Fluorescent and Iodized Emulsion for Preoperative Localization of Pulmonary Nodules

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Objective: This study was conducted to develop a fluorescent iodized emulsion comprising indocyanine green (ICG) solution and lipiodol (ethiodized oil) and evaluate its feasibility for use in a clinical setting.

Background: ICG use for the preoperative localization of pulmonary nodules is limited in terms of penetration depth and diffusion.

Methods: First, fluorescent microscopy was used to investigate the distribution of ICG-lipiodol emulsions prepared using different methods. The emulsions were injected in 15 lung lobes of 3 rabbits under computed tomography fluoroscopy guidance; evaluation with imaging and radiography was conducted after thoracotomy. Subsequently, the emulsions were used to preoperatively localize 29 pulmonary nodules in 24 human subjects, and wedge resections were performed using fluorescent imaging and C-arm fluoroscopy.

Results: The optimal emulsion of 10% ICG and 90% lipiodol mixed through 90 passages had even distribution and the highest signal intensity under fluorescent microscopy; it also had the best consistency in the rabbit lungs, which persisted for 24 hours at the injection site. In human subjects, the mean diameter of pulmonary nodules was 0.9 ± 0.4 cm, and depth from the pleura was 1.2 ± 0.8 cm. All emulsion types injected were well localized around the target nodules without any side effects or procedure-related complications. Wedge resection with minimally invasive approach was successful in all pulmonary nodules with a free resection margin.

Conclusions: A fluorescent iodized emulsion prepared by mixing ICG with lipiodol enabled accurate localization and resection of pulmonary nodules.

Keywords: indocyanine green, lipiodol, localization, pulmonary nodule, wedge resection

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Detection of small pulmonary nodules has increased with the widespread use of chest computed tomography (CT) screening. Video-assisted thoracoscopic surgery (VATS, http://links.lww.com/SLA/B635) is the minimally invasive procedure of choice in cases involving unsuccessful diagnosis through percutaneous transthoracic needle biopsy or requirement for the resection of pulmonary nodules. However, the usefulness of VATS to identify nodules is limited by the clinicians' inability to inspect the lesions visually or palpate them directly during the procedure.[1]

Many methods have been developed for the preoperative localization of pulmonary nodules in patients undergoing VATS, including the use of hookwires, microcoils, radiotracers, radio contrasts, and dyes.[2–5] Each method is currently in clinical use but has unique drawbacks: hookwires are frequently dislodged; microcoils and radiocontrast agents cause high probability of radiation exposure; radiotracers cannot easily identify the precise resection margin; and dyes are rapidly diffused and do not remain at the lesion.

To overcome the disadvantage of each method, a combination of hookwire and lipiodol (ethiodized oil) or ^{99m}Tc-phytate has been developed by our group for use as a dual-marker system.[6,7] Recent reports have indicated newly developed combinations of dye and contrast agent to replace hookwires such as methylene blue and lipiodol, India ink and lipiodol, or methylene blue and agar .[8–10]

Near-infrared (NIR) fluorescent imaging with indocyanine green (ICG) has been introduced as a useful tool for real-time visualization of the lymphatic flow and tissue perfusion during surgery .[11,12] Recently, some reports have indicated that ICG fluorescent imaging was successful in the preoperative localization of pulmonary nodules .[13–17] However, we have shown that an aqueous ICG solution developed for clinical use may diffuse into the lung tissue without remaining around the lesion; in addition, NIR fluorescent imaging had difficulty to determine the resection margins of deep-seated nodules due to limited depth penetration .[11,12]

This study aimed to optimally develop a fluorescent iodized emulsion comprising a mixture of ICG solution and lipiodol using in vitro and in vivo approach, and evaluate its effectiveness and feasibility for preoperative localization of pulmonary nodules in human subjects through clinical trial.

METHODS

Preparation of Fluorescent Iodized Emulsion

First, to determine the optimal number of mixing times, emulsions were prepared by mixing 0.5-mL ICG solution

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JR and JWL equally contributed to this work.

Authors' contribution: HKK and HSY designed the study. JR and JWL conducted the in vitro experiments. JR, YHQ, and BHC conducted the in vivo studies. JWL and HSY evaluated localization in patients. BKS conducted the histopathologic examination of patient tissues. HKK, KNH, and YHC performed surgeries. BMK provided the intraoperative color and fluorescence-merged imaging system. JR and JWL analyzed the data. JR, JWL, and HKK drafted the manuscript. All authors contributed to the manuscript drafts and preparation.

