Effects of the Antimicrobial Peptide OH-CATH on *Escherichia coli*

CHEN Xin-xin[1,2], YU Guo-yu[1,3], ZHAN Yan[2], ZHANG Yun [1], SHEN Ji-hong [4,*], LEE Wen-hui[1, *]

(1. *Key Laboratory of Animal Models and Human Diseases Mechanisms, Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming Yunnan   650223, China*；2. *Cell Biology and Genetics Section of Kunming Medical College, Kunming Yunnan   650031, China*；
3. *Biochemistry Section of Kunming Medical College, Kunming Yunnan   650031, China*；4. *Department of Urology, the First Affiliated Hospital of Kunming Medical College, Kunming Yunnan   650032, China*)

Abstract: OH-CATH is a novel cathelicidin identified from king cobra. It showed strong antibacterial activity against various bacteria in the presence of 1% NaCl and no haemolytic activity toward human red blood cells even at a high concentration. OH-CATH might serve as model molecules for the development of antimicrobial drugs. Understanding the action mechanism of OH-CATH and the reason for its selectivity against microbes is very important for this purpose. The bactericidal effect of the king cobra antimicrobial peptide OH-CATH on Gram-negative *Escherichia coli* (ATCC 25922) is observed by scanning electron microscopy (SEM) and transmitted electron microscopy (TEM). The SEM and TEM results suggested that the bactericidal mechanism of OH-CATH against *Escherichia coli* happened in three steps. Firstly, OH-CATH attached to the negatively charged bacterial wall by positively charged amino acid residues. In the second step, the accumulated OH-CATH aggregated and damaged the bacteria membrane in a pore-forming manner. In the last step, with the damage of cell permeability, the contents of the cells were released and eventually cells died.

Key words: Antimicrobial peptide; OH-CATH; *Escherichia coli*; Electron microscopy

抗菌肽 OH-CATH 对大肠杆菌 *Escherichia coli* 的作用

陈欣欣[1,2]，余果宇[3]，张　彦[2]，张　云[1]，申吉泓[4,*]，李文辉[1,*]

(1. 中国科学院动物模型与人类疾病机理重点实验室，云南 昆明　650223；2. 昆明医学院 细胞生物学及遗传学教研室，云南 昆明
650031；3. 昆明医学院 生物化学教研室，云南 昆明　650031；4. 昆明医学院第一附属医院 泌尿外科，云南 昆明　650032)

摘要： OH-CATH是眼镜王蛇中新发现的cathelicidin家族抗菌肽。它在1% NaCl存在的条件下对多种细菌都有较强的抗菌活性，同时，在高浓度下对人红细胞无溶血活性。OH-CATH是开发新型抗菌药物的优良模板。阐明OH-CATH的作用机理及其对微生物的选择性，对研发以OH-CATH为先导结构的药物研发有十分重要的意义。本文利用扫描电镜以及透射电镜对OH-CATH与革兰氏阴性菌－大肠杆菌ATCC 25922相互作用的效应研究。结果揭示：OH-CATH对大肠杆菌的作用涉及到3个步骤。首先，OH-CATH借助其带正电的氨基酸残基附着到细菌带负电荷的细胞壁；然后，附着的OH-CATH在达到一定浓度后发生聚集，以孔道形成的方式破坏细菌的膜结构；最终，由于细菌膜的损坏，膜的渗透性被破坏，胞内内含物释放造成细菌死亡。

关键词： 抗菌肽；OH-CATH；大肠杆菌；电镜
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It has been widely recognized that antimicrobial peptides play an important role in protecting organisms from invading of microorganisms (Boman, 1991, 1995; Zasloff, 1992; Nicolas & Mor, 1995). Antimicrobial peptides have successfully been isolated from plants (Broekaert et al, 1995), invertebrates (Vizioli & Salzet, 2002), vertebrates (Hancock & Lehrer, 1998; Li et al, 2001), and human (Harder et al, 1997; Li et al, 1995; Li et al, 2001; Zhao et al, 2008a,b). Because of the growing problem of multi-drug resistant pathogenic organisms,

there is increased interest in the pharmacological application of antimicrobial peptides to treat infection. Currently, large efforts are being made to increase the potency and specificity of these peptides so that they become toxic to microbes and not to humans. In order to achieve this goal in an efficient manner, it is important to elucidate the mechanism of action of these peptides and the reason for their selectivity against microbes (da Silva & Teschke, 2003).

