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Antibacterial activity of erythrocyte from grass carp (*Ctenopharyngodon idella*) is associated with phagocytosis and reactive oxygen species generation

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ABSTRACT

Red blood cells (RBCs) are widely accepted as their primary function in respiration. Recent studies in mammals have revealed a vital role in immune responses of RBCs; however, little is known about immune function of teleost erythrocytes. Here we demonstrated that RBCs from grass carp (*Ctenopharyngodon idella*) were capable of binding and aggregating the bacteria with apparent morphological alterations. The phagocytosis by teleost RBCs (erythrophagocytosis) was visualized by confocal, scanning and transmission electron microscopy. Hb-Fe^{II} of hemoglobin (Hb) could quickly be auto-oxidated to Hb-Fe^{III} (methemoglobin/metHb) in the presence of oxygen (O_2), and release superoxide radical ($O_2^{-\cdot}$) which could be spontaneously dismutated into H_2O_2 that could further oxidize Hb-Fe^{III} to transient $HbFe^{IV}$-OH (ferryl-Hb). Furthermore, bacterial extracellular proteases and pathogen-associated molecular patterns (PAMPs) binding to Hb could synergistically activate pseudoperoxidase, subsequently facilitated the generation of reactive oxygen species (ROS) which were toxic to the bacteria. Our results indicated that erythrocyte pertains anti-bacterial activity using unique ROS generation pathway via oxidation of hemoglobin and associated with its phagocytosis.

1. Introduction

Pathogen and host coevolve during the long evolutionary history and developed many strategies to fight against each other. For instance, pathogenic microorganisms have encoded various virulence factors that facilitate the invasion and replication in their hosts [1,2]. On the other hand, hosts have gained various immune defense capabilities against pathogen infections, that including highly toxic reactive oxygen species (ROS, such as superoxide anion $O_2^{\cdot-}$) and reactive nitrogen species (RNS, such as nitric oxide NO$^{\cdot}$) [3,4]. It is well-known that microbicidal mechanisms of macrophages comprise of the activation of NADPH oxidase and inducible nitric oxide synthase (iNOS) in facilitating the ROS and RNS synthesis, respectively. The NADPH oxidase consists of five subunits, including two membrane proteins ($p91^{phox}$ and $p22^{phox}$) and three cytosolic proteins ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), different subunit plays dissimilar roles in the generation of ROS [5]. The activated cytosolic proteins ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) can move to the cellular membrane where they make a complex with two membrane proteins ($p91^{phox}$ and $p22^{phox}$), so as to generate ROS [5].

Red blood cells (RBCs) are well-known for their major function in exchange of gases during respiration. However, RBCs are also found to participate in entrapping and killing the invaded pathogens [6–8]. Concerning the antimicrobial mechanisms of human RBCs, previous studies indicated that oxyhemoglobin (Hb-Fe^{II}) of hemoglobin (Hb) could quickly be auto-oxidated to Hb-Fe^{III} (metHb/methemoglobin) in the presence of oxygen (O_2), and release superoxide radical ($O_2^{-\cdot}$), which can be spontaneously dismutated into H_2O_2. Consequently, Hb-Fe^{III} could be oxidized to transient ferryl-Hb ($HbFe^{IV}$-OH) while

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exposure to H_2O_2. Further, the transient $HbFe^{IV}$-OH can be reduced to Hb-Fe^{III} by pseudoperoxidase (POX) and that generates $HO_2^{\cdot}$ [9,10]. The reactive oxygen species ($O_2^{-\cdot}$, H_2O_2, $HO_2^{\cdot}$) generated during the Hb oxidative process are toxic to the invaded pathogens [9–12]. Besides, microbial proteases (not the human proteases), such as proteinase K from *Tritirachium album* and Subtilisin A from *Bacillus licheniformis* were able to activate the antimicrobial activity of human Hb [9,12]. The pathogen-associated molecular patterns (PAMPs) like LPS from the gram-positive bacteria could bind to human Hb and elicits a broad spectrum of antimicrobial ROS to kill the invaded pathogens [9]. Although, the antimicrobial activity of RBCs has been identified and reported in human RBCs, little information is available in regards to the underlying antimicrobial mechanism of piscine RBCs.

Aquaculture is a key sector of the global food system and makes a significant contribution to the production of protein-rich food for human beings [13]. Grass carp (*Ctenopharygodon idella*) is one of the commonly cultured fish worldwide [14,15]. Conversely, the bacterial diseases outbreaks have caused devastating economic loss in grass carp culture. Usually, in fish RBCs composed of 95% of total peripheral blood cells, however, there is still no conclusive evidence to validate that teleost RBCs in mediating the immune response in combating against the infectious disease.

Therefore, we sought to investigate the underlying antimicrobial mechanisms of the teleost RBCs using grass carp as a model. To accomplish this goal, we applied various molecular and microscopical approaches to analyze the role of teleost RBCs and its antimicrobial mechanisms against antigens. The results gained from the present study will shed new light on the erythrocyte fish immune mechanisms and pave the way for developing new effective strategies against fish diseases.

2. Materials and methods

2.1. Biologicals and reagents

Grass carp (100–250 g) juveniles were obtained from a fish farm located in Hubei Province, China, and maintained at 25–26 °C in a recirculating freshwater system for at least 2 weeks acclimation to the laboratory condition before carrying out experiments. *Aeromonas hydrophila*, *Staphylococcus aureus,* and *Escherichia coli* were maintained and cultured according to the routine protocols in our laboratory [16]. Fluorescein isothiocyanate (FITC) conjugated rabbit IgG was purchased from the Guge Company (China), secondary antibody donkey anti-rabbit IgG antibody was purchased from Gene Co., LTD. (Shanghai, China). Lipopolysaccharide (LPS; from *E. coli* serotype 055: B5), Lipoteichoic acid (LTA; from *S. aureus*), peptidoglycan (PGN; from *Micrococcus luteus*), Subtilisin A (from *Bacillus licheniformis*) and latex beads were obtained from Sigma and Proteinase K (from *Tritirachium album*) from ComWin Biotech.

2.2. Isolation and purification of RBCs

The grass carp RBCs were isolated and purified as described previously with minor modification [17]. Briefly, blood samples were collected from caudal vein using heparinized syringes and mixed with 0.7% buffer saline (0.7 g NaCl in 100 ml H_2O) containing heparin sodium (0.1 mg/ml). After washing with 0.7% buffer saline by centrifugation, the cell suspension was layered onto a 34%/51% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient and centrifuged at 400 g for 30 min at 4 °C. The RBC pellets were collected and washed three times and finally resuspended with 0.7% buffer saline.

2.3. Bacterial adhesion assay

An aliquot of purified RBCs (1×10^6 cells/ml) was incubated with either FITC-labeled *S. aureus* or *Green fluorescent protein*-expressing *E. coli* (GFP-*E. coli*) in 1:10 ratio for 30 min under constant rotation at room temperature (RT). Later, the suspensions were washed with sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) for four times to remove unattached bacteria and observed under fluorescent microscopy (Leica, Germany). Another aliquot of RBCs (1×10^6 cells/ml) was incubated with *S. aureus* (1×10^7 CFU/ml) as described above and scanning electron microscope (SEM) images were obtained in a Hitachi SU8010 SEM (Hitachi, Japan).

