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Detection of hypochlorous acid fluctuation *via* a selective near-infrared fluorescent probe in living cells and *in vivo* under hypoxic stress†

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Hypoxic stress is a common concern in medicine and biology, which can induce the cellular injury and death by excess production of reactive oxygen species (ROS). Hypochlorous acid (HOCl), one of the ROS, plays a crucial role in the oxidative damage to tissue proteins in the pathogenesis of various diseases. Therefore, excess production of HOCl might be an important factor for the damage caused by hypoxic stress. However, most of the present methods cannot meet the demand of real-time detection on account of the labile and reactive chemical properties of HOCl. Herein, we designed a near-infrared fluorescent probe, Cy-HOCl, for the selective imaging of HOCl in cells and *in vivo*. Cy-HOCl includes two moieties: a 4-amino-3-nitrophenol group as the response unit and a near-infrared heptamethine cyanine fluorophore as the fluorescent modulator. Cy-HOCl exhibits excellent selectivity and sensitivity towards the detection of HOCl. The hypoxic response behavior of Cy-HOCl is evaluated in cells to clarify the relationship between HOCl and hypoxia. The probe is also applied to measure HOCl in *ex vivo*-dissected organs of an acute ischemia mouse model as well as for real-time monitoring the changes of HOCl in the hypoxic zebrafish model.

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1. Introduction

Hypoxic stress is a common concern in medicine and biology, which can induce excess production of ROS¹ to cause cellular injury and death.^{2,3} ROS is a collective term that includes oxygen radicals, such as hydroxyl radical (OH•), superoxide anion (O₂•[−]) and peroxy radical (RO₂•), as well as some non-radical derivatives of oxygen (O₂) such as ozone (O₃), hydrogen peroxide (H₂O₂) and hypochlorous acid (HOCl). HOCl partially dissociates into hypochlorite anion in a solution at physiological pH.⁴

Within a definite concentration range, HOCl can act as an effective bactericide due to its strong oxidizability. On the other hand, HOCl is also a common byproduct of cellular metabolism, which plays a vital role in many biological processes.^{5,6} In organisms, HOCl can be synthesized from hydrogen peroxide and chloride ions under the catalysis of myeloperoxidase (MPO), which is mostly stored in azurophilic granules of leukocytes (including macrophages, neutrophils and monocytes).^{7,8} HOCl is extremely reactive and non-selectively modifies all biomolecules, especially proteins, giving rise to significant inactivation of proteins and severe damage to cells.⁹ The additional generation of HOCl can give rise to inflammation-associated injury, such as lung injury,¹⁰ hepatic ischemia reperfusion injury,¹¹ rheumatoid arthritis¹² and atherosclerosis,¹³ *via* the same processes utilized in the destruction of the invading microorganisms. More importantly, the increasing level of ROS in cells under hypoxic stress has been proved,¹ but the relationship between hypoxia and HOCl has not been fully demonstrated to date. We suppose the phenomenon of intracellular HOCl changes is one of the major sources of damage under hypoxic stress. Therefore, establishing an ideal and simple fluorescence method for detection of HOCl to explore the relationship between HOCl and hypoxia is still necessary.

To date, detailed research of the oxidation biology of HOCl has been restricted by the difficulty in monitoring such a small