Cathelicidins are cationic host defense peptides that play an important role in innate immune system. Members of the cathelicidin family all include N-terminal signal peptides and proregions highly homologous to cathelin, a cathepsin L inhibitor, then followed by highly divergent C-terminal antimicrobial domains (Bals & Wilson, 2003; Lehrer & Ganz, 2002). Up to now，most of the identified cathelicidins were from mammalian species (Zanetti et al, 1990, 1993, 1995). Five cathelicidins termed fowlicidin-1, 2, 3, B1 and myeloid antimicrobial peptide 27 were characterized from chicken (Xiao et al, 2006; Goitsuka et al, 2007). Cathelicidins were also found in fish species of Atlantic hagfish *Myxine glutinosa*, rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar*. Hagfish cathelicidins were considered as ancient members of the cathelicidin family (Chang et al, 2005, 2006; Uzzell et al, 2003).

Recently, we cloned three cDNA sequences coding for elapid cathelicidins from constructed venom gland cDNA libraries of *Naja atra*, *Bungarus fasciatus* and *Ophiophagus hannah*. The open reading frames of the cloned elapid cathelicidins were all composed of 576bp and coded for 191 amino acid residue protein precursors. Each of the deduced elapid cathelicidin has a 22 amino-acid-residue signal peptide, a conserved cathelin domain of 135 amino acid residues and a mature antimicrobial peptide of 34 amino acid residues. Unlike the highly divergent cathelicidins in mammals, the nucleotide and deduced protein sequences of the three cloned elapid cathelicidins were remarkably conserved. OH-CATH, the deduced mature cathelicidin from king cobra, showed strong antibacterial activity against various tested bacteria with minimal inhibitory concentration of 1−20g/mL in the presence of 1% NaCl. Meanwhile, the peptide showed no haemolytic activity toward human red blood cells even at a high dose of 200 g/mL. Elapid cathelicidins might serve as model molecules for the development of antimicrobial drugs (Zhao et al, 2008c).

In the present work, the bactericidal effect of the king cobra antimicrobial peptide OH-CATH on Gram-negative *Escherichia coli* (ATCC 25922) is observed by scanning electron microscopy (SEM) and transmitted electron microscopy (TEM). The bactericidal mechanism of OH-CATH against *E. coli* was reported based on the obtained SEM and TEM images.

1　Materials and Methods

1.1　Materials

Escherichia coli ATCC 25922 was obtained from The First Affiliated Hospital of Kunming Medical College, China. Yeast extract and Tryptone for bacterial culture were products of Oxoid Ltd. Company (England). All other reagents used were analytic grade from commercial sources.

1.2　Peptide synthesis

King cobra antimicrobial peptide OH-CATH with amino acid sequence of KRFKKFFKKLKNSVKKRAK-KFFKKPRVIGVSIPF was synthesized by solid-phase synthesis on an Applied Biosystems Model 433A peptide synthesizer according to the manufacturer's standard protocols. After cleavage and side-chain deprotection, the crude synthetic peptide was purified on a Vydac 218TP510 C18 reverse phase-HPLC column (25×1 cm. Separations Group, Hesperia, CA, USA) and eluted at a flow rate of 2mL/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Identity of the peptide was confirmed by automated Edman degradation with a protein sequencer and mass spectrometry analysis. Fast atom bombardment-mass spectrometry was carried out on an Autospec-3000 spectrometer, equipped with a high-field magnet (Lee et al, 2005). The purity of the synthetic peptide was >95% and then used for evaluating biological activities.

1.3　Sample preparation for SEM and TEM

Overnight culture of *E. coli* cells (OD_{600nm}=1.2) in LB medium was diluted 100 times with fresh LB medium and gave a total culture volume of 20mL. Then, the bacteria were continue to culture at 37 ℃ for additional 4 hours to get mid-logarithmic-phase organisms (OD_{600nm}=0.7). OH-CATH was added to the culture medium to reach a final concentration of 12g/mL and cultured under the conditions as mentioned above. At different time intervals, aliquots were removed and optical density was measured at 600 nm for both OH-CATH treated and untreated samples. The samples prepared for SEM and TEM were as follows: 1) SEM samples, aliquots of 1 mL culture medium at various