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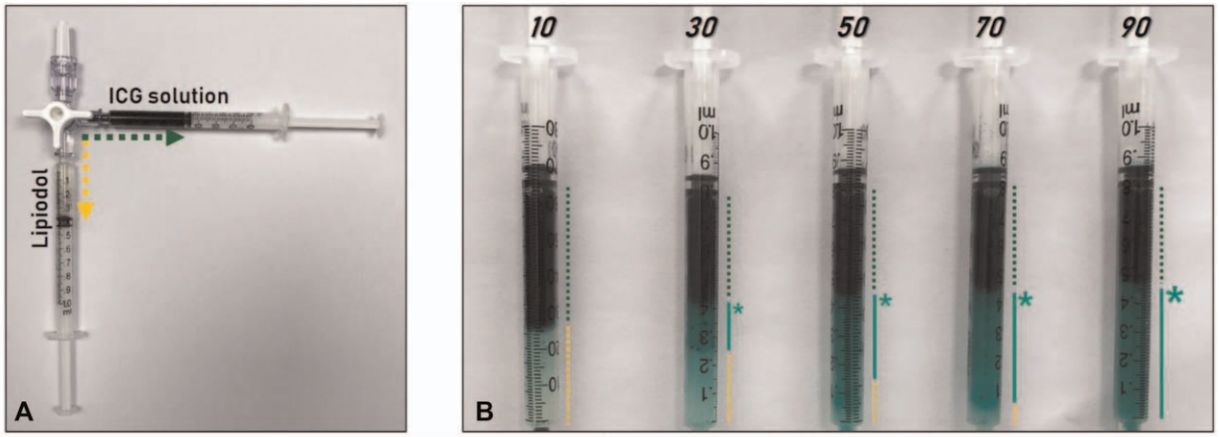


	Each material of emulsion (mL)			Percentage of lipiodol in the emulsion (%)
	ICG solution (1.5mg)	Lipiodol	Total (mL)	
1	1.5	1.5	3	50
2	1.2	1.8	3	60
3	0.9	2.1	3	70
4	0.6	2.4	3	80
5 C	0.3	2.7	3	90

FIGURE 1. Preparation of fluorescent iodized emulsions to determine the optimal number of mixing times and mixing ratio. (A) Fluorescent iodized emulsions were prepared using two 1-mL syringes and a 3-way stopcock for each type. Indocyanine green (ICG) solution and lipiodol at 0.5-mL volume each were mixed through the stopcock for a total volume of 1 mL. (B) Fluorescent iodized emulsions, each of 1-mL total volume, were prepared using an equal volume of 0.5 mL each iodized oil and ICG solution at concentration of 5 mg/mL at different mixing times of 10, 30, 50, 70, or 90 passages: the dark green portion with dotted dark-green line indicates the unmixed ICG solution; the light green area at the middle section with light green line and asterisk indicates the mixed emulsion; the near-yellow area with dotted yellow line located at the bottom of the emulsion indicates the unmixed lipiodol. (C) Fluorescent iodized emulsions each of 3-mL total volume were prepared using different volumes of ICG solution (1.5 mg) and lipiodol to yield final lipiodol proportions of 50%, 60%, 70%, 80%, or 90%. (D) Five emulsions at postmixing: the dark green area with dotted dark-green line indicates the unmixed ICG solution; the light green area with light green line and asterisk indicates the fully mixed emulsion.

(5 mg/mL; Daiichi-Sankyo Co., Tokyo, Japan) and 0.5-mL lipiodol (ethiodized oil, 480 mg iodine/mL; Andre Guerbet, Bloomington, IN) using pumping method with a 3-way stopcock (Fig. 1A) to yield a total volume of 1 mL. Five fluorescent iodized emulsions were prepared using a range of mixing times from 10 to 90 passages at interval of 10 passages. Visual evaluation of the consistency was conducted shortly after mixing.

Next, to determine the optimal mixing ratio of the fluorescent iodized emulsion, 5 emulsions were prepared using different volumes of ICG (1.5 mg) and lipiodol mixed for 90 passages. Lipiodol comprised 50%, 60%, 70%, 80%, or 90% of the 3.0-mL final volume of each of the emulsion types (Fig. 1C).

Fluorescence microscopy was used to verify the distributions of ICG and lipiodol in 4 different fluorescent iodized emulsions. First, 2 types of emulsion were prepared by mixing the same volumes of ICG solution at higher concentration of 2.5 mg/mL and lipiodol for different numbers of mixing times. One emulsion was mixed for 10 passages (10 in Fig. 1B), and the other was mixed for 90 passages (90 in Fig. 1B); the other 2 types of emulsion were prepared by mixing ICG solution at lower concentration of 0.5 mg/mL and lipiodol at different ratios for 90 passages. One emulsion comprised 50% ICG solution and 50% lipiodol (1 in Fig. 1C), and the other comprised 10% ICG solution and 90% lipiodol (5 in Fig. 1C). One droplet of each emulsion was placed on a separate glass slide and each slide was examined using a fluorescent microscope (EVOS FL Auto Imaging System, Life Technologies, MA); comparison of all slides was done.

Animal Study

This study was approved by the Institutional Animal Care and Use Committee of Korea University College of Medicine (IACUC approval number: KOREA-2016-0220). Five New Zealand white rabbits (DooYeol Biotech Co. Ltd., Seoul, Korea; body weight, 2.5–3.0 kg) were used in the study. All rabbits were anesthetized with xylazine (5 mg/kg intramuscularly; Rompun, Bayer Korea Inc., Seoul, Korea) and alfaxalone (5 mg/kg intravenously; Alfaxan, Jurox Pty Ltd., NSW, Australia).

