이학석사 학위논문

Imaging H_2S in Hypoxic Condition Using [^{99m}Tc]Tc-gluconate

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Imaging H_2S in Hypoxic Condition Using [^{99m}Tc]Tc-gluconate

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Abstract

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Objectives: Hydrogen sulfide is the third gasotransmitter that is known to be produced endogenously in hypoxia, various cancers and inflammations. H_2S can be produced through both nonenzymatic and enzymatic pathways. It is reported that cystathione γ-lyase (CSE) localized in the cytosol in normoxia is translocated into mitochondria in hypoxia, and then metabolized to L-cysteine to produce H_2S. Thus, production of H_2S increases in hypoxic condition. Furthermore, it was reported that the endogenous H_2S could be imaged by [^{99m}Tc]Tc-gluconate. In the president study, we tried to detect H_2S generated by hypoxic cells in vitro, and tried to image H_2S generated by hypoxic tissue in vivo using [^{99m}Tc]Tc-gluconate.

Methods: Gluconate (0.3 M, 0.1 mL) was labeled with ^{99m}Tc in the presence of stannous chloride as a reducing agent. Radiochemical purity of [^{99m}Tc]Tc-gluconate was checked by radio TLC using two instant thin-layer chromatography strips (eluted with acetone and normal saline). In vitro cell uptake study of [^{99m}Tc]Tc-gluconate was performed in hypoxic and normoxic conditions using colon carcinoma cell line CT26. Hydrogen sulfide level was measured by a modified methylene blue method (absorbance was measured at 670 nm). For in vivo imaging study, acute

hindlimb ischemia-reperfusion model was established in BALB/c mice after 3 h of ischemia and 3 h of reperfusion. [^{99m}Tc]Tc-gluconate (12.5 MBq) was intravenously injected into mice through tail vein and the lower limb uptake was imaged with SPECT/CT.

Results: The labeling yield of [^{99m}Tc]Tc-gluconate was almost quantitative (99.8 ± 0.1%). *In vitro*, [^{99m}Tc]Tc-gluconate uptake to hypoxic cell was higher than normoxic cell in all the time. At 120 min of incubation, radioactivity in hypoxic and normoxic CT26 cells reached 109,772 ± 6,889 and 25,587 ± 1,884 CPM/mg, respectively, representing the H_2S level in hypoxia was 40% higher than normoxia. *In vivo*, SPECT/CT imaging of [^{99m}Tc]Tc-gluconate showed five times higher uptake in ischemic limb than normal limb. The SUVmean of ischemic limb were 0.39 ± 0.03, and 0.07 ± 0.01 in normal limb.

Conclusions: We demonstrated that [^{99m}Tc]Tc-gluconate is a novel imaging agent for endogenous hydrogen sulfide that is generated in hypoxic cells and tissues. Thus, it might be used for imaging various diseases related to hypoxia in clinical field.

Keyword：[^{99m}Tc]Tc-gluconate, SPECT/CT, Ischemia/Reperfusion injury, Hydrogen sulfide.

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LIST OF ABBREVIATIONS

H_2S, hydrogen sulfide

DTPA, diethylenetriaminepentaacetic acid

ITLC-SG, instant thin layer chromatography-silica gel

FBS, fetal bovine serum

HSA, human serum albumin

HBSS, Hank's balanced salt solution

PET, positron emission tomography

SPECT, single photon emission computed tomography

IVIS, in vivo imaging system

ROI, region of interest

RT, room temperature

INTRODUCTION

Gasotransmitters is a small signaling molecule of gas that is freely permeable to membranes [1]. As gaseous signaling molecules that are either synthesized endogenously in an organ, tissue or cell or received from outside which can be induced certain physiological or biochemical changes [2]. Gasotransmitters are a subfamily of endogenous molecules of gases including nitrogen oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) that is the most recently found [3]. H_2S plays an important role in various physiological processes such as regulation of inflammation, vasodilation, oxygen sensing, angiogenesis, hypoxia and reperfusion injury, etc [4].

H_2S can be easily oxidized to some sulfur-containing substances, that is hydrogen sulfide ions (HS^-) and sulfide ions (S^{2-}) in aqueous solution [5]. Here is the dynamic equilibrium in the sequential reactions:

$$H_2S \rightleftharpoons HS^- + H^+ \rightleftharpoons S^{2-} + 2H^+$$

Around 20% of H_2S is undissociated and the rest is dissociated as HS^- and H^+ under the physiological conditions of pH 7.4 and 37□C. HS^- could later be decomposed to H^+ and sulfide ion (S^{2-}) at alkaline pH, therefore S^{2-} is not significantly found in vivo, however, there is no exact ratio and the amount

of the individual components of all three molecules of sulfide. H_2S can be produced endogenously in mammalian tissues through both nonenzymatic and enzymatic pathways [2, 5, 6]. The nonenzymatic pathway occurs when cysteine thiols interact with H_2S to form stable persulfides at pH 8.4 [6]. For now, the contribution of nonenzymatic pathway to overall H_2S production is unclear inside the cells. For the enzymatic pathway, there are three main enzymes: CBS(cystathionine β-synthase), CSE (cystathionine γ-lyase), and recently found 3-MST (3-mercaptopyruvate sulfurtransferase) [7]. Using L-cysteine as substrate those three enzymes could generate H_2S endogenously (Figure1).

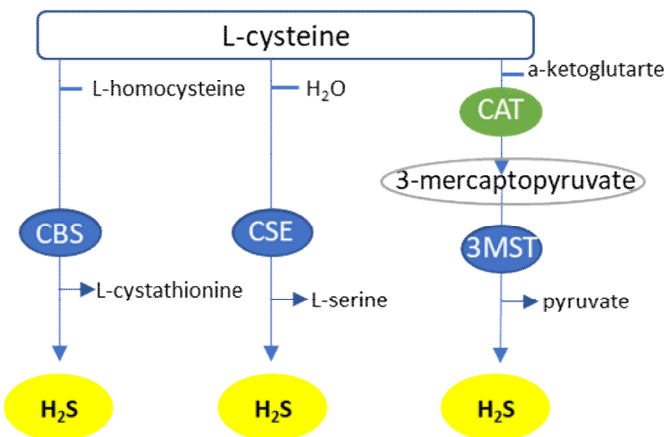

Figure 1. Scheme of enzymatic production of H_2S

Recent reports demonstrate that CSE proteins were localized in the cytosol in normal condition however it started to translocate from cytosol into mitochondria and then metabolize L-cysteine to produce H_2S when hypoxic condition is induced (Figure 2)[7,8]. Production of H_2S is regulated by the

changes of the partial pressure of oxygen and it will be increased in hypoxic condition [8]. Along with the partial pressure of oxygen, H_2S interacts with different ion channels on membranes to play a physiological role in regulating the vascular smooth muscle [9]. Increased H_2S levels may act on pre-constricted blood vessels to be expanded, so that can promote diversion from an oxygen-deprived state to an oxygen-supplied state [10].