2.4. Phagocytic activity assay

To evaluate the phagocytic ability of the teleost RBCs, 5×10^6 cells/ml in individual tubes were incubated with the various bacteria or latex beads at 1:10 ratio. Following the incubation for 1 h at 28 °C, the cells were washed four times with PBS and the phagocytosis of RBCs was evaluate in four different approaches, viz. Group A, RBCs incubated with *S. aureus* was observed under a light microscope; Group B and C, RBCs incubated with either *S. aureus* or latex beads (0.5 μm in diameter) were observed under SEM and transmission electron microscopy (TEM, H-7000FA, Hitachi, Japan), respectively; Group D, RBCs incubated with FITC-conjugated *A. hydrophila*, *S. aureus,* and GFP-*E. coli* were further nuclei stained with 500 μl of 4',6-Diamidino-2-phenylindole (DAPI, Guge Company) and observed under a confocal microscope (LSM 510 META, Carl Zeiss AG, Germany).

2.5. Bactericidal activity of ROS generated from RBCs

The hemolysis assay was performed as described previously, with minor modifications [11]. Briefly, 1 ml of saline-washed RBCs (5×10^6 cells/ml) were incubated with *A. hydrophila* (10^7 CFU/ml) at RT and the supernatants were collected at every 10 min after centrifugation at $1000 \times g$ for 5 min. The Hb contents released from the hemolysed RBCs were quantified spectrophotometrically at a wavelength of 404 nm using a microplate reader (Molecular Devices, USA).

Antimicrobial activity of the RBCs was analyzed via the methods previously described with minor modification [9]. Briefly, every 1 ml of RBCs (5×10^6 cells/ml) were incubated with *S. aureus, A. hydrophila,* and *E. coli* for 60 min at 28 °C. Thereafter, the infected cells were spread on the nutrient agar plates after 10 fold serial dilution using sterile saline and incubated overnight at 28 °C. The bacterial survival was determined by counting the bacterial colonies on the agar plate.

The superoxide anions produced by RBCs were measured and monitored by cypridina luciferin analog, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo [1,2-alpha] pyrazin-3-one (MCLA) as chemiluminescence (CL) probe (Tokyo Chemical Industry Co., Tokyo, Japan) as previously described, with minor modification [9]. Briefly, 200 μl of RBCs (5×10^6 cells/ml) were incubated with or without bacteria (*S. aureus, A. hydrophila,* and *E. coli*, 10^5 CFU/ml) for 30 min, then mixed with MCLA at 20 μM final concentration and immediately measured using a Single Tube Multimode reader (GloMax Multi Jr). The levels of ferryl-Hb were detected after adding 2 mM sodium sulfide (Na_2S) to the 200 μl of RBCs mixtures at 620 nm wavelength using a microplate reader (Molecular Devices, USA).

2.6. Purification of hemoglobin (Hb) from RBCs

The Hb purification was performed according to the protocol described previously, with minor modification [18]. Briefly, the purified RBCs was frozen at −80 °C and thawed at RT twice, followed by centrifugation at $10{,}000 \times g$ for 30 min. The supernatant was collected and analyzed for the purity by standard Bradford assay kit (Beyotime Biotechnology, Beijing, China) and SDS-PAGE method. The purified Hb was stored at −80 °C, until use.

2.7. Oxidization of Hb

The oxidization kinetics of grass carp Hb was performed as described previously [12]. Briefly, the purified Hb solution (0.5 mg/ml) pH was adjusted to 6.0 using NaH_2PO_4 (50 mM stock solution) and exposed to air at 28 °C for 30 min and continued to oxidize in the same condition at 1, 2, 4, 8 and 12 days, to allow autoxidation. Then the absorbance of the Hb solutions were measured from 350 to 700 nm with a spectrophotometer (Tecan, Austria). To determine whether grass carp fish metHb-Fe^{III} can be oxidized to ferryl-Hb. The Hb solution (200 μl) in 96-well plate were treated with 0 (control), 0.01, 0.05, 0.1, 0.5, 1 and 2 μmol of H_2O_2 for 5 min, and subjected to spectrophotometric measurement by scanning between 350 and 700 nm at an interval of 1s.

2.8. Proteases coordinate the structural alteration of metHb

The structural alterations of the metHb were determined using protease enzymes as described previously [9]. Based on our pilot study, 200 μl of the metHb (0.5 mg/ml) solutions in 96 wells were individually mixed with 10 ng of proteinase K or subtilisin A at the optimal ratio of 10:1 (μg: ng) and treated with 0.8 μmol H_2O_2. The structural alterations of each mixtures were measured for every 10 min time intervals at the wavelength range of 500–700 nm using a microplate reader (Molecular Devices, USA). To determine whether the metHb-Fe^{III} can be oxidized to ferry-Hb-Fe^{IV}, 2 mM sodium sulfide was added into above reaction mixture as described previously [19], and the spectral changes were monitored at 620 nm wavelength. The superoxide produced from metHb was measured by using the MCLA assay as previously described, with a minor modification [9]. Briefly, 20 μM MCLA and 10 mM H_2O_2 in PBS (pH 7.4) were added to 200 μl of metHb (0.5 mg/ml). The superoxide produced were immediately analyzed in a Single Tube Multimode reader (GloMax Multi Jr) and data presented as fold increase.

The effect of PAMPs on the proteolytic activation of metHb was tested using proteinase K, subtilisin A, and LPS as described for 30 min [9], with molar ratios of LPS: metHb at 4:1 to 1:1 and subjected to Tris-Tricine SDS-PAGE analysis.

2.9. The antimicrobial activity of ROS generated from metHb

The antimicrobial assay was performed to detect the bactericidal activity of the ROS produced as previously described, with minor modification [9]. Briefly, *A. hydrophila*, *S. aureus,* and *E. coli* (10^5 CFU/ml) were added individually to the suspension of 200 μl of metHb with 0.8 μmol H_2O_2 for 60 min at 28 °C. Subsequently, 50 U of superoxide dismutase (SOD) was added to specifically quench the superoxide anions. The samples were pour-plated on nutrient agar plates after 10 fold serial dilution in PBS and incubated at 37 °C overnight to count the survival bacteria.

2.10. Binding of MetHb with microbes

The binding assay was performed to distinguish the adherence ability of MetHb to microbes according to a previously described protocol [16]. Briefly, the test bacteria *A. hydrophila*, *S. aureus,* and *E. coli* were gathered by centrifugation at for 10 min at 6000 × *g*, washed thrice and resuspended in PBS buffer to a final concentration of 10^8 CFU/ml. An aliquot (1 ml) of each bacterial species alive and another batch of aliquot were killed by incubating at 65 °C for 1 h. The metHb at a final concentration of 0.5 mg/mL was added respectively to both alive and killed bacterial tubes and incubated for 60 min at 28 °C. After incubation, the reaction mixtures were washed 4 times with PBS and collected by centrifugation for Western blot analysis. Briefly, following the membrane transfer of proteins by SDS-PAGE, the membranes were blocked in TBST containing 5% skim milk at RT for 1 h and continued incubation with 100 μl of anti-grass carp Hb rabbit serum (1:1000 diluted) at 4 °C for overnight. After washing 3 times with TBST, the membranes were incubated with 100 μl of secondary antibody infrared dye-linked goat anti-rabbit IgG antibody (1:10,000 diluted) at RT for 1 h. The signals were visualized by Odyssey CLx (LI-COR, Inc., USA).