culture times were removed to 1.5 mL eppendorf tubes and centrifuged at room temperature for 4 minutes (2000 rpm). After discard the supernatant, 1 mL fresh LB medium were added and vortex thoroughly to wash the bacterial pellets and centrifuged again at 2000 r/min for 4 minutes. After carefully removal of the supernatant, bacterial cells were chemically pre-fixed with 3.5% glutaraldehyde in 0.1mol/L PBS buffer (pH7.4) at 4℃ overnight. The pre-fixed bacterial cells were collected on a poly lysine treated microscopy coverslips and washed three times with Milli-Q water. The cells on the coverslips were post-fixed with 2% osmic acid for 1 hour and subsequently dehydrated with 30%, 50%, 70%, 80%, 90%, 100% graded ethanol and acetone series. The samples were lyophilized, coated with Pa/Pt and examined by SEM on a Hitachi 300-N instrument. 2) TEM samples, aliquots of 1.5 mL culture medium at various culture times were removed to 2mL eppendorf tubes and centrifuged at room temperature for 4 minutes (2000 r/min). After discard the supernatant, 1.5mL fresh LB medium were added and vortex thoroughly to wash the bacterial pellets and centrifuged again at 2000 r/min for 4 minutes. After carefully removal of the supernatant, bacterial cells were chemically pre-fixed with 3.5% glutaraldehyde in 0.1mol/L PBS buffer (pH7.4) at 4℃ overnight. The pre-fixed bacterial cells were post-fixed with 1% osmic acid for 1 hour and subsequently dehydrated with 30%, 50%, 70%, 80%, 90%, 100% graded acetone series. Epoxy resin (Epon-618) was used to embed the post-fixed bacterial samples for 12-16 hours at room temperature. Ultra thin section of the embedded samples were prepared by LKB-II Ultracut instrument and double stained with uranyl acetate and lead citrate. The samples were lyophilized then examined by transmitted electron microscopy on a Hitachi TEM-1011 instrument. The optical density (OD) at 600 nm was recorded to evaluate the growth of the bacteria.

2　Results and Discussion

OH-CATH is a new member of cathelicidin antimicrobial peptides characterized from Elapidae king cobra snake. Our previously results indicated that the peptide have a MIC value of 2g/mL and 8g/mL against *E. coli* ATCC 25922 under NaCl-free and in the presence of 1% NaCl conditions, respectively. Meanwhile, OH-CATH showed no hemolytic activity toward human red blood cells up to 200g/mL, suggestion that this molecular might be served as a good model for the developing of new antibacterial agents (Zhao et al,

2008c). Understanding the antibacterial mechanism of OH-CATH against bacterial pathogens should do a great help for the design of more potent antibacterial and better specificity for OH-CATH derived peptide(s) against microbes while maintain weak cytotoxic to human cells. Recently, atomic force microscope (AFM) has been used to study antimicrobial peptide mechanisms both on model membrane and intact cells. AFM is a powerful imaging tool capable of achieving high resolution images under physiological conditions and gives 3-D reconstruction images (Li et al, 2007). Nevertheless，SEM and TEM are still used to investigate the mechanisms of antimicrobial peptides with microbes (Matsuzaki et al, 1997).

Gram-negative *E. coli* ATCC 25922 strain was widely used as a control for evaluation of antibacterial activity for antibacterial peptides. AFM images of *E. coli* ATCC 25922 indicated that they showed characteristic rod shape with distinctive peritrichous flagella and pili structure (Li et al, 2007). Previously, we have demonstrated that OH-CATH have a MIC value of 2 g/mL in NaCl-free LB media with bacterial inoculation of 5×10^5 cfu/mL at 37℃ for 16 hours (Zhao et al, 2008c). In our experiment conditions, one absorption unit at 600nm approximately equal to 1×10^9 cfu/mL. To investigate the interaction of OH-CATH with *E. coli* 25922, a lower OH-CATH concentration of 12g/mL was used to treat *E. coli* 25922 at a beginning concentration of about 7×10^8 cfu/mL and incubated for 21 hours. It should be noted even the OH-CATH concentration used in the present work was 6 times higher than its MIC for *E. coli* 25922 (12 g/mL *vs* 2 g/mL), but the bacterial inoculation of *E. coli* 25922 in the present work was 1000 times higher than that for MIC determination (7×10^8 cfu/mL *vs* 5×10^5 cfu/mL). Obviously, the OH-CATH concentration used in the present work was comparatively lower at a beginning bacterial inoculation of 7×10^8 cfu/mL and allow us to investigate the time-dependent interaction of OH-CATH against *E. coli* ATCC 25922. *E. coli* ATCC 25922 growth kinetics of both OH-CATH treated and un-treated bacterial cells were shown in Tab. 1. At each determined incubation intervals, lower antibacterial dosage treated samples showed a declined bacterial cell growth compared with un-treated samples, indicating that OH-CATH do affect the *E. coli* ATCC 25922 growth. The determined OD values of OH-CATH treated samples at different incubation intervals also suggested that the OH-CATH at the fixed concentration of 12g/mL could not fully kill the

tested bacteria and the bacteria could still grow.