To evaluate the consistency of 3 emulsions at different mixing ratio of 70%, 80%, or 90% lipiodol, a 0.2-mL aliquot of each emulsion was injected into 3 pulmonary lobes of one rabbit using a 26-G needle under fluoroscopic computed tomography (CT)

guidance (Brilliance 64, Philips, Amsterdam, the Netherlands) (n = 2; Fig. 3A). CT-guided injections were performed as previously described.[7]

To optimize the injection time of the optimal emulsion comprising 90% lipiodol mixed for 90 passages, 0.2-mL aliquots of the emulsion were injected into 3 pulmonary lobes of one rabbit under fluoroscopic CT guidance at different time points of 6, 12, and 24 hours prethoracotomy (n = 3).

The fluorescence intensity in freshly excised lung surfaces was assessed using our custom-manufactured intraoperative color and fluorescence-merged imaging system (ICFIS), as previously described (Fig. 3B) .[18] All lobes were then subjected to x-ray imaging using a mammography machine (Selenia Dimensions, Hologic, Marlborough, MA). The ICFIS and mammographic images of the same lobe were compared by the experimenter to visually confirm the consistency of the emulsion.

Study in Human Subjects

This study was approved by the Institutional Review Board of Korea University Guro Hospital, and informed consent was obtained from each patient (KUGH 16343-001). Between February 2016 and July 2018, 24 patients scheduled to undergo wedge resection for confirmed primary lung cancer, biopsy, or additional resection of unidentified nodules during major pulmonary resection due to lung cancer were enrolled in the study.

One of 2 board-certified radiologists performed fluoroscopic CT-guided nodule localization in each of the patients during the morning of the day of surgery. CT fluoroscopy units (Brilliance 64; Philips, Amsterdam, the Netherlands) were used under low-dose protocol (120 kVp and 30 mAs) to reduce the patients' radiation exposure. A 21-G Chiba needle (Biopsy Needles, M.I. Tech, Seoul, Korea) was introduced into the nodule, and 0.3 mL of the emulsion was injected at the medial border of the nodule.

Single-port VATS is standard procedure at our hospital[19]; however, multi-port VATS was performed according to the patient factors or surgeon's preference. Pinpoint thoracoscope (Novadaq Technologies Inc., Mississauga, ON, Canada) or Firefly fluorescence imaging, da Vinci Si system (Intuitive Surgical, Inc., Sunnyvale, CA) with intraoperative C-arm fluoroscopy (Koninklijke Philips, N.V., Amsterdam, the Netherlands) were used. After acquiring the fluorescent image of ICG through fluorescence thoracoscopy, the precise location and resection margin of the radiopaque lesion were confirmed via C-arm fluoroscopy, and the nodule was resected with an endostapler. An adequate resection margin is considered to measure >2 cm or be equal to the size of the mass itself. In our study, the in vivo and ex vivo (back table) resection margin was verified using both NIR fluorescence imaging system and fluoroscopy intraoperatively and confirmed through pathology in the frozen and permanent sections in all cases.

RESULTS

Optimization of Fluorescent Iodized Emulsion In Vitro

First, with regard to number of the mixing time, there was a trend of increase in the mixed portion with increasing number of the mixing time (Fig. 1B). Although the fluorescent iodized emulsion mixed through 90 passages contained the highest mixed portion, all the emulsions contained some portion of unmixed ICG solution, and there was no difference in the unmixed ICG solution portion among emulsions mixed from 90 to 200 passages (data not shown).

Second, with regard to mixing ratio, there was a trend of an increase in the mixed portion with increasing volume of lipiodol through 90 passages (Fig. 1D). The emulsion comprising 90% lipiodol and 10% ICG yielded the maximum mixed portion and contained no unmixed ICG solution, which was a persistent finding at 12 and even 24 hours postmixing, without any changes (data not shown).

In the comparison of different numbers of the mixing time (Fig. 2A, B), fluorescent microscopy revealed that the emulsion

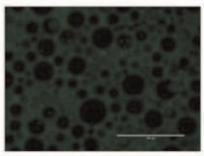
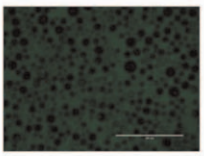
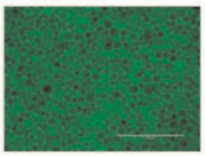
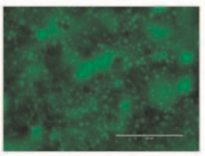
	A	B	C	D
Total volume	1 mL	1 mL	3 mL	3 mL
The volume ratio of the emulsion (ICG : Lipiodol)	1 : 1 (0.5 mL : 0.5 mL)	1 : 1 (0.5 mL : 0.5 mL)	1 : 1 (1.5 mL : 1.5 mL)	1 : 9 (0.3 mL : 2.7 mL)
ICG concentration	2.5 mg/mL	2.5 mg/mL	0.5 mg/mL	0.5 mg/mL
The number of mixing times	10 passages	90 passages	90 passages	90 passages
Fluorescence microscope image				

FIGURE 2. Fluorescent microscopy evaluation of the 4 types of emulsions prepared using different mixing times and mixing ratios. (A) A total emulsion volume of 1 mL was prepared by mixing 0.5 mL each of indocyanine green (ICG) at higher concentration of 2.5 mg/mL and lipiodol through 10 passages. (B) A total emulsion volume of 1 mL was prepared by mixing 0.5 mL each of ICG at higher concentration of 2.5 mg/mL and lipiodol through 90 passages. (C) A total emulsion volume of 3 mL was prepared by mixing 1.5 mL each of ICG at lower concentration of 0.5 mg/mL and lipiodol through 90 passages. (D) A total emulsion volume of 3 mL was prepared using different volumes of 0.3 mL ICG at lower concentration of 0.5 mg/mL and 2.7 mL lipiodol through 90 passages.