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reactive oxygen metabolite in real time, motivating investigators to develop a selective and sensitive tool for the tracking of HOCl, to provide reliable and accurate HOCl signals both *in vivo* and *in vitro*. Due to its outstanding high sensitivity, selectivity, and real-time analysis, fluorescence imaging is an attractive method for monitoring biomolecules in living systems. Previously, a number of bioanalytical methods for the detection of HOCl have been developed.¹⁴ Most were developed on the basis of the conjugation of an organic fluorophore with a HOCl-recognizing moiety, such as *p*-methoxyphenol, *p*-aminophenyl ether, thiol, N-heterocyclic carbene borane, dibenzoyl hydrazine, hydroxamic acid, ferrocene and oxim derivatives.^{15–39} Although these fluorescent probes can detect and quantify HOCl concentrations in biological samples by the changes of fluorescence intensity, the detection is often hampered by background fluorescence due to the excitation and emission wavelengths of these probes locating in the visible region; the method is not suitable for imaging in deep structures. Near-infrared (NIR) fluorescence can deeply penetrate tissue and effectively avoid background noise.⁴⁰ The existing NIR fluorescence probe for detection of HOCl is limited by the “on-off” fluorescence response leading to a poor signal-to-noise ratio.⁴¹ Therefore, imaging of HOCl in living cells and animals using a desirable “off-on” NIR fluorescent probe is another considerable issue to be addressed.

Herein, we design and synthesize the “off-on” NIR fluorescent probe Cy-HOCl for the detection of HOCl in living cells and *in vivo*. Cy-HOCl includes two moieties: a 4-amino-3-nitrophenol group as the response unit and a near-infrared heptamethine cyanine fluorophore as the fluorescence modulator. The fluorescence of Cy-HOCl is quenched due to photo-induced electron transfer (PET) between response unit and fluorophore. The response unit can be specifically eliminated with HOCl and induce the fluorescence to “turn on”. The probe exhibited a specific response for HOCl and provided high sensitivity and selectivity toward HOCl in cells. The results demonstrated that the level of HOCl increased in hypoxic cells, hypoxic zebrafish models, and acute liver ischemia mice models. To the best of our knowledge, Cy-HOCl was the first fluorescence tool for imaging and quantifying the relationship between the level of HOCl and hypoxic stress in cells and *in vivo*. We also confirmed that the hypoxic stress not only caused the apoptosis of cells, but also the damage of tissue. We anticipate that our Cy-HOCl probe will be a powerful tool for investigating the pathological and physiological roles of HOCl in living cells and *in vivo*.

2. Experimental

2.1 Synthetic routes of probe Cy-HOCl

2.1.1 Synthesis of *N*-ethyl-2,3,3-trimethylindolinium iodide 3. 2,3,3-Trimethyl-3*H*-indolenine (12 g, 75 mmol) and iodoethane (11.5 g, 75 mmol) were mixed in 40 mL anhydrous acetonitrile in a 250 mL round flask. The mixture was refluxed for 12 h, then removed from heat and cooled. The precipitate was filtered through a Büchner funnel and the solid product was

washed with diethyl ether and dried in vacuum to afford a pink product (10.9 g, yield: 90%). ¹H NMR (DMSO-*d*₆, 500 MHz) δ (ppm): 8.04–8.02 (t, 1H), 7.91–7.89 (t, 1H), 7.66–7.62 (m, 2H), 4.57–4.53 (m, 2H), 2.92 (s, 3H), 1.58 (s, 6H), 1.49–1.46 (t, 3H). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ (ppm): 196.5, 142.4, 141.1, 129.4, 124.1, 115.8, 54.6, 43.8, 40.1, 22.4, 14.9, 13.3. MS (ESI⁺): *m/z* C₁₃H₁₈N⁺ calcd 188.1434, found [M⁺] 188.1435.

2.1.2. Synthesis of compound 2. A solution of 40 mL of anhydrous *N,N*-dimethylformamide (DMF) and 40 mL of anhydrous CH₂Cl₂ was placed in a 250 mL round-bottom flask. The solution was chilled to –10 °C and then stirred for 20 min. Phosphorus oxychloride (37 mL) in 35 mL of anhydrous CH₂Cl₂ was added dropwise into the above solution through a constant pressure drop of liquid funnel. 4-Cyclohexanone (10 g, 101.9 mmol) was added into the mixture in batches; the solution immediately changed from colorless to yellow. Then the solution was slowly heated to 45 °C for 3 h, cooled, poured over ice, and allowed to stand overnight. The yellow solid was collected through a Büchner funnel and dried in vacuum (12.9 g, yield: 85%). ¹H NMR (DMSO-*d*₆, 500 MHz) δ (ppm): 8.12 (s, 1H), 8.08 (s, 1H), 7.14 (s, 1H), 2.38–2.71 (m, 2H), 2.53–2.45 (m, 2H), 2.28–2.26 (m, 2H). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ (ppm): 191.8, 162.9, 148.5, 145.7, 142.4, 37.2, 31.7, 30.6. MS (ESI[–]): *m/z* C₈H₉ClO₂ calcd 172.0553, found [M – H][–] 171.0481.

