# Supplementary Information

## Co, N-doped carbon dots nanozymes based on untriggered generation ROS approach for anti-biofilm activities and anti-bacteria treatment in vivo

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## 1. Experimental Sections

### 1.1 Reagents and materials

Co $(NO_3)_2 \cdot 6H_2O$, EDTA-Na, methyl alcohol were obtained from Sigma. 1,2-diaminobenzene (OPD) obtained from J＆K China Chemical Ltd. 2', 7'- dichlorofluorescin diacetate (DCFH-DA) Kit, Live/Dead Cell Staining Kit (Calcein-AM/PI) were obtained from Sigma. 2,2,6,6-tetramethylpiperidine (TEMP) were purchased from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Bacteria Genomic DNA Extraction Kit was obtained from Solarbio. Bacteria were purchased from Nanjing Benji Biotechnology Co., LTD. The BALB/c mice (4~5 weeks old, 18~20 g) mice obtained Hualan Biology. Animal experiments were approved by the Use Committees of Henan Normal University.

### 1.2. Apparatus and instruments

A TU-1900 double beams UV-vis spectrophotometer (Beijing Purkine General Instrument Co. LTD., China) was used to implement kinetic experiments and measure absorbance. The LS-55 from PerkinElmer (U.K) were used to obtain fluorescence spectra and intensity. The JEM-2100 transmission electron microscopy (TEM) was used to image the morphology and size of CoNCDs. Nicolet iS10 FTIR spectrophotometer was used to record the FT-IR spectrum of CoNCDs. The chemical state of the surface of CoNCDs was analyzed using X-ray photoelectron spectroscopy (XPS, America Thermo Fisher Scientific ESCALAB250Xi spectrometer with Al Kα radiation of 1486.6 eV and 1253.6 eV). The zeta potential was measured by Malvern Nano-ZS90. An Olympus spectral confocal multiphoton microscope (FV1200-MPE) was used in cell image experiment. Electron spin resonance (ESR) spectra were characterized by a Bruker A300 spectrometer (Germany). Fluorescence spectra were acquired with a Shimadzu RF-6000 spectrofluorometer (Shimadzu, Japan).

### 1.3 Synthesis of NCDs

As reference materials, N-doped carbon dots (NCDs) were prepared as follows: 2.2335g ethylene diamine tetraacetic acid disodium salt (EDTA-Na) was calcined in a tube furnace at 300 °C for 2 h at a heating rate of 5 °C $min^{-1}$ under $N_2$ exposure. The

supernatant was passed through a micropore filter (0.22 μm) and sequentially dialyzed using an MD44 (1000 Da MW cut-off) dialysis tubing. Finally, the purified solution was lyophilized to obtain the NCDs powder.

**1.4 Transmission electron microscopy (TEM)**

The CoNCDs and NCDs were identified by transmission electron microscopy (TEM). Briefly, CoNCDs were diluted with PBS. The solution was dropped on carbon-coated 400 mesh copper grids (Electron Microscopy Sciences), then were dried and analyzed using TEM.

**1.5 Dynamic light scattering (DLS)**

The hydrodynamic Diameter of CoNCDs and NCDs were assessed by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). CoNCDs and NCDs were diluted with dd$H_2O$ into a volume of 1 ml in a polystyrene cuvette (DTS0012). Three independent measurements were performed to generate the intensity-based size distribution profile.

**1.6 Kinetic Analysis of CoNCDs**

Kinetic measurements were conducted at 37 °C in 400 μL micro cuvette. 100 μL CoNCDs solution (12.5 μg $mL^{-1}$), 100 μL OPD, $O_2$ was incubating in cuvette for 1 h using TU-1900 double beams UV-vis spectrophotometer at 420 nm monitoring the absorbance change in time course mode. The kinetics data were obtained by varying the concentration of OPD while keeping the other's concentration constant. The Michaelis–Menten constant was calculated using the Lineweaver–Burk plot.

**1.7 Detection of $^1O_2$**

2 mL CoNCDs (1 mg/mL) nanozyme was mixed with 0.01 mM DPBF. After adding $O_2$ for 1 – 5 min, and the absorb of DPBF was detected using a UV-vis spectrophotometer.