2.11. ELISA-based binding assay

The analysis of polysaccharide and the bacterial binding were performed by Enzyme-linked immunosorbent assay (ELISA) as described previously, with minor modification [20]. Briefly, a 96-well microtiter plate was coated with 100 μl of TBS appropriately containing 20 μg of LPS, LTA, PGN or 10^7 CFU/ml of bacteria (*S. aureus, A. hydrophila,* and *E. coli*) at RT for overnight and extended for 1h at 60 °C to fix the ligands. Then, the plates were blocked with 200 μl of TBS containing goat serum (Boster biological technology, China) at 37 °C for 2 h. Subsequently, 100 μl of metHb (from 4 ng to 8 μg in TBS) was added to each well and further incubated at RT for 3 h. Later, 100 μl of rabbit anti-grass carp Hb antibody (1:5000 diluted in TBS) was added to each well and incubated at RT for 3 h. Finally, the wells were added with 100 μl of HRP conjugated goat anti-rabbit IgG (diluted 10,000 times in TBS) and incubated at 37 °C for 3 h. After that, 200 μl of color development solution (Beyotime Biotechnology, Beijing, China) was added to each well and incubated at RT for 15 min. During each step, the plate was washed by TBST four times. To stop the reaction, 50 μl of 2 M H_2SO_4 was added to each well and the absorbance was measured using an ELISA plate reader at 450 nm. Wells without bacteria was used as the negative control. The ELISA results were obtained from two independent experiments conducted in triplicates.

2.12. Statistical analysis

All data were presented as mean ± SE. The statistical significance was assessed by one-way analysis of variance (ANOVA) using SPSS 17.0, followed by the least significant difference (LSD) post-hoc test to compare individual groups. $P < 0.05$ considered as significant while $P < 0.01$ as an extremely significant difference.

3. Results

3.1. The adhesion of bacteria to the teleost erythrocytes

To detect the ability of the RBCs grass carp to attach and seize the bacteria, we analyzed the RBCs incubated with FITC-labeled *S. aureus* and GFP-labeled *E. coli* by fluorescent microscope. Numerous green fluorescence signals were recorded over the surface of the individual RBC cells incubated with both bacteria (Fig. 1A). Furthermore, SEM analysis of the RBCs incubated with *S. aureus* showed that the bacteria had tightly bound to RBCs and formed distinct convex vesicle structures over the surface of the RBC (Fig. 1B1). RBC surface-based interaction on the bacteria could be widely seen with the formation of multiple small projections stretching out like tentacles and capturing the group of *S. aureus* (Fig. 1B2-4). These images confirmed that the teleost RBCs potentially have the capability to clutch the trapped bacteria.

3.2. Phagocytic activity of teleost RBCs

To test the phagocytic potential of teleost RBCs, FITC-labeled bacteria: *A. hydrophila*, *S. aureus,* and *GFP-E. coli* were individually incubated with the RBCs. Confocal microscopy photomicrographs revealed that the RBCs had engulfed a bunch of all the three tested bacteria and localized inside the cytoplasm close to the nucleus (Fig. 2A), and the internalization of *S. aureus* could be observed clearly under the optical microscope (Fig. 2Ba). To better characterize the phagocytosis of grass carp RBC, we performed SEM analysis to the RBCs incubated with *S. aureus.* We could notice that some bunches of *S. aureus* have adhered onto the surface of the single RBC, and formed invagination on the RBC cells surface with the partially engulfed

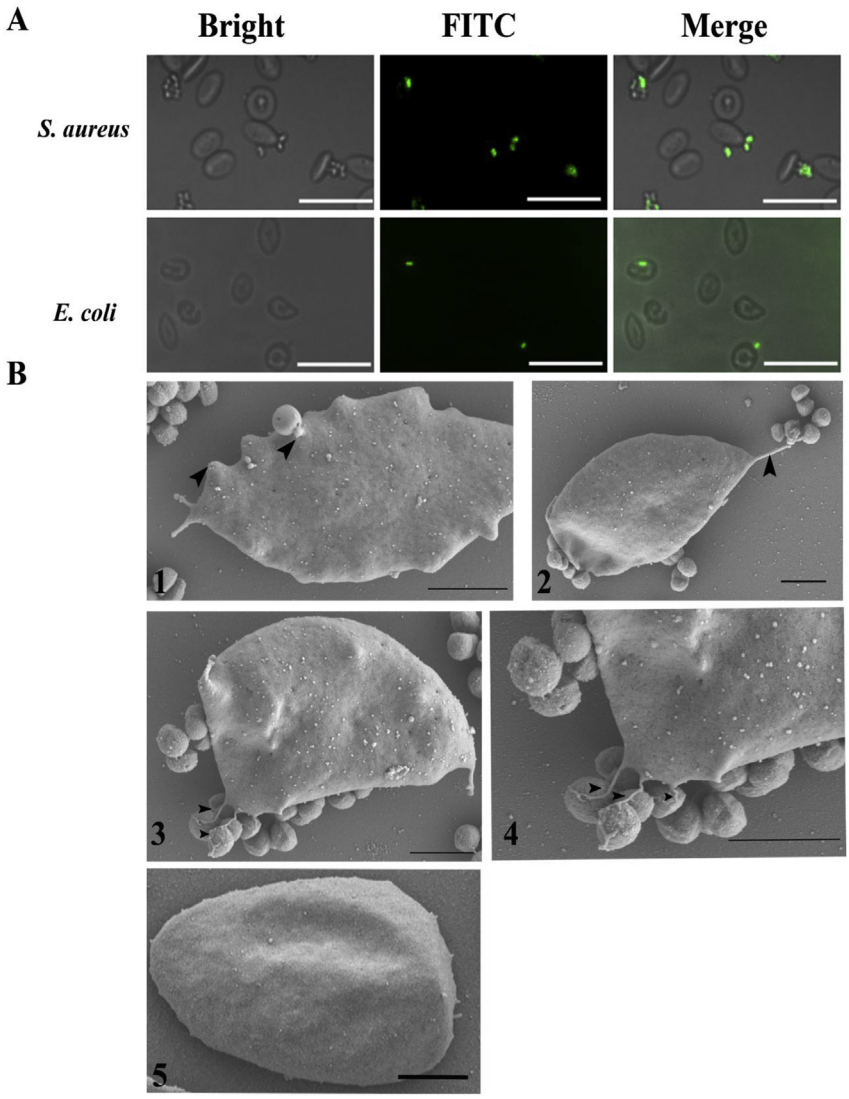

Fig. 1. The cell surface of erythrocytes with bacterial adherence. **(A)** Fluorescence microscopy images of FITC-labeled *S. aureus* and GFP-*E. coli* adhered to the cell surface of erythrocytes. The ruler represents 25 μm. **(B)** Scanning electron micrograph of grass carp erythrocyte used to detect the adhesion of *S. aureus*; **(B1)** The representative erythrocytes was induced to form some swelling or depression (black arrow) induced by *S. aureus.* **(B2)** The red blood cells capture the bacteria by forming a pili-like filaments (black arrow). **(B3)** Erythrocytes formed multiple filaments to capture several bacteria (arrowheads), protruding extracellularly in close proximity, and the bacterial clusters could be seen around the surface. **(B4)** Partial magnification of B3. **(B5)** Normal uninfected grass carp erythrocyte. The ruler represents 2.0 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bacteria which might be further consumed by RBCs (Fig. 2Bb). To further confirm the phagocytic activity, we incubated the RBCs with the latex beads and examined under TEM microscope. It was apparent that the beads were also visualized inside of the RBCs (Fig. 2Bc). These data suggest that the teleost RBCs have the ability to bind to various bacteria and latex beads by adjusting their shapes.