2.1.3. Synthesis of compound 1. Compounds 3 (0.84 g, 2 mmol) and 2 (0.17 g, 1 mmol) were redissolved in 100 mL mixed solution of *n*-butyl alcohol and benzene (7:3, v/v) in a 250 mL round flask, refluxed for 3 h, and dried in vacuum to obtain a green solid. The crude product was purified by silica gel chromatography using EtOAc/CH₃OH (8:1, v/v) as eluent to afford compound 1 as green solid (0.682 g, 70% yield). ¹H NMR (CDCl₃-*d*₁, 500 MHz) δ (ppm): 8.43–8.40 (d, 1H), 7.41–7.39 (m, 2H), 7.28–7.15 (m, 6H), 6.09–6.07 (d, 2H), 5.01 (s, 1H), 4.14–4.15 (m, 4H), 3.01–2.97 (m, 4H), 2.68–2.63 (m, 2H), 2.04 (m, 6H), 1.79 (m, 6H), 1.42–1.39 (m, 3H), 1.27–1.24 (m, 3H). ¹³C NMR (DMSO-*d*₆, 125 MHz) δ (ppm): 172.2, 171.2, 150.3, 144.6, 141.6, 141.2, 128.9, 125.5, 122.3, 110.9, 100.6, 60.4, 49.5, 39.9, 38.0, 34.2, 28.1, 21.1, 14.2, 12.5. MS (ESI⁺): *m/z* C₃₄H₄₀ClN₂⁺ calcd 511.3137, found [M⁺] 511.3137.

2.1.4. Synthesis of probe Cy-HOCl. 4-Amino-3-nitrophenol (0.77 g, 5.00 mmol) and NaH (60% in mineral oil, 0.208 g, 5.00 mmol) were dissolved in anhydrous DMF (30 mL). The mixture was stirred at 25 °C for 15 min under Ar atmosphere. Then, compound 1 (0.424 g, 0.83 mmol) was introduced into the above mixture. The reaction mixture was further stirred for 24 h at 25 °C. The solvent was removed under reduced pressure and the crude product was purified by silica gel chromatography using EtOAc/CH₃OH (4:1, v/v) as eluent to afford probe Cy-HOCl as a green solid (0.293 g, 50% yield). ¹H NMR (CH₃DO, 500 MHz) δ (ppm): 8.53 (s, 1H), 8.00–7.97 (d, 1H), 7.66–7.36 (m, 6H), 7.26–7.21 (m, 2H), 7.10–6.84 (m, 2H), 6.65–6.60 (m, 3H), 6.19–6.16 (m, 2H), 4.15–4.14 (m, 4H), 2.75–2.74 (m, 4H), 1.92 (m, 6H), 1.41–1.28 (m, 14H). ¹³C NMR (CH₃DO, 125 MHz) δ (ppm): 171.9, 163.4, 149.7, 142.2, 141.7, 141.6, 141.1, 130.1, 129.4, 128.4, 124.8, 124.6, 122.0, 121.6, 121.0, 110.3, 108.7, 99.4, 65.2, 38.7, 30.3, 26.6, 23.8, 21.0, 10.9. MS (ESI⁺): *m/z* C₄₀H₄₅N₄O₃⁺ calcd 629.3486, found [M⁺] 629.5806.