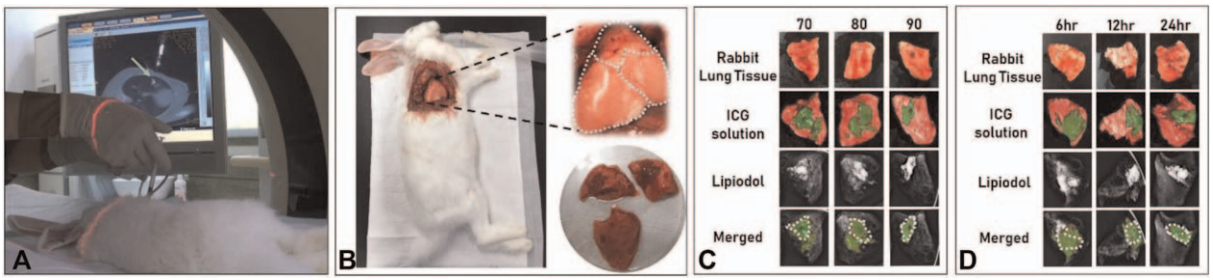


FIGURE 3. Imaging of the rabbit lungs under treatment with fluorescent iodized emulsions prepared at different mixing ratios and injection times. (A) Fluorescent iodized emulsion was injected into the rabbit lung under computed tomography guidance. (B) The lobes of the rabbit lung were resected; fluorescent and x-ray images of the sections were acquired. (C) Postthoracotomy fluorescence and x-ray images of ex vivo lung tissue at 12 hours after injection of the emulsions with different mixing ratios. In the merged images, the green area indicates ICG and the white dotted line indicates lipiodol. (D) Postthoracotomy fluorescence and x-ray images of ex vivo lung tissue at different time points (6, 12, or 24 h) after injection of the emulsion. In the merged images, the green area indicates ICG and the white dotted line indicates lipiodol.

mixed through 90 passages was more evenly distributed than that through 10 passages. In the comparison of different mixing ratios (Fig. 2C, D), the emulsion containing 90% lipiodol exhibited increased water-in-oil formation, compared with that containing 50% lipiodol, and the emulsion with ICG solution at a lower concentration of 0.5 mg/mL (Fig. 2C, D) had higher signal intensity than that at higher concentration of 2.5 mg/mL (Fig. 2B, C), which was attributed to a quenching effect. In summary, the emulsion containing 10% ICG solution at 0.5 mg/mL and 90% lipiodol that was mixed through 90 passages yielded the most even distribution, highest proportion of water-in-oil formation, and highest signal intensity among all tested emulsions.

Optimization of Fluorescent Iodized Emulsion and Localization Time In Vivo

Aliquots of the emulsions were injected in 15 pulmonary lobes of 5 rabbits under fluoroscopic CT guidance. In all resected lobes, all emulsions were clearly detected under ICFIS (green area, 15/15: 100%) and mammography (white area or dotted line, 15/15: 100%) after thoracotomy (Fig. 3C, D).

With regard to mixing ratio, the emulsion containing 90% lipiodol exhibited the highest consistency in the rabbit lung (Fig. 3C), followed by emulsions containing 80% or 70% lipiodol, which yielded similar consistencies.

With regard to localization time, the optimal emulsion comprising 90% lipiodol mixed through 90 passages was detectable for up to 24 hours after injection; in addition, the consistency of the emulsion in the rabbit lung remained similar (Fig. 3D).

Validation of the Use of Fluorescent Iodized Emulsion in Patients

Fluoroscopic CT-guided nodule localization was performed for 29 nodules in 24 patients (13 men, 11 women) (Table 1). The patients' mean age was 61.9 ± 10.2 years (range, 36–80 yrs). The mean nodule diameter was 0.9 ± 0.4 cm (range, 0.3–1.4 cm); the mean depth of the nodules from the pleural surface was 1.2 ± 0.8 cm (range, 0.3–5.9 cm).

The mean procedure time for localization was 15.8 ± 6.4 min (range, 6–30 min). None of the patients developed side effects related to the use of emulsion, and only one patient experienced procedure-related complication. In one case, a small pneumothorax (1/29, 3.4%) occurred during localization, but no additional management was needed before surgery. Localization was successful (28/29, 96.6%) in all lesions (Fig. 4A, B) except one involving a superficially located nodule and intraprocedural leakage of the emulsion into the pleural space (Fig. S2, http://links.lww.com/SLA/B626, Case 1). The average time between localization and surgery was 215 ± 110.6 minutes (range, 47–396 min), except in one patient with mild sore throat and suspected afebrile upper respiratory infection before localization, who wished to delay the operation soon after localization (Fig. S2, http://links.lww.com/SLA/B626, Case 3). The patient underwent surgery on day 6 postlocalization without additional localization.