3.3. RBCs produce bactericidal ROS

To demonstrate the ability of teleost RBCs to produce bactericidal ROS, we incubated the *A. hydrophila* with RBCs and the Hb concentration that released was measured at regular time intervals. The spectrophotometric profiles revealed that the magnitude of Hb from the RBCs gradually increased in a timely manner indicating that the RBCs have been suffered in hemolysis (Fig. 3A). Therefore, we asked whether Hb released from the RBCs could activate and affect the survival of bacteria. To determine the effects of Hb on bacteria, we incubated RBCs with *A. hydrophila, E. coli,* and *S. aureus* for 60 min, the survival rates of three bacteria were significantly decreased to about 40% for *E. coli* and approximately 25% for both *A. hydrophila* and *S. aureus*, indicating that the Hb released from teleost RBCs had a strong antibacterial activity (Fig. 3B). In addition, we quantified the levels of ROS and ferryl-Hb-Fe^{IV} which are normally responsible for the bactericidal activity by RBCs. We found that the luminescence signals had been significantly increased in both ROS and ferryl-Hb-Fe^{IV} of the RBCs (Fig. 3C and D), indicating that ferryl-Hb-Fe^{IV} and ROS might involve in the antibacterial activity.

3.4. The oxidation of Hb

To determine whether the Hb could undergo structural alteration during oxidation and surge in the production of ferryl-Hb and free radicals. We purified the grass carp Hb from the RBCs and analyzed its purity using 15% of Tris-Tricine SDS-PAGE stained with Coomassie-blue. The results showed a major band of around 14 kDa which was identical to the molecular weight of Hb (Fig. 4A). Subsequently, we analyzed the oxidation reactions of the purified grass carp Hb molecule and turnover of oxidation intermediates by autoxidation or induced by exposing to H_2O_2 with the spectrum analysis. The typical spectral profiles of the Human Hb for Hb-Fe^{II} was 544 nm and 577 nm, and for metHb-Fe^{III} was 407 nm and 630 nm. As shown in Fig. 4B, a major peak at 407 nm (Hb-Fe^{III} spectrum) was observed and two smaller peaks at 544 nm and 577 nm (Hb-Fe^{II} spectrum) in grass carp Hb, indicating that metHb-Fe^{II} was quickly oxidized to metHb-Fe^{III} within 30 min of exposure. Likewise, Hb molecule exposed to atmospheric air for different day periods, where the peak for metHb-Fe^{III} 630 nm could not be seen and 407 nm was lesser at day 0. Then gradually increasing profiles of metHb-Fe^{III} from day 1–2 and so on at day 4 and reached its extreme