2.2 Cytotoxicity of Cy-HOCl

The cytotoxicity of probe Cy-HOCl was assessed by MTT assay. Human normal liver cells (LO2 cells) were seeded into a 96-well cell culture plate at a final density of 8×10^3 cells per well. Different concentrations of Cy-HOCl (0.1 μM , 1 μM , 10 μM , and 100 μM) were added to the wells. The cells were incubated for 24 h at 37 °C under 5% CO_2 . Subsequently, MTT was added to each well (final concentration 5 mg mL^{-1}) for an additional 4 h incubation at 37 °C under 5% CO_2 , then formazan crystals formed, which were dissolved in 150 μL DMSO. The amount of MTT formazan was quantified by absorbance (OD) at 570 nm using a microplate reader (Tecan, Austria). Calculation of IC_{50} values were done according to Huber and Koella. The results are the mean standard deviation of six separate measurements.

2.3 Zebrafish line imaging

Four to five pairs of zebrafish were placed in crossing tanks for spawning overnight. Embryos settled to the bottom of the tank, then were collected using a sieve and transferred to Petri dishes for culture. They were screened, incubated at 27 °C, 0.4% CO_2 and grown in egg water (10% NaCl; 1.63% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4% CaCl_2 ; 0.3% KCl). At 22 h post-fertilization, PTU was added to prevent melanin formation and yield optically transparent fish. 3 d post-fertilization, the embryos were seeded to into 96 well plates at 1 embryo per well. The embryos were soaked in 5 μM of Cy-HOCl for 30 min and were imaged for uptake using confocal laser scanning microscope imaging (Japan Olympus Co., Ltd) at 405 nm.

2.4 Liver ischemia mice model and imaging of liver ischemia tissue sections

Six to eight-week-old BALB/c mice were obtained from Binzhou Medical University. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals; protocols were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University, Yantai, China. Approval Number: No. BZ2014-102R. Mice were group-housed on a 12 : 12 light–dark cycle at 22 °C with free access to food and water. BALB/c mice, 20–25 g, were selected and divided into different groups. Mice without any treatment were used as the control group. Mice were fasted 12 h with free access to water before liver ischemic surgery. First, the mice were anesthetized by intraperitoneal injection of 3% pentobarbitalum natricum (80 mg kg^{-1}) dissolved in saline, then the abdomens of mice were shaved and disinfected used 75% ethanol. Left and middle lobe liver were

separated after dissection of the abdominal cavity; to close the portal vein and hepatic artery, we used a non-invasive vascular clip. After 0.5 min, the whitened left and middle lobe liver was observed to certify that liver ischemia was successful. Persistent ischemia was maintained for 0.5 min, 5 min, 10 min, 30 min and 60 min, then ischemic liver tissue was dissected into frozen sections. The entire process of mouse ischemia was conducted on a 37 °C heating plate. Afterward, the liver ischemia tissue sections were treated with 5 μM Cy-HOCl for 30 min and washed with PBS buffer (pH = 7.4) to remove excess Cy-HOCl. Then, the sections were imaged under a laser scanning confocal microscope.