Although one patient experienced leakage of the emulsion during localization that was subsequently detected intraoperatively (Fig. S2, http://links.lww.com/SLA/B626, Case 1), all emulsions were clearly localized around the target nodules and detectable under fluorescence imaging system (28/29, 96.6%) (Fig. 4C and E) except for one lesion that was located at large depth of 5.9 cm below the pleural surface (Fig. S2, http://links.lww.com/SLA/B626, Case 2).

TABLE 1. Characteristics of the Patients and Pulmonary Nodules Included in the Study

Patients (n = 24)	
Male:female, n	13:11
Age, yrs	61.9 ± 10.2 (range, 36–80)
Single nodule, n	19
Multiple nodules, n	5
Nodules (n = 29)	
Diameter, cm	0.9 ± 0.4 (0.3–1.4)
Depth from the pleura, cm	1.7 ± 1.3 (0.3–5.9)
CT appearance, n	
Solid	14 (48.3%)
Part solid GGO	3 (10.3%)
GGO	12 (41.4%)
Location, n	
RUL	8 (27.6%)
RML	2 (6.9%)
RLL	5 (17.2%)
LUL	6 (20.7%)
LLL	8 (27.6%)

CT indicates computed tomography; GGO, ground-glass opacity; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe.

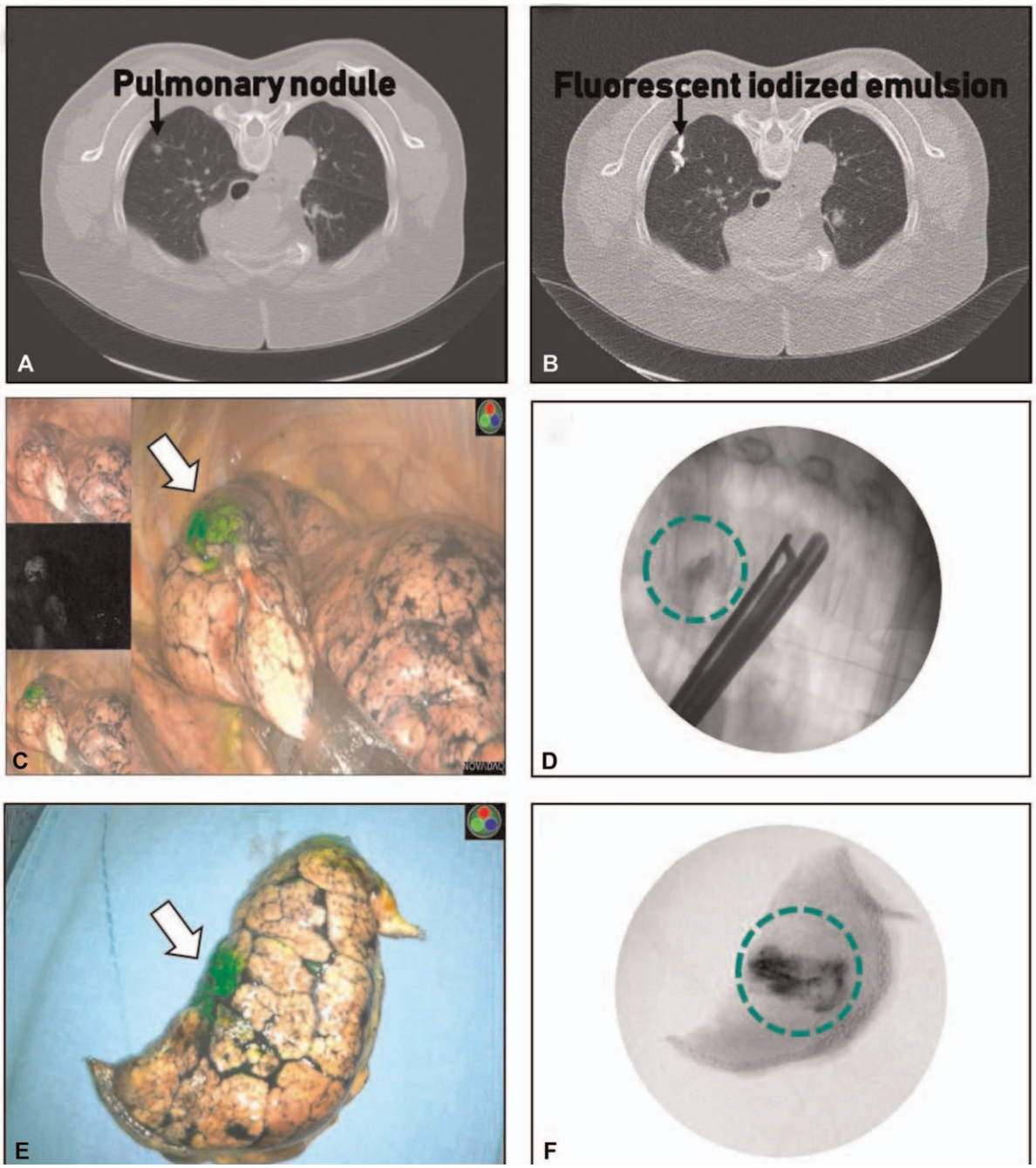


FIGURE 4. Successful resection of the pulmonary nodule after preoperative localization with fluorescent iodized emulsion. (A) Computed tomography (CT) image of the pulmonary nodule. (B) CT-guided injection of the emulsion at the region of the pulmonary nodule. The shortest depth from the pleura of the mass was 1.6 cm. However, the emulsion was injected vertically because presence of the bone prevented direct access. The vertical pleura was at 3.5-cm depth from the injection site and the emulsion was intentionally spilled on the lung surface. (C) Intraoperative detection of the fluorescence signal using a fluorescence imaging system. (D) Intraoperative detection of lipiodol using C-arm fluoroscopy. The nodule was grasped with the endostapler and C-arm fluoroscopy was performed to confirm adequate resection margin. (E) Detection of the ICG signal in an ex vivo specimen. (F) Detection of lipiodol in an ex vivo specimen; the specimen also exhibited an adequate resection margin.

TABLE 2. Operation, Disease, Pathology, and Further Procedures

Operation (n)	Disease (n)	Pathology	n	Additional Procedure	n
Biopsy (12)	Benign (10)	Inflammation	3	Lobectomy	3
		Anthracofibrotic nodule	2		
		Focal necrosis	2	Lobectomy	1
		Intrapulmonary lymph node	2	Lobectomy	2
		Langerhans cell histiocytosis	1	Lobectomy	2
	Malignancy (2)	Adenocarcinoma	1		
		Small cell carcinoma	1		
Curative intent (17)	Primary lung cancer (3)	Adenocarcinoma	3	Lobectomy	1
	Metastatic lung cancer (14)	Metastatic cancer	11	Segmentectomy	1
		Squamous cell carcinoma	2		
		Adenocarcinoma	1		