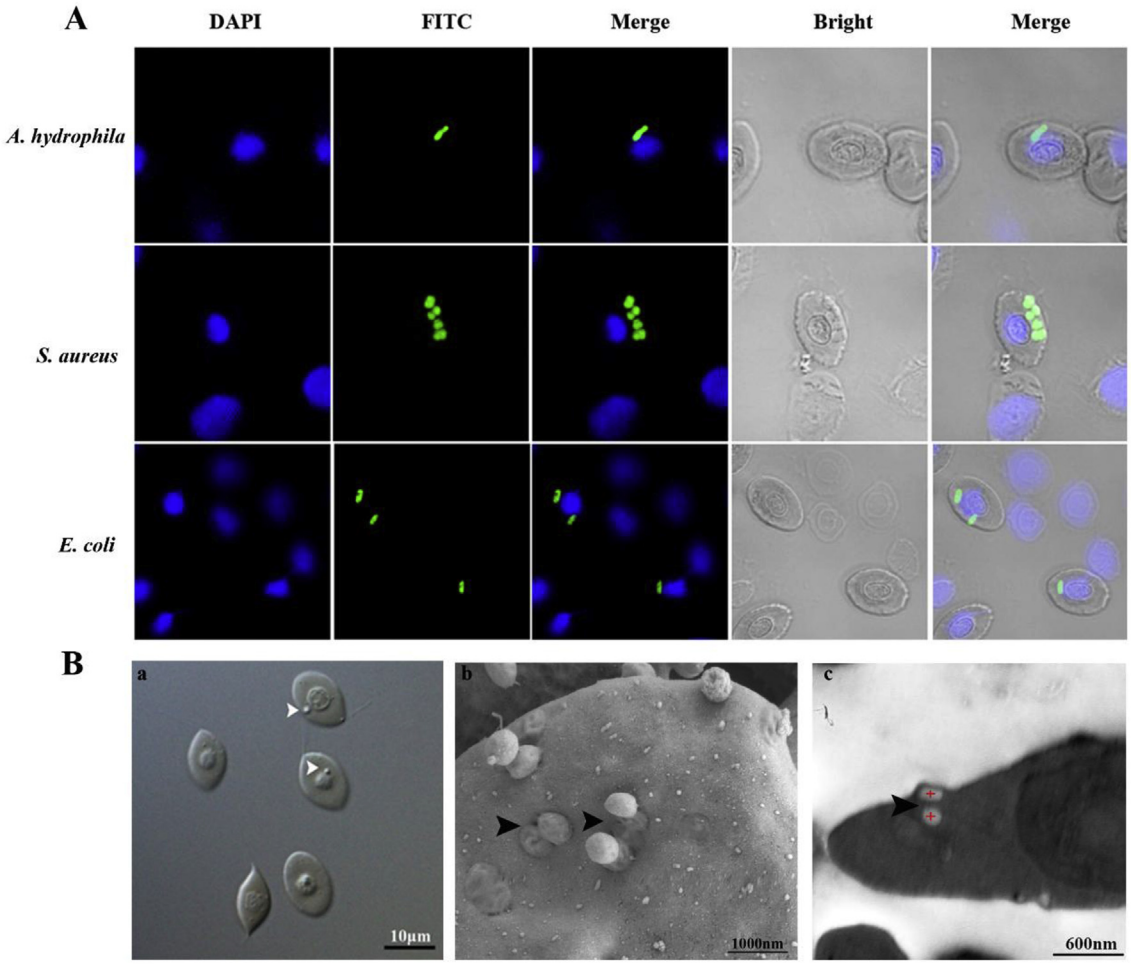

Fig. 2. Phagocytosis by RBCs. **(A)** Immunofluorescence confocal microscopic images show erythrocyte ingested with FITC-labeled bacteria: *A. hydrophila*, *S. aureus,* and GFP-*E. coli.* DAPI fluorochrome marked the nucleus of RBCs. **(Ba)** Optical microscopic analysis of phagocytic RBCs, the white arrow shows the internalized of *S. aureus*, × 100. **(Bb)** Scanning electron micrograph of RBCs incubated with *S. aureus,* the black arrow shows the *S. aureus* was in the process of phagocytoses. **(Bc)** Transmission electron micrograph shows the RBC incubated with 0.5 μm diameter beads, two beads adhered to the surface (marked with red crosses). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spectrum level on day 12. Besides, we could also see the decreasing peak patterns at 544 and 577 nm (Hb-Fe^{II} spectrum) and completely disappeared after day 8 (Fig. 4C). Exposure of the Human $HbFe^{III}$ (metHb) to H_2O_2 further catalyzed into $HbFe^{IV}$(ferryl-Hb) thru pseudoperoxidase (POX) catalytic cycle [9]. To test this in grass carp Hb, we treated metHb-Fe^{III} molecule with different molar concentrations of H_2O_2 for 5 min and the spectral analysis data showed the characteristic absorbance peaks proportional to the increasing concentration of the H_2O_2 from 0.5 μM and completely oxidized at higher levels, and little changes were observed in control or at low concentrations of H_2O_2 (Fig. 4D). Overall, the above evidence suggests that grass carp Hb proteins could be able to completely autoxidized when exposure to air or induced by H_2O_2 by generating more harmful free radicals during these oxidation processes.

3.5. Proteases coordinate the structural alteration of metHb

It has been reported that microbial proteases and PAMPs could structurally alter the Hb molecule [9,19]. To test this mechanism in purified grass carp metHb, we treated 100 μg metHb with microbial proteases subtilisin A and proteinase K, and the spectral changes were monitored. The binding of metHb to the proteases under the effect of H_2O_2 stimulated the decomposition immediately and continued to increase as time extends in both the protease treated samples, as evidenced by the characteristic peak change in the spectra, compared to the metHb treated alone with H_2O_2 (Fig. 5A–C). However, the microbial proteases disintegrated the metHb that compelled us to find out that the breakdown of metHb was productive for the cells. Therefore, we verified the disintegration and conversion of metHb by using sodium sulfide, and the free radicals that produce during the breakdown process by MCLA assay. Our results showed that both the microbial proteases had equally transformed metHb into transient ferryl-Hb with significantly increasing the conversion ratio (Fig. 5D). Analysis of the free radicals data indicated that addition of microbial proteases significantly increased the superoxide production, comparatively subtilisin A produced more than proteinase K (Fig. 5E). Besides, we also analyzed the binding ability of grass carp metHb to the PAMPs (LPS, LTA, and PGN) that originated from the various bacterial origin by ELISA assay. As shown in Fig. 5F, each of the microbial PAMP could able to bound to metHb, among them LPS was found to have the high affinity. These results indicated that the bacterial proteases can alter the oxidative structure of metHb and buildup ROS production.

It is already verified that the horseshoe crab and human respiratory proteins specifically elicits an antimicrobial activity by microbial proteases that enhanced by microbial virulence factors, such as LPS and proteases [11]. Based on the above studies, we validated the proteolytic profile and the superoxide production levels of grass carp metHb with two combinations of PAMPs, in particular, LPS with proteinase K and

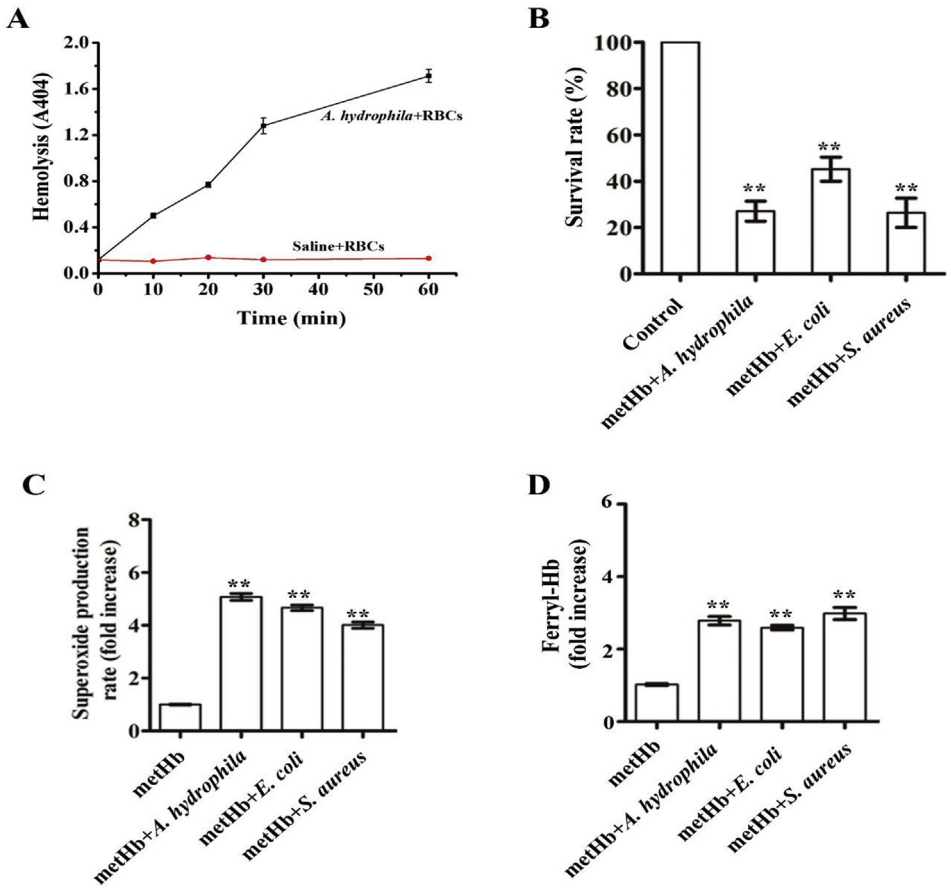

Fig. 3. ROS produced by red blood cells kill microbials. **(A)** Hemolysis assay of RBCs incubated with *A. hydrophila* analyzed at different time intervals. Black line represents treated by *A. hydrophila*, the red line represents treated by saline. **(B)** Survival chart of *A. hydrophila, S. aureus* and *E. coli* (10^5 CFU/ml) after incubated with 5×10^6 RBCs for 1 h. The bacteria in saline without any treatment as control. **(C)** The RBCs incubated with *A. hydrophila* , *E. coli* and *S. aureus* (10^5 CFU/ml) produced superoxide which determined by MCLA-CL assay, showed a significant increase of superoxide production based on bacterial concentration. **(D)** The quantity of Ferryl-Hb produced by bacteria stimulation which was detected at 620 nm by the addition of 2 mM sodium sulfide (Na_2S). *, $P < 0.05$; **, $P < 0.01$. Data represent the mean ± S.E. of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

LPS through subtilisin A in various sequential orders. We noted that there was no dosage effect of LPS when treated alone with metHb even on higher concentration. Though both the microbial protease, proteinase K and subtilisin A could able to act solitarily on metHb. However, the potential combination of proteases with LPS displayed an increasing proteolytic profile of metHb upon increasing the LPS concentration (Fig. 5G). Simultaneously, we measured the extracellular superoxide anion levels that produced during the metHb catalysis by MCLA-CL assay and the effects were consistent with the protein profiles, where the superoxide production levels were significantly reduced in the proteinase K treatment than subtilisin A (Fig. 5H). The above studies imply the potential role of PAMPs that act as a catalyst over the microbial proteases and metHb mixtures in inducing the proteolysis and localized superoxide production.