3. Results and discussion

3.1 Design strategies for probes Cy-HOCl

The synthetic approach of fluorescence probe Cy-HOCl is outlined in Scheme 1. The synthetic details of compounds were discussed in the Experimental section. All compounds were characterized by ^1H NMR, ^{13}C NMR and MS. A heptamethine cyanine dye was employed as the fluorophore unit to emit the detection signal located in the near-infrared region (NIR). Due to extremely low tissue auto-fluorescence and absorption in the NIR spectral range, optical imaging with NIR fluorophores shows high sensitivity and deep tissue penetration. In previous work, we found that the oxidative cyclization reaction of 4-amino-3-nitrophenyl with HOCl was highly rapid and specific.⁴² Based on this molecule, 4-amino-3-nitrophenol was chosen as the response group conjugated to the fluorophore unit to obtain the HOCl detection probe. The integration of 4-amino-3-nitrophenol into the fluorescence platform quenches the fluorescence of the fluorophore. The mechanism for this fluorescence quenching is a photoinduced electron transfer (PET) process from the excited fluorophore to a strong electron-withdrawing group (donor-excited PET; dPET).^{43,44} Finally, we obtained new probe Cy-HOCl for detection of HOCl by oxidative cyclization reaction. The reaction between Cy-HOCl and HOCl results in the 4-amino-3-nitrophenyl group being released as a leaving group. This ends the PET effect and induces recovery of fluorescence. The proposed detection mechanism is outlined in Scheme 2. Using this probe, we could real-time monitor the fluctuation of HOCl in living cells and *in vivo* during hypoxic stress.

3.2 Spectroscopic properties

We investigated the spectral response of Cy-HOCl toward HOCl under simulated physiological conditions (10 mM HEPES buffer, pH = 7.4). The maximum absorption wavelengths of the probe

Scheme 1 The synthetic route of probe Cy-HOCl. (a) Iodoethane, acetonitrile, refluxed 12 h, 90%; (b) DMF, CH_2Cl_2 , POCl_3 , 45 °C, 3 h, 85%; (c) V_n -butyl alcohol/ V_{benzene} = 7 : 3, refluxed 3 h, 70%; (d) NaH, 4-amino-3-nitrophenol, DMF, 25 °C, 24 h, 50%.

Scheme 2 Proposed detection mechanism of probe against HOCl.

Fig. 1 Spectral properties of Cy-HOCl. (a) Absorption spectral changes of Cy-HOCl (10 μM) in the presence of various concentrations of NaClO (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 μM) in HEPES (pH 7.4, 10 mM) at 37 $^{\circ}\text{C}$. (b) Fluorescence emission spectral changes of Cy-HOCl (10 μM); spectra were acquired 10 min after addition of various concentrations of NaClO (0–45 μM). (c) The plot of the linear relationship between fluorescence intensities and NaClO concentrations (0–45 μM). (d) Plot of fluorescence intensity of Cy-HOCl (10 μM) vs. reaction time in the presence of 30 μM NaClO. Data were obtained at λ_{ex} = 543 nm, λ_{em} = 625 nm.

Cy-HOCl locate at 480 nm and 780 nm (Fig. 1a). In the presence of Cy-HOCl and HOCl, Cy-HOCl exhibited a significant decline at the wavelength of 780 nm. Simultaneously, obvious enhancement of the absorption peak at 480 nm was caused by the addition of HOCl. The fluorescence spectra of Cy-HOCl were next measured to investigate the fluorescence response of the probe towards HOCl. As shown in Fig. 1b, the addition of HOCl (from 0 to 45 μM) triggered the increase of NIR fluorescence emission centered at 625 nm; therefore, the emission at 625 nm was selected as the optimal fluorescence intensity for quantifying the fluorescence response of our probe to HOCl. The calibration curve is shown in Fig. 1c. There exists a good linearity with the concentration of HOCl ranging from 0–45 μM . The regression equation was $F_{625\text{nm}} = 1.19 \times 10^4 [\text{HOCl}] (\mu\text{M}) + 6.19 \times 10^4$ ($r = 0.9961$). Under the given experimental conditions, the limit of detection ($3\sigma/\text{slope}$) toward HOCl was determined to be 10 nM. The results suggested that Cy-HOCl had high sensitivity for HOCl. The probe Cy-HOCl has potential for application in bioassays.

3.3 Kinetic Investigations of Cy-HOCl toward HOCl

Due to the rapid metabolism and unstable properties of HOCl in biological systems, immediate response offers the possibility of direct detection without cumbersome pretreatment processes.