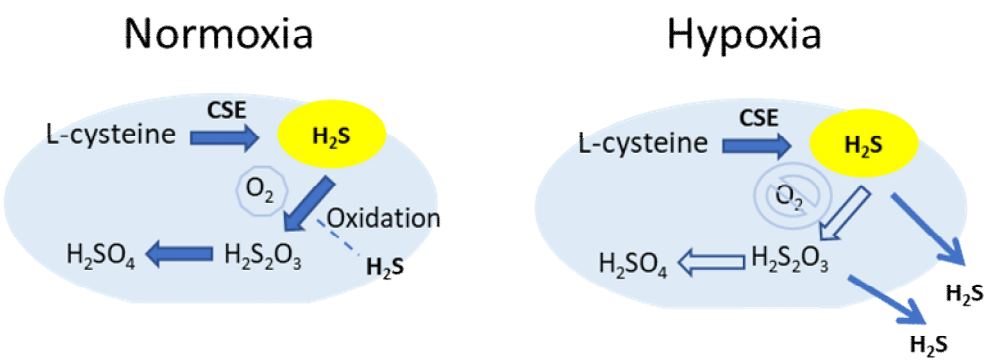

Figure 2. Biological pathway under normoxic and hypoxic condition

Common methods of detecting H_2S are the methylene blue assay, ion-selective electrodes, amperometric sensors, and gas chromatography [11] and it has various strengths and weaknesses. But, all generally fall short to detect H_2S inside of intact living organisms [12]. Also there are a few reports of *in vivo* imaging H_2S using fluorescence and luminescence. Unfortunately, imaging deep mammalian tissue is still an issue to solve for H_2S detection. Fluorescence probe has a lack of sensitivity and depth penetration due to background autofluorescence, light scattering and etc. [13]. Hence, in this study, we attempted to use radionuclide for imaging endogenously produced H_2S.

Single-photon emission computed tomography (SPECT) is an in vivo imaging modality. When a radiotracer that emits gamma ray is injected into body, it can be measured directly by SPECT cameras [14]. Although there are many radioisotopes for in vivo imaging, Technetium-99m [^{99m}Tc] is the most commonly used isotope for diagnosis [15]. ^{99m}Tc has a short half-life of 6 hours, and is inexpensive and available in many nuclear medicine departments. It can be obtained conveniently from ^{99}Mo/^{99m}Tc generator at low cost. Therefore, using ^{99m}Tc-labeled agent for diagnosis of hypoxic condition will be more suitable in clinical use than using any other radio isotopes.

The very first reported radionuclide for imaging H_2S is ^{64}Cu-labeled cyclen, which was imaged by using Positron Emission Tomography (PET) [16]. However, ^{64}Cu is only available in limited institutions that have high-energy cyclotron, and the production cost of ^{64}Cu is high. Recently, we developed ^{99m}Tc-labeled agents for the quantification of H_2S in vivo. Presence of H_2S, the ^{99m}Tc-labeled agents form insoluble fraction and precipitate, and it makes endogenously produced H_2S imageable. Various α-hydroxy acids can be labeled with ^{99m}Tc such as D-gluconic acid, D-glucaric acid, and glucoheptonic acids (Figure 3).

This work is designed to evaluate whether [^{99m}Tc]Tc-gluconate is proper agent for imaging H_2S in hypoxic condition, since it showed the highest percentage of insoluble fraction formation with H_2S. Also we attempted to

compare *in vivo* imaging endogenously produced H_2S using fluorescent probe.

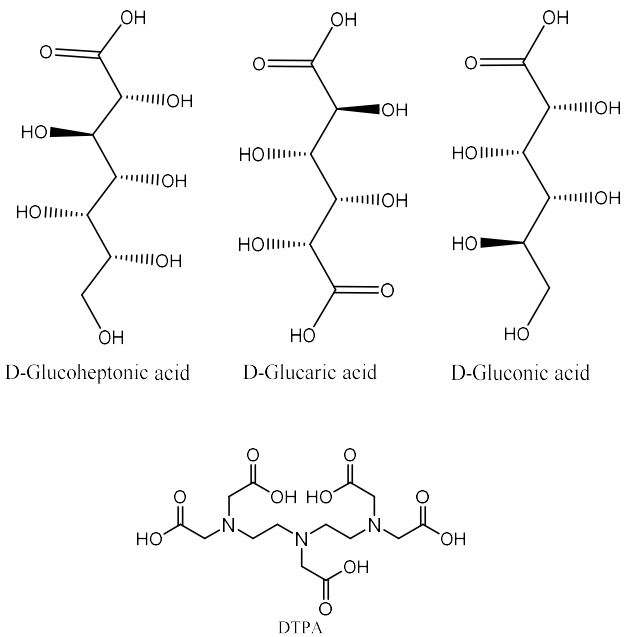

Figure 3. Chemical structure of α-hydroxy acids and DPTA

MATERIALS AND METHODS

General methods and materials

All chemicals, reagents and solvents used in the experiments were analytical grade, purchased from Sigma Aldrich and TCI. ^{99m}Tc was acquired from ^{99}Mo/^{99m}Tc-generator purchased from Sam Young Unitech Co. (Seoul, Korea). Instant thin layer chromatography (ITLC) plates were purchased from Aglient Technologies (Santa clara, CA, USA) The Bio-Scan AR-2000 scanner (Bioscan Co., WI, USA) was used for the ITLC plates scanning. Single photo emission computed tomography/computed tomography (SPECT/CT) animal images were taken by using NanoSPECT/CT^{Plus}(Mediso, Budapest, Hungrary) and analyzed with InVivoScope program. All animal studies were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (SNUH-IACUC).

^{99m}Tc-labeling of gluconate and DTPA

N_2 gas purged distilled water was used for the labeling experiment. For the [^{99m}Tc]Tc-gluconate labeling, 0.3 M D-Gluconic acid sodium salt 100 uL was prepared and then added 10 uL of sodium ascorbic acid (25 mg/mL)

and 50 μL of $SnCl_2 \cdot 2H_2O$ (1 mg/mL in 0.05 M HCl) then vortexed. 140 μL of [^{99m}Tc]$NaTcO_4$ (approximately 370 MBq) eluted in normal saline from the generator was added to the mixture for the labeling at room temperature for 20 mins, (total pH 4.5).

Radiochemical quality control testing of [^{99m}Tc]Tc-gluconate by two instant thin-layer chromatography strips were measured with radio TLC. A drop of labeled mixture was spotted near the bottom of the each ITLC strips and then placed in the desired solvent. In this case organic and aqueous solvent, which is acetone and saline, was used. As the solvent migrates up, soluble radiochemical species are carried with it, while insoluble compounds remain at the origin. The radioactivity at each place on the strip can be measured with radio TLC scanner (Bioscan Co., WI, USA). When it was eluted with acetone, reduced-hydrolyzed ^{99m}Tc impurity and ^{99m}Tc-labeled gluconate remained at the origin and unlabeled free ^{99m}Tc moved up to the solvent front. Later, in contrast when it was eluted with saline, only reduced-hydrolyzed ^{99m}Tc stayed at the origin and [^{99m}Tc]Tc-gluconate moved to the solvent front.