3.6. Bactericidal activity of ROS generated by MetHb

It has been reported that metHb is activated by microbial cellular factors and succumb to catabolize into transient ferryl-Hb by elevating the production of superoxide radicals that could cause a deleterious effect to microbes [5,8,9,11,21]. To demonstrate and to clarify the ability of grass carp metHb whether the ROS generated from MetHb has the ability to kill bacteria, we incubated metHb with *A. hydrophila, E. coli,* and *S. aureus* individually for 60 min and detected formation of transient ferryl-Hb and the release of superoxide radicals. Spectrophotometry analysis distinguished a significant increase in the formation of ferryl-Hb on all the three bacteria tested (Fig. 6A). Simultaneously, production of the superoxide radicals was significantly increased more than three folds of the metHb alone in all the three bacterial treatments (Fig. 6B). To detect the ability and specificity of metHb to the bind with the different bacterial pathogens, we have incubated *A. hydrophila, E. coli,* and *S. aureus* with metHb at gradient concentration. The ELISA assay exposed that the metHb has the capability to bind with both gram-positive and gram-negative bacteria without any significant differences (Fig. 6C). The binding ability of metHb to the bacteria prompted us to analyze whether this skill is only specific for bacteria or for the microbial cellular factors. Therefore, we heat killed the three above mentioned bacteria and incubated with a metHb in alive and killed form, then the cellular extracts were electrophoresed. Western blot showed that the metHb has the ability to attach all the three bacteria either it's dead or alive, however the binding was stronger in the alive bacteria when compared to the dead one (Fig. 6D).

To recognize that both the binding ability and the production of bactericidal superoxide metabolite from the metHb, we carried out the antibacterial assay on *E. coli, A. hydrophila,* and *S. aureus* for 1 h with or without superoxide dismutase (SOD). Based on the data, we found that metHb showed a powerful antibacterial activity against all the three bacteria by killing nearly 80% of *A. hydrophila* and *S. aureus,* and about 60% of *E. coli.* To influence the bacterial survival, SOD was added to quench the superoxide release from metHb during oxidation. However, there was a slight death was recorded due to the effect of superoxide metabolite (Fig. 6E). These results indicate that the grass carp fish hemoglobin molecules have intrinsic potential toxicity factors, which resulted in significant upregulation of ROS when alerted by specific pathogens.

4. Discussion

Since, from the discovery of phagocytosis in 1882 [21,22], the ability to phagocytosis of large foreign bodies or invaded microorganism regulated by host cell types that including polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes but there were no considerable reports on erythrocytes. It is well known that

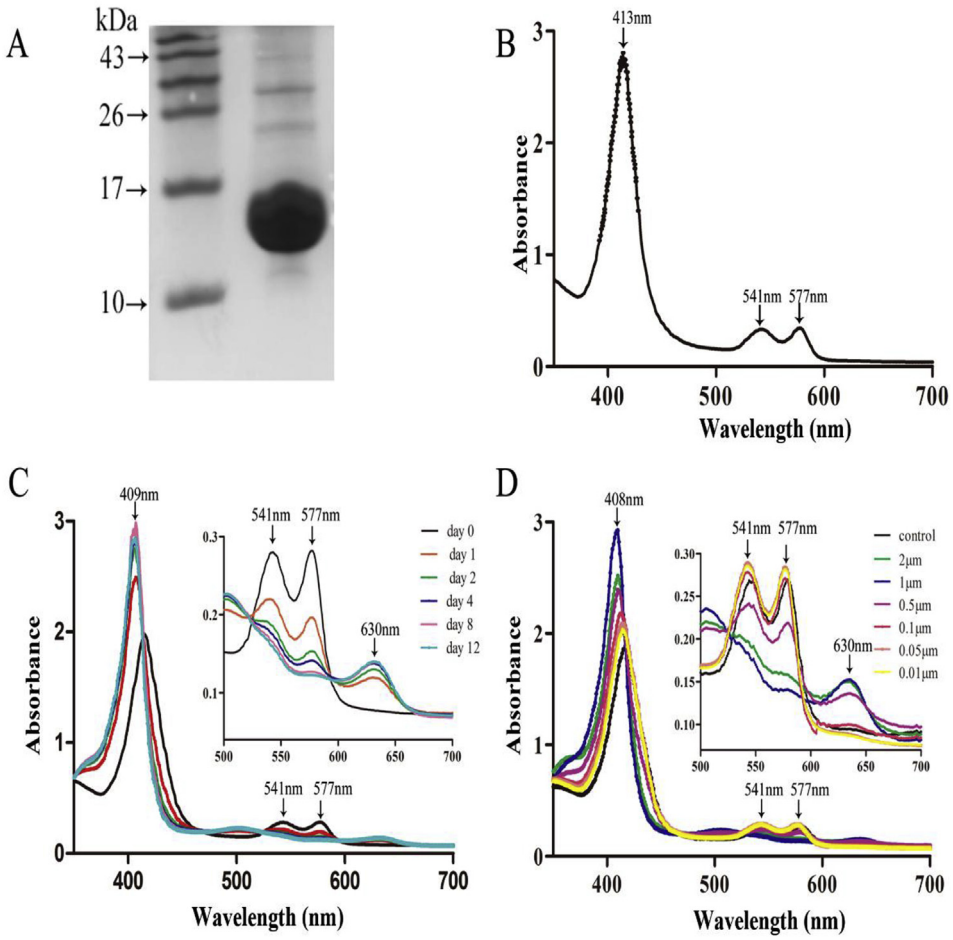

Fig. 4. Oxidation of Hb to metHb. **(A)** Purified hemoglobin from RBCs, was resolved by 15% reducing Tris-Tricine SDS-PAGE, the hemoglobin sample contains a major band around in 14 kDa. **(B)** Hemoglobin kept open at 28 °C for 30 min for its autoxidation, the arrows indicate the characteristic absorption peak of metHb. **(C)** Hemoglobin kept open at 28 °C continued for 12 days, then detected the change of spectrum curve, the arrows indicate the characteristic absorption peak of metHb.**(D)** Hemoglobin was added with different concentration of H_2O_2 for 5 min then the spectroscopic carried out to analyze the change of spectrum curve, the arrows indicate the characteristic absorption peak of metHb.

leukocytes play a key role in innate immune response, which are central regulators of immune responses and one of the most active secretory cell types in the body [5,22]. In contrast to leukocytes, the erythrocytes are generally accepted as their main function is in respiration [21]. Interestingly, in 1953, Nelson had observed that the human erythrocytes had also participated in the immune responses [6]. Contemporary studies have revealed that RBCs are also involved in the immune system, modulating T cell proliferation [23], survival by enhancing cytokine secretion [24], pathogen binding [25], endothelial nitric oxide synthase (eNOS)-like protein activity [26], hormone binding [27], and complement receptor (CR1) - dependent immune complex clearance [28,29].