However, all lesions, including the deep-seated lesion, were detectable with mobile C-arm fluoroscopy (29/29, 100%) (Fig. 4D and F); moreover, emulsion staining remained stable even in the nodule of the patient who underwent operation at day 6 postlocalization (Fig. S2, http://links.lww.com/SLA/B626, Case 3).

A total of 20 nodules were resected with single-port VATS and the remaining with multiport VATS; 12 nodules were resected for diagnostic purpose (10 benign lesions, 2 cases of primary malignancy) and 17 for treatment purpose (14 metastatic lung cancers, 3 primary lung cancers) (Table 2). All nodules had a free resection margin, and the mean length of the resection margin was 1.9 ± 0.7 cm (range, 1.1–3.4 cm), which indicated an adequate resection margin defined as that of >2 cm or the size of the mass itself, and no patient underwent further procedure due to a close resection margin. In all 5 primary malignant nodules, further procedures such as segmentectomy or lobectomy were not performed due to previous ipsilateral or contralateral segmentectomy or lobectomy due to primary lung cancer in 3 patients, old age with poor pulmonary function in one patient, and inability to discriminate between primary tumor and metastasis with frozen biopsy in one patient.

A total of 14 patients underwent wedge resection of the nodules alone, and the mean operation time for resection procedure was 42.8 ± 5.3 minutes (range, 15–58 min); 9 patients underwent lobectomy and 1 patient underwent segmentectomy as planned additional procedure. In all patients, the mean time of surgery was 142.8 ± 55.3 minutes (range, 35–225 min).

None of the patients had major complications during the hospital stay. The mean duration of indwelling chest tube in the patients who underwent only wedge resection was 2.4 ± 0.7 days (range, 2–4 d), and mean duration of hospital stay in those patients was 3.4 ± 0.7 days (range, 3–5 d). In all patients including patients who underwent further procedure, the mean duration of indwelling chest tube was 3.0 ± 1.1 days (range, 2–5 d), and mean duration of hospital stay was 4.0 ± 1.1 days (range, 3–6 d).

Reports have indicated that lipiodol and ICC had no effect on the pathologist's ability to analyze the specimen ,[20,21] which agrees with the findings in all cases of our study. Histopathologic examination confirmed the colocalization of ICG with lipiodol (lipid component), which was engulfed by the macrophages in the tumor environment (Fig. S1, http://links.lww.com/SLA/B626).

DISCUSSION

We developed a fluorescent iodized emulsion comprising an ICG solution and lipiodol to overcome diffusion and depth limitations of wedge resection in pulmonary nodules undergoing preoperative localization, using in vitro approach and in vivo rabbit model. We additionally validated the feasibility and usefulness of the emulsion through clinical trial in human subjects.