For [^{99m}Tc]Tc-DTPA labeling DTPA kit vial (Mallinckrodt., made in Netherlands) was obtained from Saehan. Freshly eluted [^{99m}Tc]$NaTcO_4$ (400 MBq) of 4 mL from the generator was added to DTPA kit. Again, the labeled product was analyzed by ITLC eluted with solvents. Similarly, when it was eluted with acetone, labeled [^{99m}Tc]Tc-DTPA stayed at the origin, on the other hand labeled [^{99m}Tc]Tc-DTPA moved to the solvent front when it

was eluted with saline.

Formation of insoluble fraction with the presence of H_2S

100 μL of [^{99m}Tc]Tc-gluconate, [^{99m}Tc]Tc-DTPA was mixed with 100 μL of 0.2 mM NaHS in 0.2 M sodium phosphate buffer (pH 7.4). The mixtures were vortexed and incubated at 37 °C for 10 min, then spotted on ITLC plates and eluted with saline. The ITLC plates were scanned by Bio-Scan AR-2000. The insoluble fraction formation was measured by calculating the percentage of remaining radioactivity at the origin.

Cell culture

CT26 (colon carcinoma cell line) cells were grown in DMEM medium (Gibco, Grand Island, NY, USA). DMEM medium contains 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibiotics containing penicillin/streptomycin (Invitrogen, Grand Island, NY, USA). Cells were incubated at 37°C humidified incubator with 5% CO_2 atmosphere.

In vitro hypoxic cell culture studies, ^{99m}Tc-Gluconate uptake.

CT26 cells were purchased from the Korean Cell Line Bank. CT26 was cultured in DMEM medium (High glucose, Gibco, Grand Island, NY, USA).) and incubated at 37°C with 5% CO_2 in a humidified incubator.

Cells were cultured under hypoxic condition (N_2 95% and CO_2 5%) and normoxic condition (Air 95% and CO_2 5%) for [^{99m}Tc]Tc-gluconate, [^{99m}Tc]Tc-DTPA uptake. Suspension 5x10^6 cells/1 mL using N_2 purged media placed in sterile glass vials. Cells were incubated for 4 h under hypoxic and normoxic condition was proceeded. [^{99m}Tc]Tc-gluconate was diluted in serum free N_2 purged DMEM medium and 1 mL was added into vials. Vials were incubated in a humidified incubator at 37°C with 5% CO_2. At each time points 30 min, 1 h and 2 h, 200 μL of samples were taken into 5 mL test tubes. Washed and centrifuged three times with cold HBSS, lysed in 0.2 mL of 1% SDS and transferred into the new 5 mL test tubes. The radioactivity of the cells was counted with a γ-counter. BCA protein assay was proceeded on the next day.

Ischemia/ reperfusion mouse modeling for *in vivo* imaging

Tourniquet induced ischemia-reperfusion method was proceeded according to the previously reported method [17,18]. Mice were anesthetized with an anesthetic cocktail consisting of 0.1 mg/g alfaxalone and 0.01 mg/g xylazine, given by intraperitoneal injection (0.005 mL/g body weight).

Anesthesia was maintained throughout the whole experiments with additional anesthetic cocktail (0.05 mL) as needed. Under the anesthesia, fur was completely removed on both hind limbs using electric shaver and hair

removal cream. The animals were restrained on a heating pad to maintain body temperature at 37°C.

Unilateral hind limb ischemia was induced by placing an orthodontic rubber band at the hip joint. After 3 h of ischemia, the orthodontic rubber band was released, and reperfusion was proceeded.

In vivo detection of endogenous H_2S by fluorescence and SPECT/CT imaging in mouse models.

For imaging endogenously produced H_2S in ischemic hindlimb, 100 μL of HSip-1 (200 μM) was intravenously injected after 0, 1, 3, 12 and 24 h of reperfusion. After 1 h of circulation, mice were sacrificed, and the fluorescence signal intensities of left and right limbs were measured. Fluorescence signals were measured by Lumina II (Perkin Elmer, Waltham, MA, U.S.A). Fluorescence images were acquired with an exposure time of 20 sec, medium bining, 2 f/stop, with open filter. Data were analyzed using Living Image software (ver. 2.5)

For SPECT/CT imaging the hindlimb underwent 3 h reperfusion. After 3 h of reperfusion, 12.5 MBq [^{99m}Tc]Tc-gluconate, 12.7 MBq [^{99m}Tc]Tc-DTPA in 100 μL saline were intravenously injected to mice. Only the lower limb was imaged with SPECT/CT by covering upper limb with 2mm thick lead plate.

The scanning parameters for whole body imaging modality employed a γ-ray energy window of 140 keV ± 10%, a matrix size of 256 × 256, an acquisition time of 5 s per angular step of 18° and a reconstruction algorithm of ordered subset expectation maximization with 9 iterations. For integrated CT, a tube voltage of 45 kVp, an exposure time of 1.5 s per projection, and a reconstruction algorithm of cone-beam filtered back-projection were used. The SPECT/CT images were represented with same scale condition by using imaging program of InVivoScope on nanoScan-SPECT/CT.

Immunohistochemical staining of hindlimb sections

200 μL of 10 mM EF5 (hypoxia marker, #CS222743, Millipore) was injected with HSip-1 (H_2S marker, SB21, Dojindo Molecular Tech.). Both ischemic and normal hind limbs were extracted right after Lumina II (Perkin Elmer, Waltham, MA, U.S.A) image. Limbs were embedded in Optimal cutting temperature (OCT) compound (Leicabiosystems, Richmond, IL, USA), snap frozen, and sectioned at 8 μm thickness using cryostat and placed on slides. Section slides were dried for 30 min at RT, then slide samples were fixed with acetone at -20°C for 10 min and then let it dry again for 30 min at RT. Fixed slides were washed three times with PBS for 5 min. After washing, all the sections were marked with a PAP pen (hydrophobic pen) and blocking solution was added. The slides were placed

in a staining tray containing small amount of water covered with a lid for overnight at 4°C. After blocking solution was removed, all the slides were washed and rinsed with 1X ttPBS (1X PBS with 0.3% Tween 20 and 2 mM sodium azide).

100 µL of staining solution Cy3-conjugated anti-EF5 (75 µg/mL) was added to slides for 6 h in a tin-foil covered staining tray with a lid at 4°C. After finishing the staining all the slides were washed three times with cold 1X ttPBS for 40 min, in dark. Mounting with ProLong™ Gold antifade reagent with DAPI (Invitrogen, USA). Fluorescence images were taken on the next day using confocal laser scanning microscope (Leica TCS SP8, Wetzlar, Hesse, Germany).