SEM images suggested that the untreated *E. coli* cells (Fig. 1A,B) had a smooth surface with average size of 0.5×2^{-4} m. The arrows in both Fig. 1A and 1B indicated the flagella or pili connections between *E. coli* cells. After treated with lower concentration of OH-CATH for 1 hour, there are litter obviously morphological changes for *E. coli* cells (Fig. 1C). Pore-forming and morphological changes were observed for *E. coli* cells being treated with OH-CATH for 2 hours, lysis of *E. coli* cells and release of fluid and debris could be observed (Fig. 1D). After treated with OH-CATH for 6 hours, elongation of cells (>9 μm) and shorten cells (<1μm) were clearly observed (Fig. 1E). Meanwhile, collapse of *E. coli* cell outer membrane and release of bacterial fluid could be seen as indicated by the arrows in Fig. 1F. The average pore sizes of bacteria treated with OH-CATH were measured to be 100−300nm (figure not shown). After treated with lower concentration of OH-CATH for 21 hours, the bacteria cells continuously grew (Tab. 1) and the morphological changes of the growing cells (Fig. 1G, H) were obviously different from the cells untreated with antibacterial peptide (Fig. 1A,B). The most obvious differences were included more fiber-like connection among cells as indicated by arrows in Fig 1G, elongation of cell body and cell surface becoming roughness for the growing cells (Fig. 1H). These images indicated that the cell growth is affected by the antibacterial peptide.

TEM images suggested that the untreated *E. coli* cells had normal structure (Fig. 1A). The ultra-structure of an untreated *E. coli* cell was shown in Fig. 1B where outer membrane, periplasm and cytoplasmic membrane could be seen. After treated with lower concentration of OH-CATH for 2 hours, some bacterial cells showed morphological changes, like bleb-like structures, were observed (Fig. 2C, D). The arrow in Fig. 2C indicated that periplasmic and/or cytoplasmic fluid was released

from damaged cells. The OH-CATH treated cells showed cytoplasmic membrane extraction resulted in the separation of outer membrane and cytoplasmic membrane as indicated by the arrows in Fig. 2D. It should be noted that SEM images of bacterial cells after treated with OH-CATH for 1 or 2 hours showed little morphological changes (Fig. 1C, D). Thus, SEM and TEM results of bacterial cells treated with OH-CATH for 1−2 hours suggested that OH-CATH should bind to the bacterial wall and reach a certain concentration then aggregate to act in a pore-formation manner to damage the cell membrane and release the cell fluid. After treated with OH-CATH for 6 hours (Fig. 2E, F), many bacterial cells were damaged by OH-CATH and bleb-like structure existed in many cells (Fig. 2E). Formation of bacterial ghost, which contains intact bacterial envelope, after treated with OH-CATH was indicated by the arrow in Fig. 2E. Bacterial ghost was formed by OH-CATH in a pore-forming manner and the opening of the bacteria was indicated by an arrow in Fig. 2F. After treated with lower concentration of OH-CATH for 21 hours (Fig. 2G, H), some new growing bacteria cells showed normal structure, together with damaged cell debris (Fig. 2G). The arrows in Fig. 2H indicated the opening of the damaged cells.

In conclusion, our results indicated that OH-CATH and Gram-negative *E. coli* ATCC 25922 interactions might happen in three steps. At beginning, OH-CATH attached to the negatively charged bacterial wall by positively charged amino acid residues. In the second step, with the attached OH-CATH reached a certain concentration, the accumulated OH-CATH aggregated and damaged the bacteria membrane in a pore-forming manner, the average pore size of *E. coli* treated by OH-CATH was measured to be around 100−300 nm. In the last step, with the damage of cell permeability, the contents of the cells were released and the cells eventually collapsed.