ICG Use for Preoperative Localization of Pulmonary Nodules

ICG is an NIR fluorescent dye with fluorescence absorption of 800 nm. Although ICG has amphiphilic properties (both hydrophilic and lipophilic), it must be dissolved in water to yield an aqueous solution for injection in patients.[22,23] In addition, ICG is the only NIR fluorescent dye currently approved for clinical use in patients to detect the sentinel lymph node (SLN), tumor margin, cardiovascular imaging, or tissue perfusion[24]; it can also be used to localize pulmonary nodules preoperatively either alone or premixed with human serum albumin (Table S1, http://links.lww.com/SLA/B626, "Movie S1. Fluorescent iodized emulsion for preoperative localization of pulmonary nodule.").[13–15,17]

NIR fluorescence imaging using ICG has several advantages compared with other conventional dyes such as methylene blue (MB). First, ICG was easily detected in the presence of color change of the lung surface to black due to anthracosis, whereas MB was difficult to detect in that situation.[13] Second, NIR light is invisible under universal thoracoscopy without fluorescence imaging system; thus, ICG does not hamper the surgical field, whereas MB is detectable with a thoracoscope, which interferes with the surgeon's field of view.[25] Third, ICG has higher tissue penetration than that of MB of 0.5 mm, and hence, ICG is more useful to localize deep-seated pulmonary nodules. Lastly, the signal-to-background ratio (SBR) of NIR is maximized because NIR has low absorption, scatter, and autofluorescence.[22,26]

Despite its advantages, ICG has limitations for use in localization of nodules. First, the ICG signal cannot be clearly detected in deep-seated lesions because of the limited penetration of NIR of up to 10 mm even though it is considered superior to MB. Second, ICG solution can easily diffuse into the adjacent normal lung tissue. Some reports have indicated that mixing an ICG solution with a nonionic water-soluble CT contrast agent (iopamidol) is effective to track the ICG injected around pulmonary nodules at preoperative localization under CT or fluoroscopy guidance with or without electromagnetic navigational bronchoscope (ENB) (Table S1, http://links.lww.com/SLA/B626)[14,15]; however, in those studies, the success rate of localization was high (35/37, 94.6% reference and 54/54, 100% reference), and the depth limitation could not be overcome because ICG mixed with the CT contrast agent shows diffusion and the aqueous component does not have capability to restrict distribution to a distinct spot. Moreover, ICG at high concentration may have a quenching effect that could decrease the fluorescence intensity; therefore, ICG at low concentration and volume should be used for the localization of nodules. To the best of our knowledge, a method to prevent diffusion of the ICG solution has not been previously reported.

Mixture of ICG and Lipiodol

In our study, ICG solution was mixed with lipiodol, an oil-based radiopaque contrast agent, which reduces the diffusion of ICG solution into the adjacent tissue, and thereby facilitating accurate nodule localization; lipiodol can also overcome the limited penetration of ICG under C-arm fluoroscopy and enable identification of deep pulmonary nodules that cannot be detected using an NIR probe.

In our in vitro studies, both materials at equal volume of 0.5 mL each were mixed completely at different mixing times of 10 to 90 passages to yield different levels of mixing among the 5 emulsion types: the emulsion mixed through 90 passages contained the highest proportion of mixed emulsion and lowest proportion of unmixed ICG solution that was present in all emulsions irrespective of the number of mixing times (up to 200 passages). Therefore, 90 passages were considered as the optimal number of mixing times. Next, the total emulsion volume was increased to achieve increased mixed portion of the emulsion; in addition, the volume of lipiodol was increased and volume and concentration of the ICG solution was reduced to achieve lowered proportion of unmixed ICG solution, which allows more water-in-oil formation, and prevents diffusion and quenching. Thus, the final product obtained was an even, almost fully mixed emulsion that was stable over time and with higher level of signal intensity of ICG. These characteristics were confirmed visually and under fluorescence microscopy.

Emulsions containing 70%, 80%, or 90% lipiodol mixed through 90 passages were mixed to near-maximum limit and with no significant visual differences. In vivo test performed with the 3 emulsions to evaluate the in vivo differences in consistency and intensity revealed that the 3 emulsions differed in terms of consistency but not fluorescent signal intensity. The results of in vitro and in vivo experiments indicated that the emulsion comprising 10% of low-concentration ICG solution (0.5 mg/mL) and 90% lipiodol in a total volume of 3 mL and mixed through 90 passages was most suitable for clinical use as compared with the other emulsion preparations.

In addition, the optimal emulsion remained without diffusion for up to 24 hours after localization in the rabbit lung. After the early morning localization procedure, most surgical operations were conducted on the same day. Although the localization was conducted several hours before the operation at different injection time points, the optimal emulsion remained stable and clearly detectable at the surgical field of the lung for up to 24 hours after localization.

Clinical Trials Using Fluorescent Iodized Emulsion

In the clinical trial, the optimal emulsion containing 90% lipiodol mixed through 90 passages yielded similarly favorable outcomes as those in the in vitro studies. All emulsions showed satisfactory localization of the nodules without related side effects or procedure-related complications, and all pulmonary nodules were resected successfully with a free resection margin.

The one nodule with leakage of the emulsion in the pleural space was of very small size of 0.6 cm and located at $\geq$0.3-cm depth from the pleura, as compared with the other lesions; nevertheless, successful resection was achieved due to high-intensity signal of ICG at the nodule and injection site. However, clinicians should use caution and consider the use of ENB to prevent emulsion leakage during the localization of nodules at superficial location.