Confocal imaging

For immune-fluorescence staining, confocal imaging was used to analyze fluorescence signal in the samples. After finishing the immunofluorescence staining samples were washed with PBS for three times, and the samples were mounted with ProLong™ Gold antifade reagent with DAPI (Invitrogen, GrandIsland, NY, U.S.A) and covered the samples with cover slide. The confocal samples were stored at 4°C and imaged on the next day. Fluorescence signals were detected using confocal laser scanning microscope (Leica TCS SP8m Wetzlar, Hesse, Germany) in the specific rage of wavelength (DAPI; 401-480, FITC; 495-519 Cy3; 550-570). Fluorescence intensities were analyzed using an LAS X system (Leica

Microsystems, Wetzlar, Germany)

Statistical analysis

Quantitative data were expressed as mean ±SD. Means were compared using Student's *t*- test provided by Excel (Microsoft, U.S.A) or GraphPad Prism (GraphPad Software, Inc.).

RESULTS

^{99m}Tc-labeling of gluconate and DTPA

For the labeling ^{99m}Tc, stannous chloride was used as a reducing agent, successfully labeled with gluconic acid and DTPA. Both reagents showed almost 99.8 ± 0.1% labeling yield (Figure 4, 5).

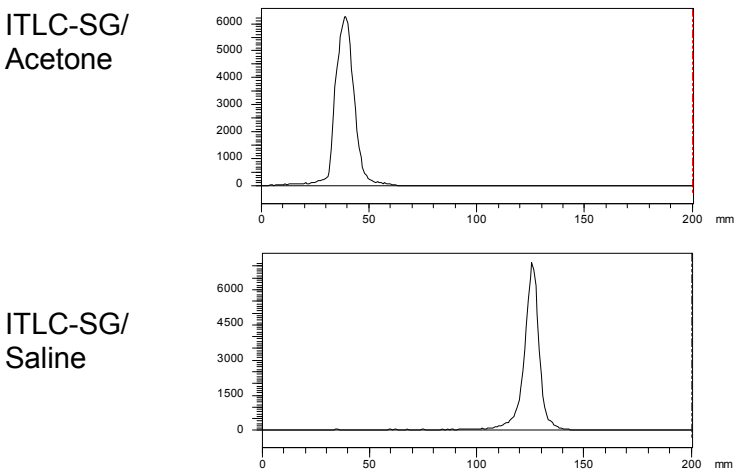

Figure 4. Chromatogram of [^{99m}Tc]Tc-gluconate

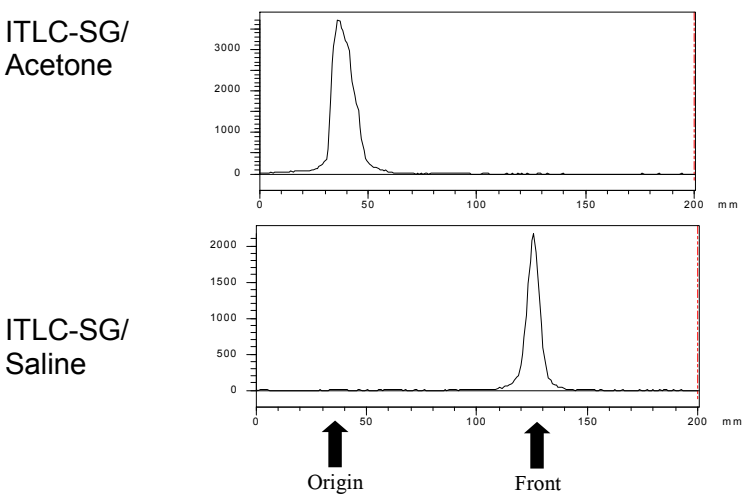

Figure 5. Chromatogram of [^{99m}Tc]Tc-DTPA

Formation of insoluble fraction with the presence of H_2S

In this experiment NaHS solution was prepared instead of using H_2S gas, to make the experiment more convenient and NaHS generates H_2S molecules in aqueous solution. [^{99m}Tc]Tc-gluconate, [^{99m}Tc]Tc-DTPA were incubated with 0.2 mM NaHS in 0.2 M phosphate buffer for 10 mins, at 37°C. 100uL of 0.2 mM sodium hydrosulfide solution was incubated with 100uL of [^{99m}Tc]Tc-gluconate, [^{99m}Tc]Tc-DTPA. The reactants were analyzed with ITLC/Saline. Compared to DTPA, gluconate showed that insoluble fraction was formed in the presence of H_2S. Significantly higher signal appeared at the origin, when its eluted with saline. Formation of insoluble fraction with H_2S was compared and [^{99m}Tc]Tc-gluconate (88.9 ± 2.4%) (Figure 6 (A)) showed much higher percentage than [^{99m}Tc]Tc-DTPA (Figure 6 (B)).

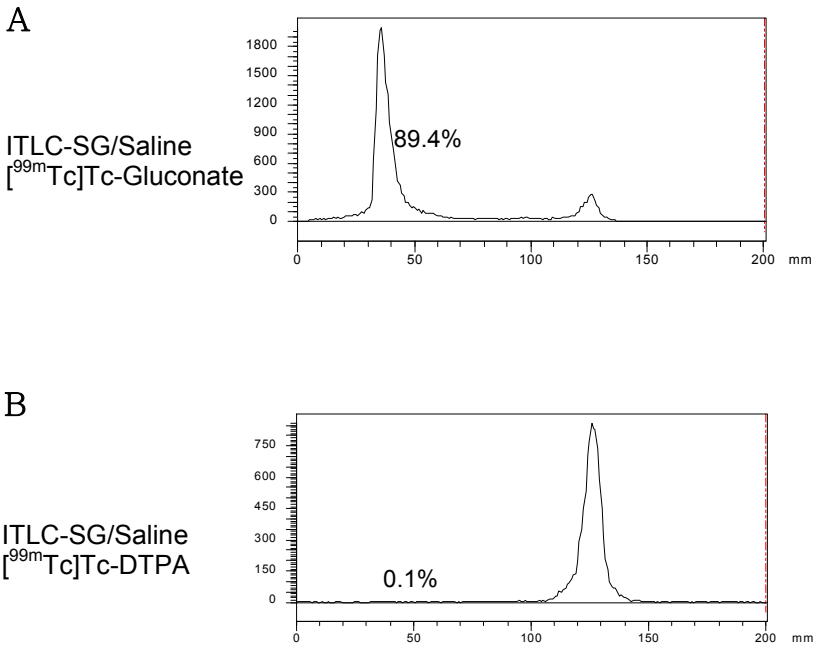

Figure 6. Comparison of insoluble fraction formation.