Our present studies reported here take the lead in determining the underlying mechanism and antibacterial activities of teleost erythrocytes. Our photo-micrographic images of grass carp erythrocytes showed that it could able to entrap the whole bacterium by establishing a series of filaments similar to pseudopods as observed in the PMNs [1,4]. Consequently, the surface structure of the RBCs was retracted inside along with the trapped antigen. These abovementioned observations were essentially in accordance with the video materials of human RBC obtained from the bacteremia patient, where the erythrocytes entrapped the whole bacteria and killed repeatedly without being injured [21]. Previous research has demonstrated that the RBCs from the mammalian immune system has the capacity to capture the microbes by using complement receptor type 1 (CR1) protein complexes through the multiple binding domains [28–31]. Our fluorescence microscopy results showed that grass carp RBCs could able to seize *S. aureus* and *E. coli* over their surfaces. Remarkably, we could observe that most of the microbes were captured by establishing a series of strands as similar to the pseudopods that interacted with the bacteria on the phagocytosis. Our results are in accordance with the Li and colleagues findings, where they demonstrated that rainbow trout peripheral blood leukocytes (PBLs) internalized FITC fluorescence containing *A. hydrophila* bacteria with their confocal, fluorescence and electron microscopy data [32]. The pseudopod-like formation of the RBCs was never reported previously, the possible explanation of this finding is that the fish is a lower vertebrate animal and the tendency of some cells may potentially differing in contrast to mammalian system. Some of the piscine RBCs might have a close relationship with the macrophages that might have evolved during the evolutionary processes, still it needs to be further verified at this conjecture.

Here, we have extensively studied the phagocytic activity of teleost RBCs by confocal microscopy with FITC-labeled three different bacteria (*S. aureus*, *A. hydrophila,* and *E. coli*). A bunch of *S. aureus* bacteria was phagocytosed by the grass carp fish RBCs were struggled to breakout the RBC from inside when observed under the optical microscope (data not shown), as Minasyan presented in videos, where the internalized bacteria try to escape from the erythrocytes of bacteremia patience [21].

Furthermore, the results of SEM and TEM analysis of RBCs with *S. aureus* and latex beads, individually, evidenced variation in the morphology during the extensive phagocytic activity that resembled that of lymphocytes and macrophages (Fig. 2B and C). Hb is known to continuously undergo redox reactions and less stable to various environmental factors when released from the erythrocytes, this phenomenon is most likely a contributing factor to the pathophysiology of the disease [33,34]. Our results showed that the binding of *A. hydrophila* disrupted the RBCs spontaneously and induced the hemolysis without any other requirements. This was also in line with studies of Jiang and coworkers, they found hemolysis occurred in rabbit and human RBCs within 5 min

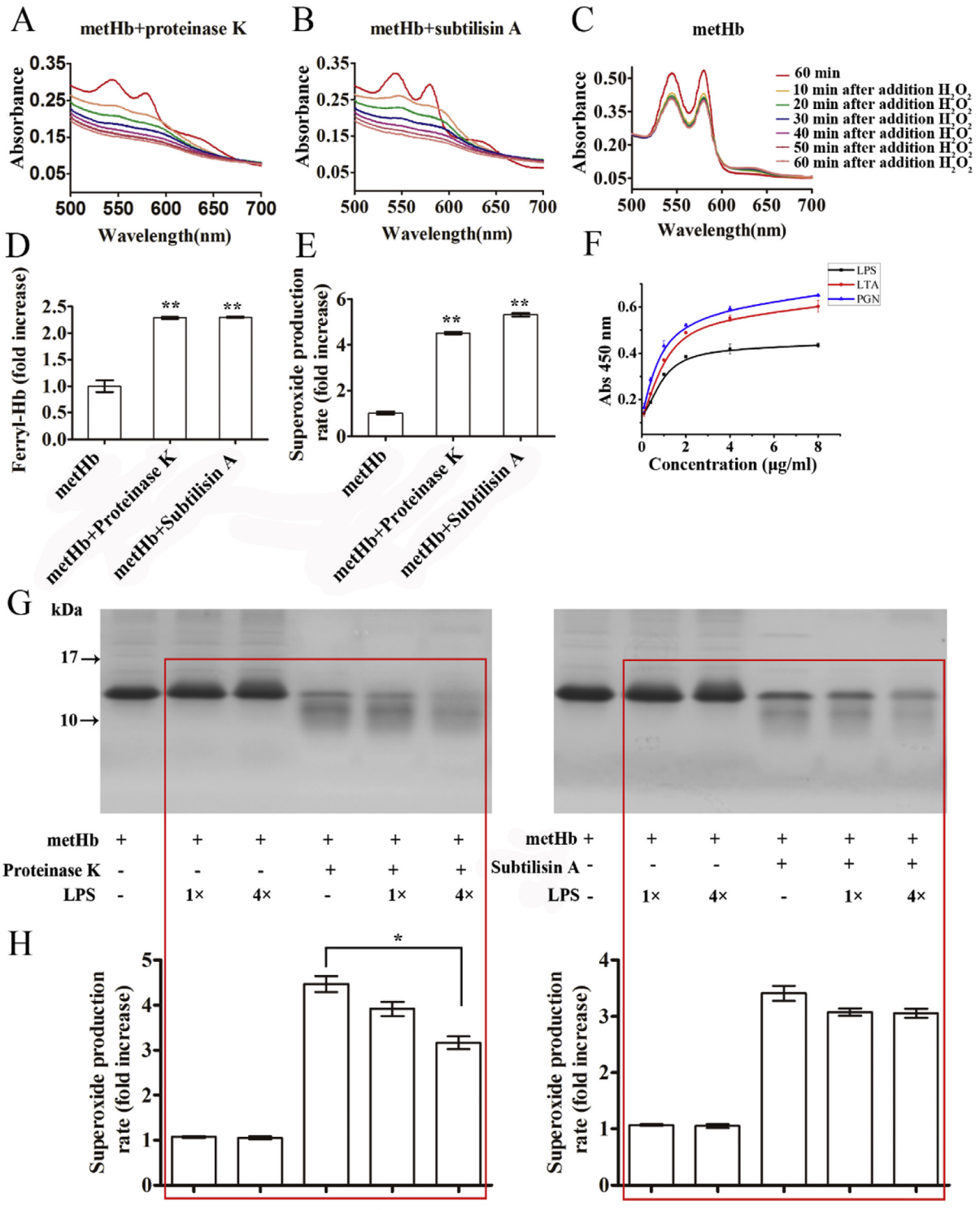

Fig. 5. Proteases effects the structural alteration of metHb. **(A)** The partial protein lysis of metHb by proteinase K and **(B)** Subtilisin A (addition of H_2O_2), the spectral changes of Hb were monitored by spectrophotometric scanning between 500 and 700 nm. **(C)** The spectral changes of Hb without any proteinase. **(D)** The Ferryl-Hb production was quantified at 620 nm after addition of 2 mM sodium sulfide (Na_2S). **(E)** MCLA-CL assay conducted on the 'D' experiment to analyse superoxide production levels. **(F)** The quantitative binding of metHb (100 ng-8 μg) to LPS determined by ELISA. **(G–H)** The potential collaboration between PAMPS and proteases on the proteolytic activation of the metHb, and followed with MCLA-CL assay. The samples analyzed by Tris-Tricine SDS-PAGE (15%). '1 × 'is 1:1 M ratio of metHb:LPS, '4 × 'is 1:4 M ratio of metHb:LPS. *, $P < 0.05$; **, $P < 0.01$. Data represent the mean ± S.E. of three independent experiments.

when encountered with *S. aureus* [11].