Tab. 1   *Escherichia coli* ATCC 25922 growth kinetics at OD$_{600nm}$

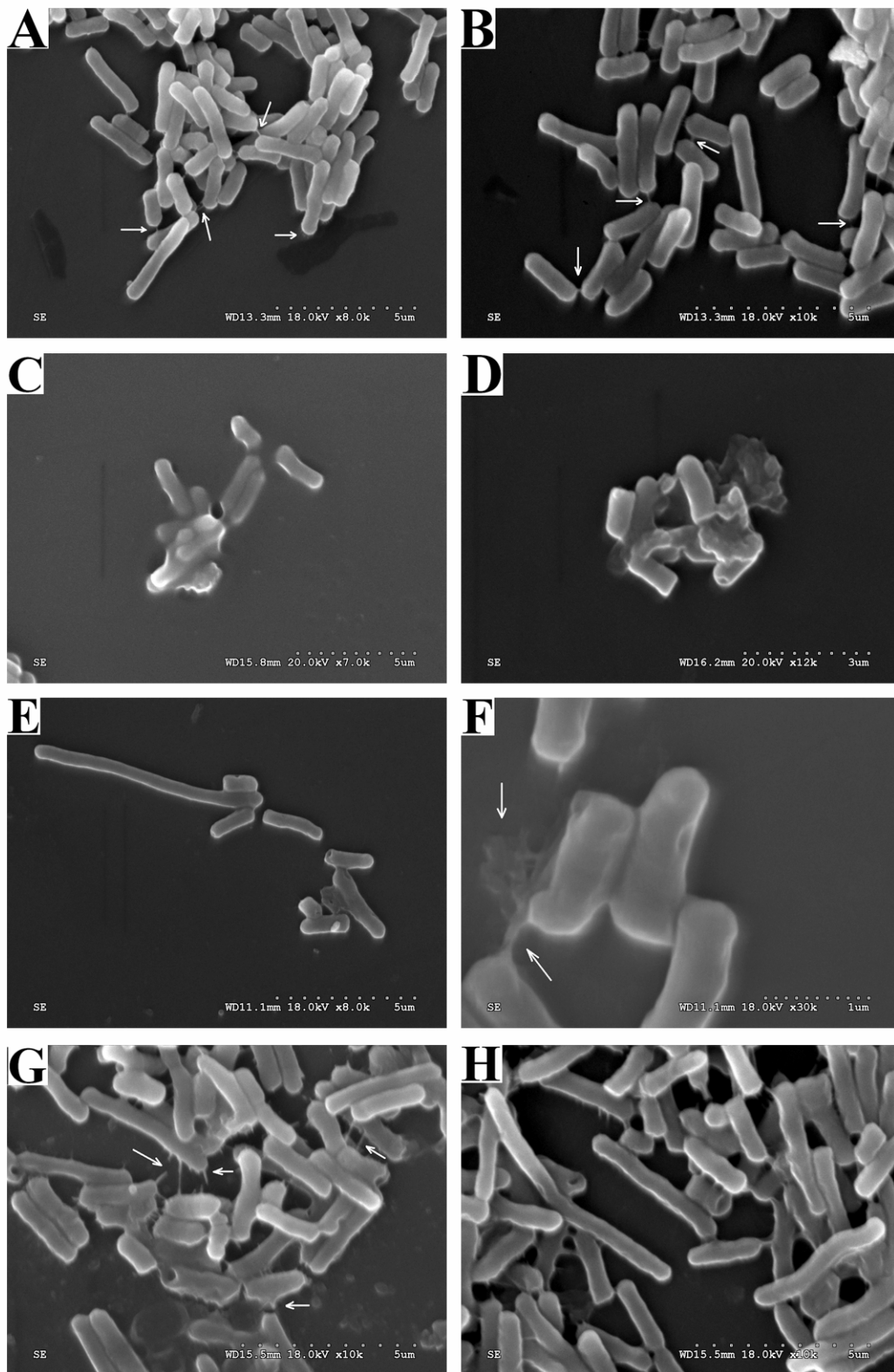| Samples	0	0.5h	1h	2h	6h	21h
OH-CATH treated	0.708	0.760	0.804	0.852	1.141	1.678
Without OH-CATH	0.708	1.104	1.340	1.548	1.906	2.506