(A) ITLC-SG data eluted with saline. [^{99m}Tc]Tc-gluconate was reacted with NaHS at 37°C for 10 min at pH 7.4. The radioactivity remaining at the origin was calculated as insoluble fraction.

(B) [^{99m}Tc]Tc-DTPA was reacted with NaHS at 37°C for 10 min at pH 7.4.

In vitro hypoxic cancer cell uptake of [^{99m}Tc]Tc-gluconate.

To confirm gluconate uptake on the hypoxic cell, cell uptake of [^{99m}Tc]Tc-gluconate and [^{99m}Tc]Tc-DTPA was performed in hypoxic and normoxic conditioned colon carcinoma cell line CT26 up to 2 h. The CPM per sample was divided by the total protein amount and were expressed as mean ± SD for three samples. At 120 min of incubation, [^{99m}Tc]Tc-gluconate uptake in hypoxic and normoxic CT26 cells increased 109,772.48 ± 6,889.49 and 25,588.32 ± 1,886.07 CPM/mg respectively. In contrast, [^{99m}Tc]Tc-DTPA uptake reached only 5,198.67 ± 466.80 and 4,770.28 ±167.79 CPM/mg at 120 min. This results demonstrate that the [^{99m}Tc]Tc-gluconate can have comparatively higher uptake to hypoxic conditioned cancer cell.

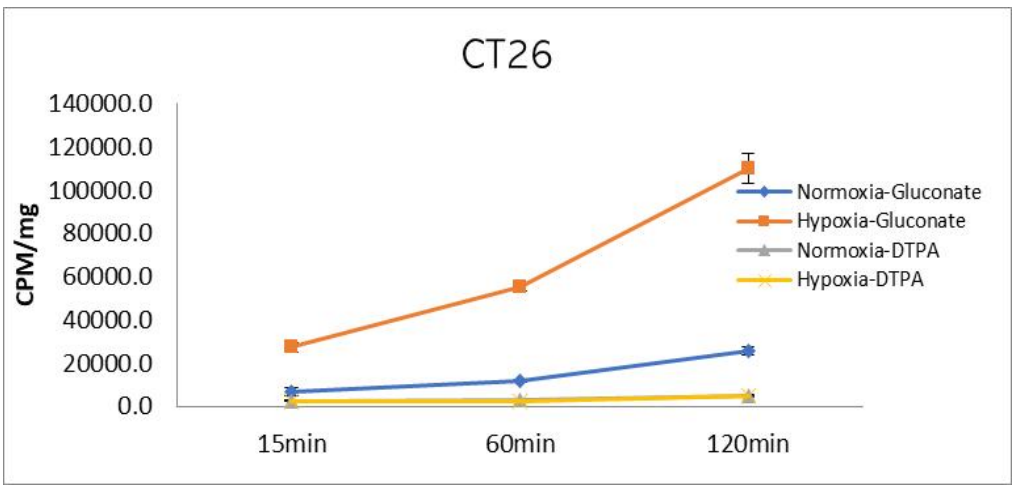

Figure 7. In vitro uptake study of [^{99m}Tc]Tc-gluconate and [^{99m}Tc]Tc-DTPA.

Cell uptake study was performed in hypoxic and normoxic condition cells. The cells were harvested at 15, 60, 120 min, and the radioactivites were counted by a gamma counter. The value was expressed as CPM/mg, each CPM values were divided by protein of the cell. (n=3)

In vivo detection of endogenous H_2S by using fluorescence probe in mouse models.

To compare the H_2S production in normal and ischemic hindlimb, H_2S detecting fluorescence probe (HSip-1) was injected intravenously in different time points (Figure 8). The fluorescence signal increased in ischemic leg up to 3 h of reperfusion time, and after 3 h the fluorescence signal decreased. As shown in Figure 8, the highest fluorescence intensity on the ischemic limbs was detected after 3 h of reperfusion which refers to H_2S production is high at 3 h after the leg was released. H_2S detection probe (HSip-1) was mainly accumulated in the ischemic legs, meanwhile only little amount of fluorescence signal observed in the normal leg (Figure 8). Interestingly, the signal decreased as the reperfusion time increased. At 24 h of reperfusion time, fluorescence intensity was almost the same in both normal and ischemic legs.

Then, immune-fluorescence staining and confocal imaging were performed with hindlimb section which had 3 h of reperfusion time. As shown in figure 9, the fluorescence intensities of EF5 (hypoxia detection) and HSip-1 (H_2S detection) in ischemic leg tissue were much higher than normal leg tissue.

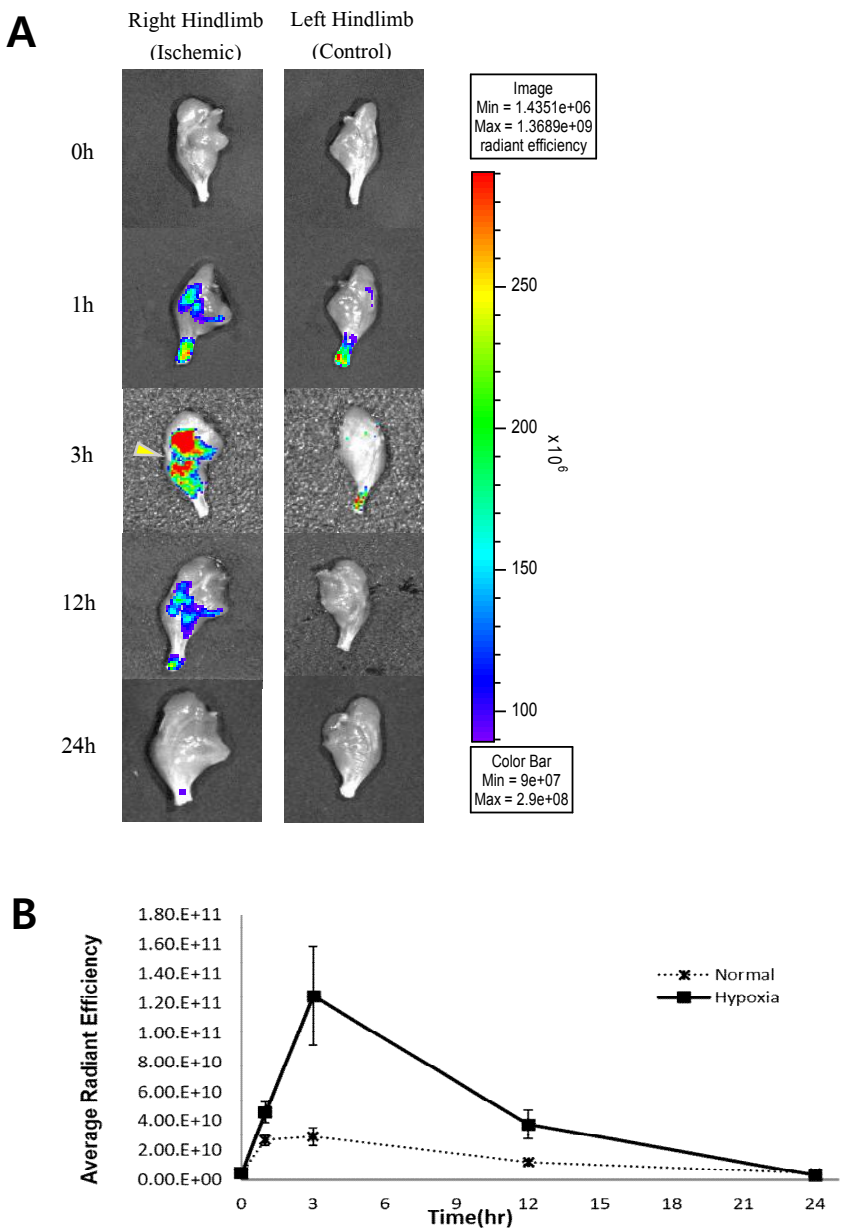

Figure 8. *In vivo* fluorescence imaging of endogenous H_2S by fluorescence probe in I/R model.