It has been noted that the extracellular hemoglobin abruptly undergoes auto-oxidization when exposed to the air [10,12,19]. In our study, the grass carp Hb (metHb-Fe^{II}) was almost started auto-oxidizing instantly when exposed to open air and prolonged exposure leads to oxidation of saturation levels ($HbFe^{IV}$) and the same effects were observed when treated with the pro-oxidant H_2O_2 for 5 min (Fig. 5). The cytotoxicity of the human Hb was characterized in lung epithelial cells (E10), where the oxidation of the human purified Hb was prepared by using 10-fold molar of H_2O_2 and analyzed its various oxidative forms

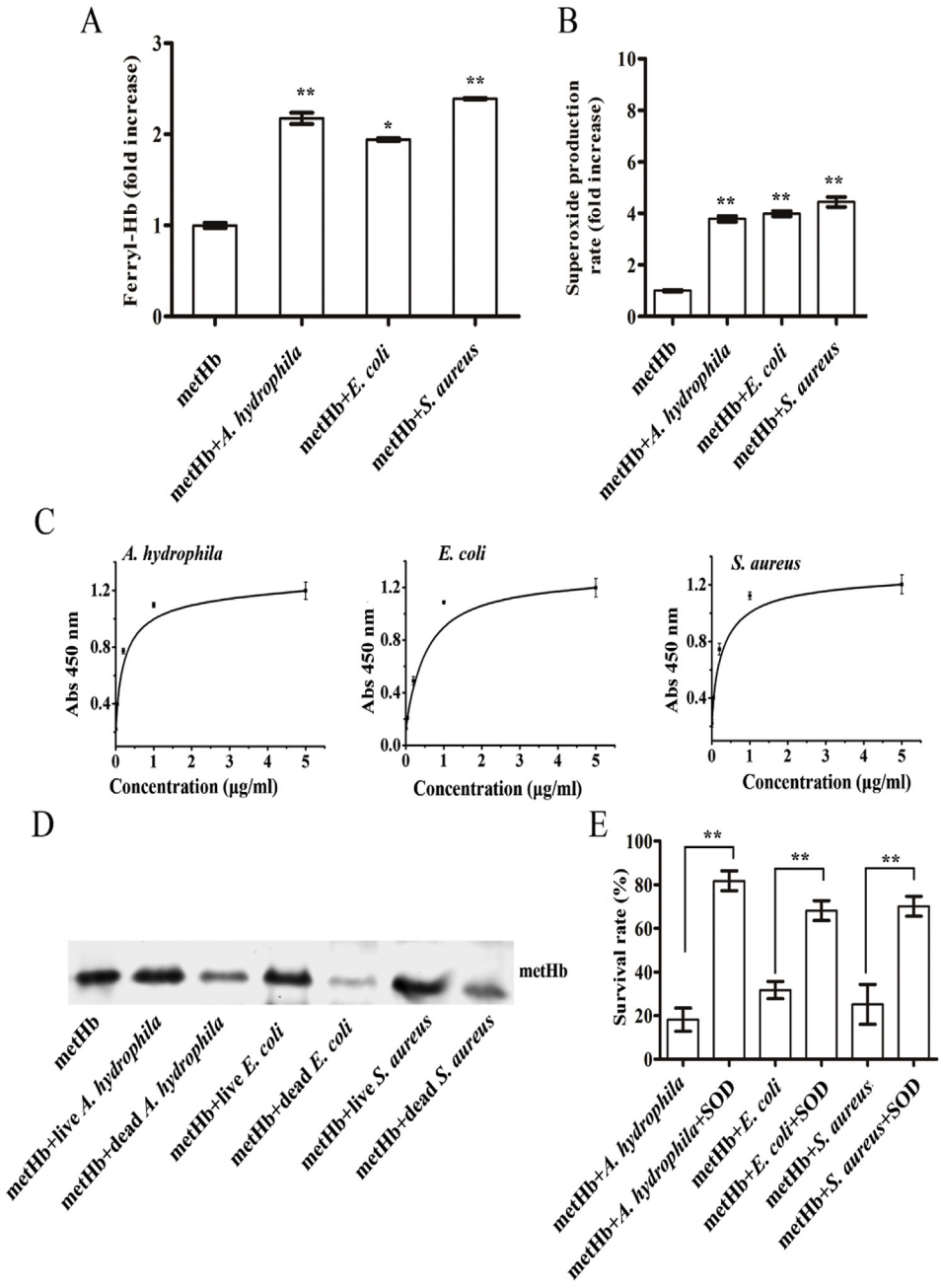

Fig. 6. Infection and produced bactericidal ROS by MetHb. **(A)** metHb was incubated with *A. hydrophila, E. coli, and S. aureus* (10^5 CFU/ml) separately for 30 min, the quantity of Ferryl-Hb (addition of H_2O_2) detected at 620 nm by the addition of 2 mM sodium sulfide (Na_2S). **(B)** MCLA-CL assay conducted on the 'A' experiment to analyse superoxide production levels. **(C)** The quantitative binding of metHb (4 ng-5 μg) to *A. hydrophila, S. aureus and E. coli* to determine by ELISA. **(D)** Immunoblotting-based binding of metHb to dead and alive bacteria. **(E)** Antimicrobial assay of metHb (0.5 mg/ml) with *A. hydrophila, S. aureus, and E. coli* (10^5 CFU/ml) for 1h, superoxide dismutase (SOD) was used to quench superoxide anions. *, $P < 0.05$; **, $P < 0.01$. Data represent the mean ± S.E. of three independent experiments.

[10].

The greater the quantity of PAMPs or proteases, greater the cleavage of metHb proteolytic profiles and that encourage the pseudoperoxidase activity capable of catalyzing the production of superoxide ion [9,11,20]. The data obtained in this study showed an addition of PAMP (LPS) together with the proteases (proteinase K or subtilisin A) had structurally hydrolyzed and increased in the production of superoxide, as observed in the studies of invertebrate hemocyanin (HMC) and vertebrate metHb [11], and in purified human metHb [9]. The reasonable explanation for this phenomenon in grass carp Hb might be the microbial protease should activate the Hb-POX cycle activity and the LPS was reacted like as detergent as mentioned by Du et al. [9]. Removal of the foreign particle or undesired cells either dead or alive from the system is the central molecular process in the maintenance of cellular homeostasis [35,36]. Interestingly, grass carp Hb had bound both to live or dead bacteria parallel to LPS and magnificently produced superoxides. Peroxidation and reactive oxygen generation were observed in the bacterial peritonitis regardless of live and dead *E. coli* bacteria [37].

In summary, we set out to detect the essential antibacterial activity of teleost RBCs and its underlying mechanism involved in killing the bacteria. We unambiguously revealed the capability of teleost RBCs in immune adherence and phagocytosis towards the pathogenic bacteria and further illustrated the transformation of cell-free Hb oxidation. We indicated the activation of POX cycle by bacterial components and the stimulated the Hb to release spontaneous superoxide radicals. Moreover, we have shown the Hb are the potent ROS producers that have significant antibacterial properties. In conclusion, presented data provide a novel clue about the teleost RBCs that it has phagocytic bactericidal hemoglobin contents, which have the ability to entrap, phagocyte, and kill the pathogens by using its powerful superoxide weapon.

Acknowledgments

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