The ICG signal was not detected in 1 patient with a nodule at 5.9-cm depth from the pleura because of limited penetration of the dye; however, the nodule was successfully resected under C-arm fluoroscopy guidance. Nodules located at 1- to 2.5-cm depth from the pleura could be detected using fluorescent imaging due to leakage of the emulsion through the needle track, despite the depth limitation, whereas 5.9-cm depth exceeded the range of detection for emulsion leakage through the needle track. Our experience suggests that intended spillage of emulsion on the lung surface could be helpful in case of the presence of nodule at >2.5-cm depth from the pleural surface.

Preoperative marking should be considered when the distance to the nearest pleural surface is >0.5 or 1 cm.[27,28] In this study, 5 of 29 nodules which were located at <0.6-cm depth from the pleura were identified without need of any fluoroscopy or NIR imaging, suggesting that preoperative localization of the nodules at <0.6-cm depth from the pleura may not be required.

C-arm fluoroscopy was not always needed in all cases. In this study, it was difficult to identify the resection margin based on the fluorescent signal alone in the 3 cases of pulmonary nodules at >2.5-cm depth from the pleura, despite a fluorescent signal detected at the pleural surface. In case of the pulmonary nodule at >2.5-cm depth from the pleura, C-arm fluoroscopy is considered as useful to identify the accurate resection margin.

In one case in our series, surgery was deferred for 6 days due to the patient's poor condition with fever of unknown cause; however, the emulsion remained stable with little diffusion and yielded sufficient fluorescence and radio signal to allow safe resection of the nodule.

The emulsion comprising 10% of low-concentration ICG solution (0.5 mg/mL) and 90% lipiodol mixed through 90 passages exhibited no diffusion during localization, and high-intensity fluorescent signal around the pulmonary nodules, and facilitated the resection of nodules with an adequate margin. Mixture of lesser proportion of ICG (10%) in relation to the larger proportion of lipiodol (90%) could allow the formation of a water-in-oil emulsion that would prevent dye diffusion and enable localization around the nodule, and the low concentration of ICG in the emulsion could increase the fluorescent signal intensity by preventing the quenching effect. Finally, lipiodol component of the emulsion could accurately indicate the resection area under C-arm fluoroscopy guidance, especially in case of deep-seated nodules.

With regard to cost-effectiveness, the cost per ampoule of ICG is about USD 10, and that per vial of Lipiodol Ultra at the time of study was about USD 170; the procedure cost by health insurance in Korea is about USD 300 including CT guidance. The method using a mixture of ICG and lipiodol has higher cost at USD 180 than that of hook-wire insertion at USD 25 and similar cost as that of the microcoil at USD 480 versus CAD 567 including procedure cost.[30] However, the required amount of ICG and lipiodol for a single localization procedure is less than the content of each ampoule and vial, respectively; therefore, after clinical validation through such a multitrial, it may be possible to request smaller quantity unit production by the manufacturers, or development of a premix product that can be easily mixed using a prefilled syringe.

Minimally invasive surgical (MIS) techniques such as VATS or robotic surgery are increasingly used to resect lung cancer.[29] Recently, several intraoperative fluorescent imaging systems such as PINPOINT (PINPOINT Endoscopic Fluorescence Imaging System, Stryker, Kalamazoo, MI), D-LIGHT (OPAL1 technology for NIR/ICG with the IMAGE1 S camera platform, KARL STORZ SE & Co. KG, Tuttlingen, Germany), Firefly fluorescence imaging system (Da Vinci Vision, INTUITIVE, Sunnyvale, CA), and VISERA ELITE II Infrared Imaging System (Surgical Endoscope System, Olympus Europa SE & Co. KG, Hamburg, Germany) have become available on the market,[22] which can extend the applicability of NIR imaging technology to various kinds of operations including the localization of pulmonary nodules. Currently, ICG is the only Food and Drug Administration (FDA)-approved NIR fluorescent contrast agent, but has several limitations mentioned previously. Obtaining national

regulatory agency such as FDA approval for newly developed fluorescent agents could be challenging and time-consuming; the proposed method by mixing ICG and lipiodol, which are already widely used in clinical settings, could be an alternative solution to enable accurate localization of the pulmonary nodule.

Limitations

Our proposed mixing method involves a simple and quick process for the preparation of emulsion that can be easily administered to patients by the clinicians. The emulsion has several advantages over other single-component dye localization materials; however, the mixing consistency may differ depending on the producer and adherence to manual mixing technique. Therefore, further studies are needed to develop a carrier that will completely incorporate the ICG and lipiodol.

In conclusion, we developed a fluorescent iodized emulsion comprising 10% of low-concentration ICG solution (0.5 mg/mL) and 90% lipiodol mixed through a 3-way stopcock with 90 passages. This emulsion was effective to overcome the diffusion and depth limitations of an ICG solution when administered alone and has the potential to facilitate accurate localization and resection of pulmonary nodules during minimally invasive procedures such as VATS or robotic surgery.

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