(A) Fluorescence images (Lumina II) at different time points; 0, 1, 3, 12 and 24 h. Left legs are normal and right legs, rubber band was applied, (n=4). (B) Fluorescence intensity of both normal and ischemic regions.

Immunohistochemical staining of hindlimb sections

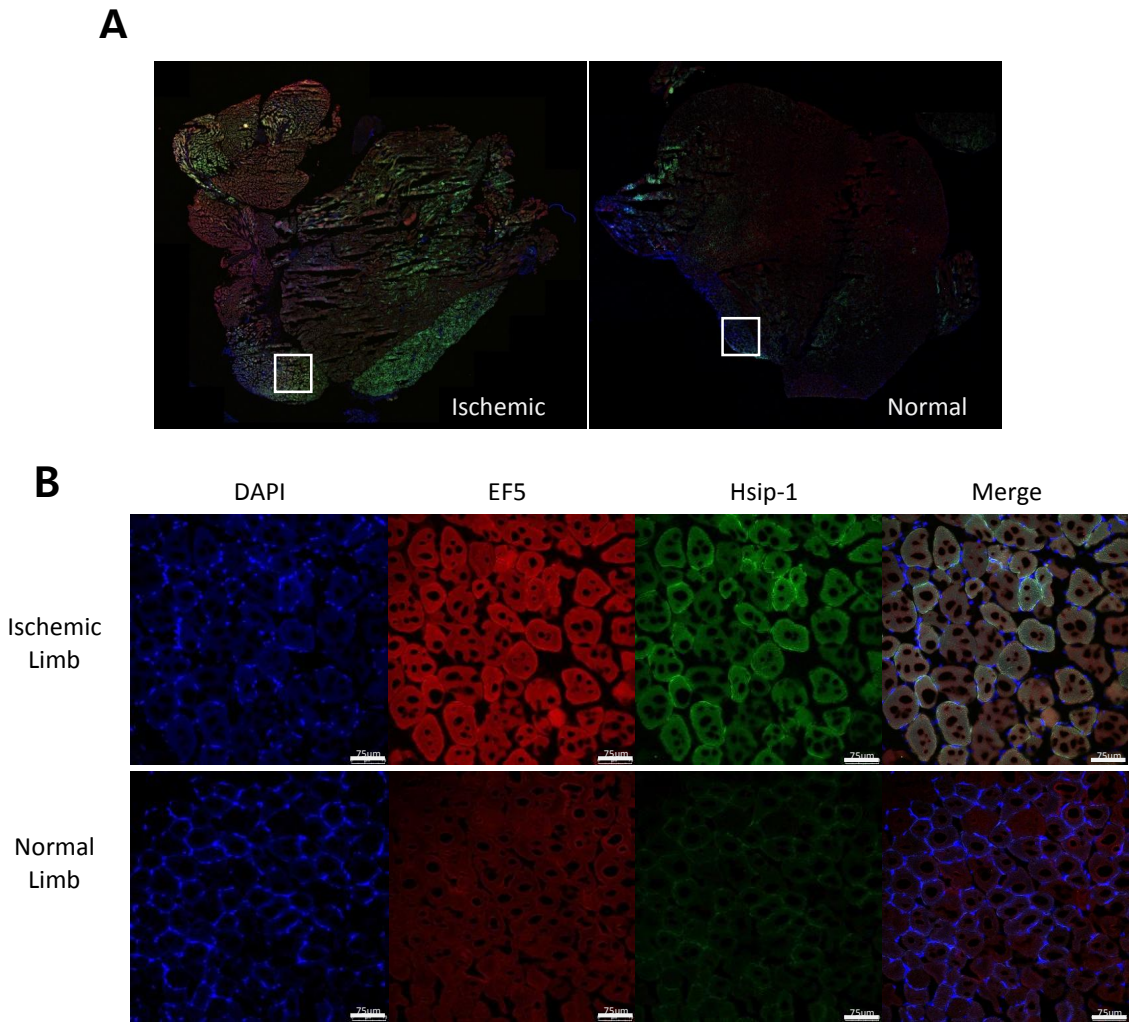

Figure 9. Immunohistochemical staining of hindlimb sections.

(A) Fluorescence images of the ischemic tissue and normal tissue. Tissue imaging was performed with confocal microscope (Leica TCS SP8m Wetzlar) Original magnification: 100x. (B) The red is EF5 (hypoxia detection), the green is HSip-1 (H_2S detection), and the DAPI (nuclei) is blue. Original magnification: 400x; scale bars, 75 μm

In vivo SPECT/CT using tourniquet induced I/R mouse model.

To confirm [^{99m}Tc]Tc-gluconate as an H_2S imaging agent *in vivo*, [^{99m}Tc]Tc-gluconate or [^{99m}Tc]Tc-DTPA was injected intravenously on the tourniquet induced ischemia mouse model. Only legs were imaged with SPECT/CT (nanoSPECT/CT, Mediso, Hungary) 1 h after the injection. The images showed higher uptake of [^{99m}Tc]Tc-gluconate on the ischemic leg (Figure 10 (A)). [^{99m}Tc]Tc-DTPA did not show significant uptake on both ischemic and normal leg (Figure 10 (B)). These experiment shows the feasibility of [^{99m}Tc]Tc-gluconate as an agent for the imaging endogenously produced H_2S.