Fig. 1　SEM images of *Escherichia coli* ATCC 25922 treated with OH-CATH for different times
(A−B) At zero time, no OH-CATH added. (C) 1 hour after OH-CATH treated. (D) 2 hours after OH-CATH treated. (E−F) 6 hours after OH-CATH treated. (G−H) 21 hours after OH-CATH treated.
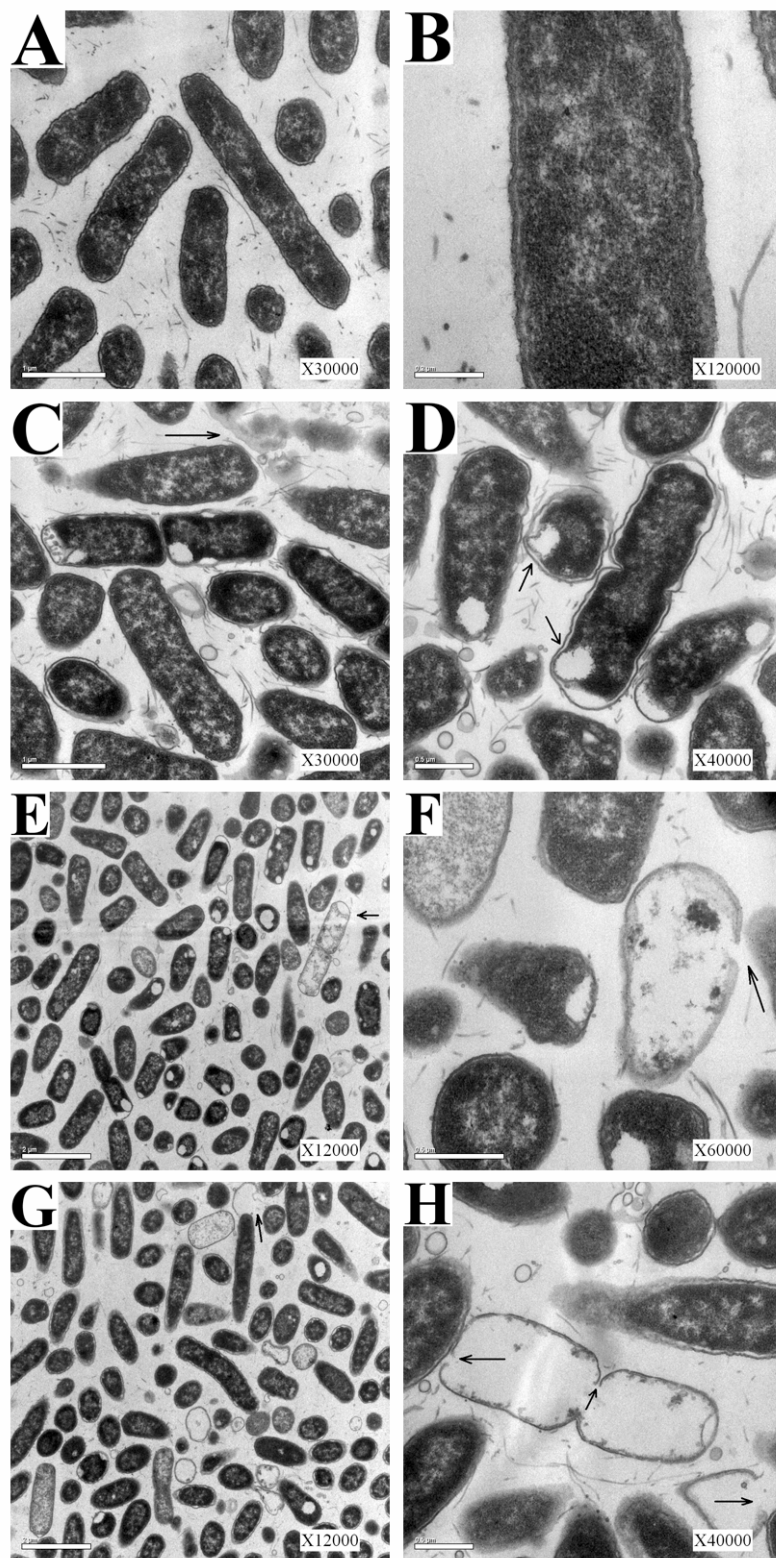
Fig. 2   TEM images of *Escherichia coli* ATCC 25922 treated with OH-CATH for different times

(A−B) At zero time, no OH-CATH added. (C−D) 2 hours after OH-CATH treated. (E−F) 6 hours after OH-CATH treated. (G−H) 21 hours after OH-CATH treated.

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