The activity of HOCl toward the reaction of Cy-HOCl was assessed. Fluorescence kinetic curves of Cy-HOCl with HOCl (at the concentration of 30 μM) for 30 min are depicted in Fig. 1d. The probe showed a fast response rate and a large fluorescence enhancement. The fluorescence signal reached a plateau in about 15 min. But no obvious fluorescence signal change was observed with HOCl absent, which demonstrated that Cy-HOCl is stable in the reaction system. The results illustrated that our probe could respond to HOCl. The fast reaction kinetics are conducive to real-time detection in cells and *in vivo*.

3.4 Selectivity of probe for HOCl

We next tested the fluorescent response of Cy-HOCl to other physiologically related species in HEPES buffer solution (10 mM, pH 7.4). As illustrated in Fig. S1 (ESI[†]), the probe was incubated with various biospecies, such as *S*-nitroglutathione (GSNO), ONOO[−], NO (NOC-5), NO₂[−], tocopherols, H₂O₂, O₂^{•−}, methyl linoleate hydroperoxide, Na₂S₄ (a donor of H₂S_n), L-cysteine (L-cys), glutathione (GSH), NaHS, ascorbic acid, L-arginine (L-arg), tyrosine, hydroxylamine (HA) and HOCl. The fluorescence intensities were obtained. No obvious fluorescence signal changes were observed in the present of these interfering species without HOCl. Only HOCl offered a remarkable increase of fluorescence emission. Our results indicated that Cy-HOCl possessed excellent selectivity toward HOCl in the presence of various biologically relevant species under physiological conditions.

3.5 Fluorescence imaging of HOCl in living cells

Since Cy-HOCl showed strong ability in detecting HOCl under simulated physiological conditions, we next assessed whether Cy-HOCl was sensitive enough to monitor physiologically relevant levels of HOCl in living cells. Before that, it was inevitable to check its biocompatibility. Cy-HOCl showed low cytotoxicity toward LO2 human hepatocytes, as determined *via* MTT assays with IC₅₀ of 360 μM (Fig. S2, ESI[†]). The results showed that Cy-HOCl could be applied for long-time cell imaging.

We applied Cy-HOCl to estimate the changes of HOCl levels in cells. Before imaging, all cells were incubated with Cy-HOCl for 30 min, then washed with Dulbecco's Modified Eagle Medium (DMEM) 3 times. The intracellular fluorescence shown in Fig. 2a is very weak. If the cells were pretreated with NaClO to increase the exogenous HOCl, there was strong fluorescence, as seen in Fig. 2b. The results certified that our probe is adequate to detect exogenous HOCl directly in living cells. LO2 cells were stimulated with lipopolysaccharide (LPS) for 16 h to induce ROS (including HOCl) expression.^{45,46} As expected, obvious

Fig. 2 Fluorescence imaging and flow cytometry assay in living LO2 cells to detect exogenous and endogenous HOCl with Cy-HOCl (5 μM), after washing with DMEM to remove the redundant probe. Images display emission collected from 600 to 700 nm upon excitation at 543 nm. (a) LO2 cells incubated with Cy-HOCl as control. (b) LO2 cells incubated with Cy-HOCl manipulated as in (a), then treated with 50 μM NaClO for 15 min for imaging. (c) LO2 cells stimulated with 1 $\mu\text{g mL}^{-1}$ LPS for 16 h, then incubated with Cy-HOCl for 15 min for imaging.

fluorescence intensity was acquired (Fig. 2c). In addition, flow cytometry studies were performed to further confirm the results. These results indicated that our probe had potential to detect endogenous HOCl in living cells. Taken together, these data indicate that our probe Cy-HOCl is suitable to real-time monitoring of exogenous and endogenous HOCl in living cells.