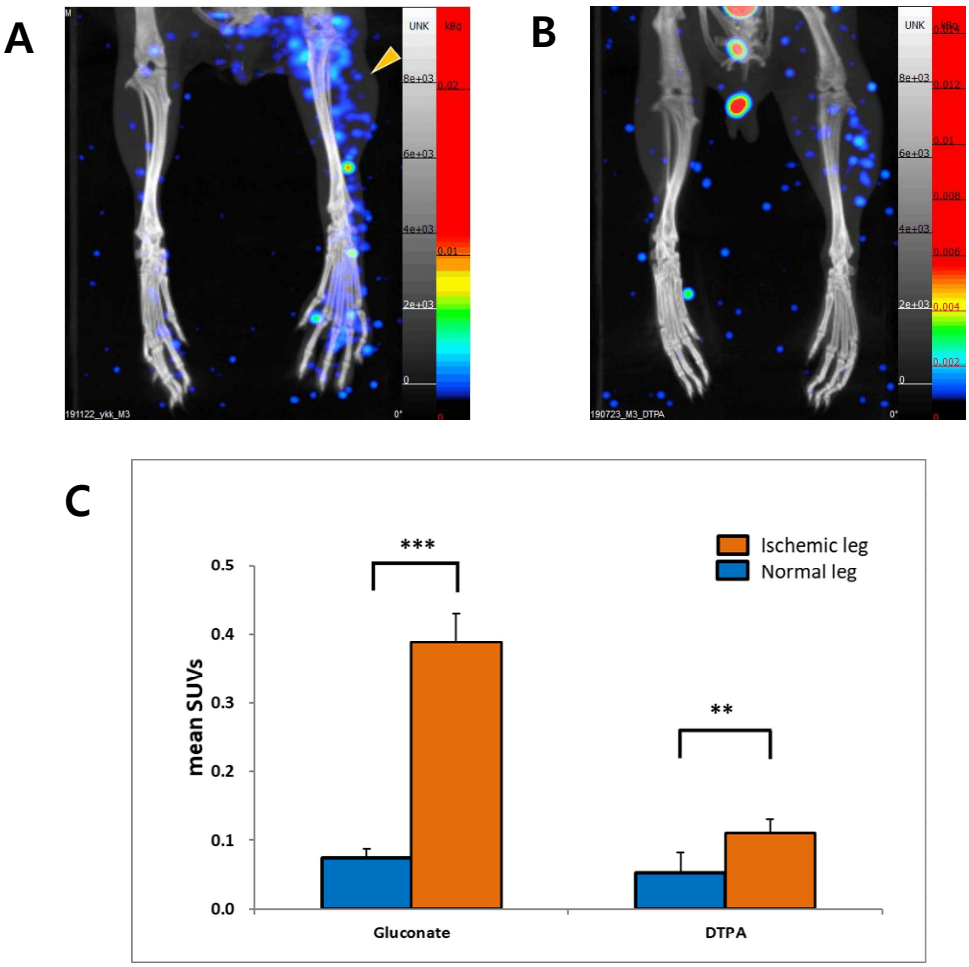

Figure 10. [^{99m}Tc]Tc-gluconate and [^{99m}Tc]Tc-DTPA imaging of mice with ischemia reperfusion model.

Balb/c mice went through 3 h tourniquet induced ischemia modeling, after 2 h of reperfusion then 12 MBq of (A) [^{99m}Tc]Tc-gluconate or (B) [^{99m}Tc]Tc-DTPA was intravenously injected. After 1 h of circulation legs were imaged by SPECT/CT. (C) Uptake on both normal and ischemic limbs mean SUV was calculated. Statistical significance was determined by unpaired Student's *t* test. **p < 0.01, ***p < 0.005.

DISCUSSION

For centuries, hydrogen sulfide (H_2S) has been known to be a toxic molecule in biological systems. However, recent studies have found that H_2S can be used to cellular regulation and signaling events similar to other gasotransmitters, such as nitric oxide, carbon monoxide. H_2S is the third gasotransmitter, used as a cell signaling molecule, and can be generated in various physiological processes. Recent reports demonstrate that when hypoxic conditions were induced, H_2S production will be increased in various cells by changes of the partial pressure of oxygen [24]. Depending on the partial pressure of oxygen, H_2S can be produced in cells, which probably means hypoxia is related to H_2S production. Since hypoxia is a common characteristic of solid tumors, it was necessary to detect and image endogenously produced H_2S under hypoxic conditions.

As shown in Figure 8, after injecting H_2S detection probe (HSip-1) the higher fluorescence intensity was observed in the ischemic-reperfusion injury legs compared to normal legs in every time point except 24 h reperfusion time. The uptake results indicate that H_2S formation is increased after 3 h of reperfusion. A report measured skeletal muscle blood flow by placing a transonic flow probe in the gastrocnemius muscles which connected to a Laser Doppler blood flowmeter and recorded. Blood flow to

the gastrocnemius muscle was measured during the ischemia-reperfusion, 3 h of ischemia and 4 h or reperfusion. When tourniquet was applied on the limb, blood flow went down to 2% of baseline and remained the same for 3 h of ischemia. When the tourniquet was released, the blood flow was rapidly increased to approximately 50% of baseline and declined to a steady state of 30% of baseline [25]. This result disprove that fluorescence probe couldn't reach gastrocnemius muscle because blood vessels were clogged when the reperfusion time was only 1 h. Even though there is a study of blood flow measurement, future works for checking and measuring blood flow during SPECT/CT imaging is needed. No exact mechanisms about formation of H_2S in reperfusion and when it forms the highest are unknown. However, interestingly, blood flow was consistent during the reperfusion time and formation of H_2S increased at 3 h of reperfusion. The fluorescence intensity *in vivo* imaging result shows that not only H_2S is produced under hypoxic condition, but also H_2S detection fluorescence probe can be used in vivo imaging. However, there are disadvantages of using fluorescence probes. First of all, it is difficult to be used for deep-tissue *in vivo* imaging due to the poor tissue penetration. Also, generally fluorescence probes have a relatively low signal-to-background ratio, due to the nonbound probes in the background noise [26]. To overcome these problems of using fluorescence probe, we tried to use radionuclide for detecting H_2S. In this study, ^{99m}Tc labeled agent was used since it is frequently used in many nuclear medicine departments, easy to access and generated inexpensively

from a generator. There are several advantages of using ^{99m}Tc, such as it allows rapid diagnosis, 6 h of short half-life that means radioisotope decays very quickly and causes very little damage to patients and no side effects [27].

Gluconic acid was successfully labeled with ^{99m}Tc (Figure 4), and also [^{99m}Tc]Tc-gluconate showed insoluble fraction formation with NaHS (Figure 6). The previous study in our group, [^{99m}Tc]Tc-gluconate have shown that it forms the highest percentage of insoluble fraction and also it was not identical with Tc_2S_7 [19]. However, identifying the chemical structure of insoluble fraction is still a task to be solved. One of possible reasons is forming insoluble polydisulfide $[Tc_3(\mu^3-S)(S_2)_3(S_2)_{3/3}]_n$ by trimerization of insoluble sulfide Tc(IV)S_2 [20]. Moreover, the possibility of chemical structure of [^{99m}Tc]Tc-gluconate is two gluconate molecules form a complex with Tc(V)O, the complex contains Tc = O core and two gluconate ligands (oxobis(gluconto)technetate(V) anion (net charge: -1) in aqueous solution) [21]. This previous report figured the chemical structure of [^{99m}Tc]Tc-glucoheptonate, and because of the molecular similarity of glucoheptonate and gluconate, [^{99m}Tc]Tc-gluconate will have the similar structure. Also because of this structure, presence of H_2S, Tc(V) can be reduced to Tc(IV) and precipitate, so that it makes possible to be image. Another chance is ^{99m}Tc can be fixed by transchelation with the sulfhydryl groups of protein [22, 23]. Nevertheless, further studies of identifying chemical structure and forming insoluble fraction mechanisms are required.

