infectious blood products and are produced from compounds known to be biocompatible. These radiopharmaceutical are also convenient in that they can be packaged in kit form and easily prepared as needed and can be used to study vascular imaging. Lack of bowel activity and low activity in kidneys and bladder for both 99mTc liposome formulations in the images was also observed.
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