3.6 Change of HOCl under hypoxia stress

Appropriate oxygen supply sustains normal cellular activities, while hypoxia results in a series of physiological dysfunctions. Hypoxic stress is accompanied by the apparent increase of lipid peroxides, oxygen radicals, and some non-radical derivatives of oxygen (O_2) such as hypochlorous acid (HOCl), which cause inactivation of proteins and consequently severe damage to cells. We performed assays in cells to explore how HOCl fluctuated under hypoxic stress. Next, we applied the probe to examine intracellular HOCl changes under different O_2 levels. LO2 cells in Fig. 3a were cultured under hypoxic conditions with 20%, 10%, 5%, 1%, and 0.1% O_2 for 4 h. Strong fluorescence images were obtained from cells cultured with 1% and 0.1% O_2 , while the fluorescence signal was obviously weaker under 10% and 20% O_2 . The degrees of hypoxia positively correlated with fluorescence intensities of Cy-HOCl, clarifying that the stress response of HOCl was strongly dependent on oxygen levels. Flow cytometry studies were performed to

Fig. 3 Confocal microscopy images and flow cytometry assay of LO2 cells for evaluating HOCl fluctuation under hypoxic stress. All cells were incubated with Cy-HOCl (5 μM) for 15 min, then washed with DMEM to remove excess probe. (a) Fluorescence imaging of LO2 cells under hypoxic conditions with 20%, 10%, 5%, 1%, and 0.1% O_2 for 4 h. (Emission from 600 to 700 nm upon excitation at 543 nm.) (b) Flow cytometry under the conditions in part (a). (c) Apoptosis analysis of LO2 cells under hypoxic stress. (Q1) Necrotic, (Q2) Late apoptosis, (Q3) Viable, (Q4) Early apoptosis.

confirm the results, seen in Fig. 3b. These data demonstrated that our probe could be applied to real-time detection of hypoxia-induced HOCl changes in living cells. The phenomena were attributed to cellular stress response caused by hypoxia. This redox imbalance causes cell apoptosis. To access the apoptosis, we performed PE Annexin V/7-AAD assay under different degrees of hypoxia. As indicated in Fig. 3c, the cells cultured under 20%, 10%, 5%, 1%, and 0.1% O_2 had apoptosis rates of 0%, 9.5%, 20.4%, 42.6% and 65.5%, respectively. The data revealed that hypoxia accelerated cell apoptosis.

3.7 Imaging HOCl in acute ischemia zebrafish model

Among the variety of animal models used to evaluate HOCl, zebrafish are an attractive model for biocompatibility, toxicity, biodistribution, teratogenicity, immune response, and long-term effect studies.^{47–49} Zebrafish is a vertebrate model, with 99% embryonic essential genes conserved in humans.⁵⁰ The ease of acquisition of zebrafish embryos from fertile adults, their small embryo size, and their optical transparency make it a suitable model for assessment of fluorescent dyes using standard imaging microscope.⁵¹ Novel fluorescent dyes can be applied to satisfy properties such as brightness and photostability of fluorescent signals detected through living tissues during whole embryo imaging.⁵² As mentioned above, however, no research has been reported to visualize HOCl in zebrafish under hypoxic stress during their early development. On the basis of the remarkable performance of probe Cy-HOCl, here we make such an attempt. We employed Cy-HOCl to examine the changes of HOCl in a zebrafish model under hypoxic stress. Zebrafish were grown in E3 embryo media under different hypoxic conditions of 20%, 10%, 5%, and 1% O_2 for 4 h. For fluorescence imaging experiments, zebrafish were incubated with 5 μM of Cy-HOCl in E3 embryo media for 30 min at 28 $^{\circ}\text{C}$, then washed with PBS (pH 7.4) to remove the remaining Cy-HOCl. Finally, we obtained the fluorescence images of zebrafish under hypoxic stress. As shown as Fig. 4, the results of the

Fig. 4 Fluorescence images for monitoring HOCl generation during hypoxic stress in zebrafish via Cy-HOCl. The zebrafish were pretreated with 20%, 10%, 5%, and 1% O₂ for 4 h, then incubated with Cy-HOCl (5 μM) for 30 min at 28 °C and washed with PBS (pH 7.4) to remove the remaining Cy-HOCl. The emission was collected from 600 to 700 nm upon excitation at 543 nm.

fluorescence intensities were all in the order of group 1% > 5% > 10% > 20% O₂. These results illustrated that HOCl should be rapidly released under hypoxic stress.