Multiple publications and studies showed [^{99m}Tc]Tc-gluconate as an imaging agent for cancer, inflammation, cerebral and myocardial ischemia, however, there is no clear uptake mechanism ecplained the present study shows the increased [^{99m}Tc]Tc-gluconate uptake when the hydrogen sulfide in hypoxic condition (Figure 8 and 10) is produced. It is possibly due to the insoluble fraction formation and it made the H_2S possible to be imaged.

Most importantly, the benefit of using [^{99m}Tc]Tc-gluconate as a H_2S imaging agent compared to fluorescence imaging probe is that [^{99m}Tc]Tc-gluconate is already approved radiopharmaceutical and been used in some countries. Thus, applying [^{99m}Tc]Tc-gluconate into clinical field will be much easier than both using fluorescence probe or developing a new imaging agent.

Targeting and imaging hypoxia region using [^{99m}Tc]Tc-gluconate was successful. It showed relatively high [^{99m}Tc]Tc-gluconate uptake and fluorescence probe uptake on the ischemic legs. Also hypoxia is a common characteristic of solid tumors, so that [^{99m}Tc]Tc-gluconate could possibly be used for imaging tumor cell. However, due to the time and procedure limitations *in vivo* cancer cell targeting with cancer model wasn't performed in this study. So, for the further study orthotopic tumor models imaging is necessary.

In summary, in this study, endogenously produced H_2S can be fixed by [^{99m}Tc]Tc-gluconate and H_2S in hypoxic condition was successfully visualized. Overall, [^{99m}Tc]Tc-gluconate is a proper agent for imaging H_2S,

which will be useful for diagnosing hypoxia.

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국문초록

서론: 황화수소는 염증 조절, 혈관확장, 산소 감지, 혈관 신생, 저산소증 및 재관류손상과 같은 다양한 생리적 과정에서 발현된다. 따라서 H_2S의 표적과 영상화는 신호전달물질의 역할을 연구하는 데 중요한 역할을 할 것이다. 현재까지 형광과 발광을 이용한 황화수소 영상화 기술은 다양하게 소개가 되었지만, 방사성 핵종을 사용하는 방법은 ^{64}Cu-cyclen이 처음이었다. 형광, 발광 probe는 독성이 있고 감도가 낮고 장기투과율이 낮아 외과적으로만 사용 가능하며 진단에 사용되기는 어려움이 있다. 따라서, 이 연구는 보다 안전한 테크네튬-99m이 표지 된 방사성의약품으로 허혈성 부위에 발현된 황화수소를 표적하기위해 연구하였다.

방법: 글루콘산을 Tc-99m 표지 물질로 사용했다. Tc-99m 표지 된 글루콘산 ([^{99m}Tc]Tc-gluconate)을 황화 수소 나트륨과 반응하여 불용성 입자 형성 확인 실험을 진행했다. 불용성 입자 생성은 얇은막 크로마토그래피 (ITLC-SG: Instant Thin Layer Chromatography)를 이용해 확인하였다. In vitro 연구를 위해 [^{99m}Tc]Tc-gluconate의 세포내 축적을 확인하기위해 대장암 세포주인 CT26가 사용되었다. 세포를 저산소 환경으로 만들기 위해 저산소 세포배양기에서 배양해 실험을 진행했다. 세포에 축적된 [^{99m}Tc]Tc-gluconate의 양은 감마카운터에 의해

측정되었다. In vivo 연구를 위해, 고무 지혈대를 이용한 허혈-재관류 마우스 모델을 제작하였다. 허혈-재관류 마우스 모델에 황화수소 표적 형광물질 (HSip-1)을 꼬리정맥 주사를통해 주입하여 황화수소의 발현을 확인하였다. 형광 신호는 IVIS Lumina II를 통해 검출하였다. 또한 허혈성 하지 모델의 뒷다리 조직으로 절단 샘플을 제작하여 면역 형광 염색을 진행했다. 이후 공초점 현미경 영상을 이용해 조직에 축적된 황화수소 표적 형광 신호를 검출 하였다. 이후 [^{99m}Tc]Tc-gluconate 가 황화수소를 영상화 하는데 사용될 수 있는지 확인하기위해 허혈-재관류 마우스 모델에 [^{99m}Tc]Tc-gluconate를 꼬리정맥 주사하여 허혈성 부위에 축적된 [^{99m}Tc]Tc-gluconate를 단일 광자 방출 컴퓨터 단층 촬영기 (SPECT/CT)를 이용하여 검출하였다.

결과: 글루콘산을 테크네튬-99m과 성공적으로 표지하였다. [^{99m}Tc]Tc-gluconate과 황화 수소 나트륨을 반응하여 불용성 입자가 형성됨을 확인하였다. 세포 실험에서 [^{99m}Tc]Tc-gluconate가 정상세포와 비교했을 때 저산소 세포에 더 높은 섭취가 측정되었다. 허혈성 하지모델에서 형광 detection probe (HSip-1)가 정상 다리에 비해 허혈-재관류 부위에 더 많이 축적됐고 높은 형광신호가 측정 되는 것을 보였다. 이를 통해 허혈-재관류 부위에 황화수소가 발현됨을 확인할 수 있었다. 나아가 허혈성 하지 부위에 [^{99m}Tc]Tc-gluconate가 결합되어 insoluble complex를 형성하여 SPECT/CT 영상이 가능함을 확인했다. [^{99m}Tc]Tc-gluconate의 축적은 재관류시간을 3시간 준 뒤 진행했으며 흡수된 선량은

정상다리보다 높았다.

결론: 허혈-재관류 부위에 황화수소가 발현됨을 확인할 수 있었다. 방사성 표지 [^{99m}Tc]Tc-gluconate가 허혈성 부위에 발현된 황화수소를 표적 하기 가능한 물질인지 확인했다. 따라서 세포내 발현된 황화수소를 표적하는 방사성 의약품으로서 [^{99m}Tc]Tc-gluconate는 사용 될 수 있는 잠재성이 있다. 하지만, 과정상 많은 제한점으로 확인하지 못한 종양모델에서의 [^{99m}Tc]Tc-gluconate 축적을 후속연구로 필요하다.

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주요어 : 저산소증, 지혈대, 허혈-재관류, 황화수소 (H_2S), 형광 영상, 단일 광자 방출 컴퓨터 단층 촬영기, 테크네튬-99m
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