3.8 Imaging HOCl in acute ischemia mice model

Ischemia of organs blocks blood supply and results in deficiency of O₂, which affects the normal functions of cells and organs. The pathogenesis of acute ischemia involves various mechanisms, including excessive production of ROS. Once acute ischemia happens, these ROS irreversibly damage biomacromolecules, which leads to cell dysfunction. We next employed Cy-HOCl to examine the changes of HOCl in the mouse model of acute liver ischemia. First, the mice were intravenously injected with Cy-HOCl (5 μM, 50 μL in 1:9 DMSO/saline v/v) for 1 h. Then, the models were divided into five groups with different ligation times of portal vein and hepatic artery, 0 min (as control), 5 min, 15 min, 30 min, and 60 min, to induce acute liver ischemia. The mice without vessel ligation were set as control. As displayed in Fig. 5a, fluorescence images were obtained using this *in vivo* imaging system. As expected, the fluorescence signals of HOCl rapidly increased with the extension of liver ischemia time and saturated at the time point of 60 min. The result demonstrated that HOCl is steeply released during acute liver ischemia. The changes of pathological morphology of liver

Fig. 5 Evaluation of HOCl in mouse model of acute liver ischemia via Cy-HOCl within 60 min. The mice in each group were intravenously injected with Cy-HOCl (5 μM, 50 μL in 1:9 DMSO/saline v/v) for 1 h prior to testing. The emission was collected from 600 to 700 nm upon excitation at 543 nm. (a) The fluorescence images of acute liver ischemia mice with different ligation times. (b and c) H&E sections and TUNEL staining of liver tissue. (d) Fluorescence images of liver tissue section by laser scanning confocal microscope. (e) The details of the portal vein and hepatic artery ligation. (f) The fluorescence images of isolated organs in acute liver ischemia mice (heart, liver, spleen, lung, and kidney). (g) Mean fluorescence intensities of Cy-HOCl in livers of (a). Data are presented as mean ± SD (*n* = 5).

tissue were checked using H&E sections (Fig. 5b) and TUNEL staining (Fig. 5c). As ligation time lengthened, the HOCl fluorescence imaging of liver tissue sections showed the enhanced fluorescence signal in Fig. 5d. The details of the organ ligation are shown in Fig. 5e. As seen in Fig. 5f, we obtained gradual enhancement of fluorescence signal over time in isolated liver tissue. The corresponding quantitative fluorescence data are shown in Fig. 5g. Over all, the high sensitivity and rapid response time made our probe a possible chemical tool for real-time detection of changes of HOCl in an acute liver ischemic mouse model.

4. Conclusions

In summary, we synthesized a NIR fluorescent probe, Cy-HOCl, for HOCl detection in living cells and *in vivo* under hypoxic stress. The probe is sensitive and biocompatible, allowing fluorescence imaging of exogenous and endogenous HOCl in living cells. We detected the changes of HOCl in living cells under hypoxic stress, as well as in zebrafish under different hypoxic conditions. Acute liver ischemia models of mice were established to evaluate the biological effects of HOCl under hypoxia stress. H&E coloring, TUNEL coloring and fluorescence imaging of liver tissue of hypoxic mice were acquired to observe the liver damage and change of HOCl. All the results revealed that HOCl increases under hypoxic stress both in living cells and *in vivo* and hypoxic stress can injure cells and tissue from oxidative damage. Our research provides evidence that HOCl may be a novel factor for hypoxia-induced injury. The new probe offers a useful tool to better detect the physiological and pathological bio-roles of HOCl in cells and *in vivo*.

Conflicts of interest

There are no conflicts to declare.

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