**1.8 Detection of •OH**

Briefly, to four 5mL centrifuge tubes each containing 0.18 mL of 12.5 mM TA and 0.3 mL of 0.2 M acetate buffer (pH=7.4), were added respectively 0.12 mL water (control), 0.12 mL CoNCDs with three different concentrations (the concentrations

are 0.25 mg $mL^{-1}$, 0.5 mg $mL^{-1}$, 1 mg $mL^{-1}$ in water respectively). The mixtures in the tubes were diluted with water to 3 mL, and which were kept in the dark at 30 °C under gentle shaking for 8h. After centrifugation of the mixtures, the fluorescence of the supernatants was recorded respectively using fluorimeter with emission wavelength of 435 nm.

**1.9 Scavenging free radicals of $^1O_2$**

1 mg/mL CoNCDs in 3.0 mL of PBS buffer with OPD (0.5 mM), three different concentrations of tryptophan were added (the concentrations are 0.3 mg $mL^{-1}$, 0.5 mg $mL^{-1}$, 1 mg $mL^{-1}$ in water respectively), and $O_2$ was injected for 1 h. The oxidized OPD was recorded at 420 nm.

**1.10 Electron paramagnetic resonance (EPR) experiments**

In a typical measurement of $^1O_2$, 2 mL of 1mg $mL^{-1}$ CoNCDs methanol solution under $O_2$ atmosphere for 1.5 h, the radical trapping agent was TEMP. Then, the resulting solution was extracted with a quartz capillary and placed in a glass tube for EPR testing.

**1.11 Biofilm inhibition effects of CoNCDs**

Gram-positive bacteria (*S. aureus, B. subtilis, S. epidermidis*) and Gram-negative bacteria (*E. coli, P. aeruginosa*) were selected to investigate biofilm inhibition effects of CoNCDs. The bacteria were diluted to $10^5$ colony forming units/mL (CFU/mL). Then added 200 μL aforementioned diluted bacteria to each well of a sterile 96-well plate. The plates were incubated at 37 ºC for 24 h, and then CoNCDs suspension in sterile PBS (20 μL) were added to the wells. After treatment with CoNCDs for 10 h, all kinds of bacteria washed three times with PBS. After the wells were dried in air for 30 min, and then 0.1% crystal violet solution was added and kept standing for 15 min. After removed crystal violet solution, each well was then washed with PBS to remove the excess remained crystal violet and dried in air for 30 min. The wells were added 30% aqueous acetic acid to extract dye staining biofilm, and then Absorbance at 595 nm was measured using a plate reader. In these quantitate assay, at least 3 replicate wells were used for each treatment, and the biofilm formation (%) was represented as their averaged values.

**1.12 Bacterial DNA damage assay**

1 mL *S. aureus, E. coli, P. aeruginosa* were diluted to $10^5$ colony forming units/mL (CFU/mL), the Bacteria Genomic DNA Extraction Kit was used to extract the DNA fragments. 10 μL DNA and 20 μL CoNCDs were incubated at 37 ºC for 10 h. Besides, 1% TAE agarose gel electrophoresis containing ethidium bromide staining was used to characterize the residual levels of DNA on the Gel Image system. The concentrations of CoNCDs were 1mg/mL, 0.5 mg/mL, 0.25 mg/mL.

**1.13 Mouse Injury Model**

Wounds from three groups of BALB/c mice were added *P. aeruginosa* (OD=0.6) 50 μL for 10 min. The three groups of 12 female mice with a 1$cm^2$ wound (4-5 weeks, 8-20g, and three mice per group) were divided into PBS, CoNCDs-0.1mg, CoNCDs-0.3mg groups. The mice in three different groups with different treatment on their wound. And wounds were photographed, then gauzes were changed with 2 days interval.

**1.14 Acute peritoneal infection Model**

*S. aureus* (OD=0.6) 500 μL were injected into the peritoneal cavity of 45 female mice (15 mice per group). Two hours later, the mice were in low spirits, rolled up together, and excreted mucous stool, indicating the acute peritonitis model was successfully built. The three groups of 45 female mice were divided into PBS, CoNCDs-0.1mg, CoNCDs-0.3mg groups. The mice were observed for 5 days to evaluate survival.

At different time intervals, 3 mice of three groups were sacrificed randomly. The abdomen was then opened and washed by 1 mL PBS. The washed peritoneal fluid was diluted 10 times with PBS to spread LB agar plates. The content of CFU in LB plates was counted after incubation for 20 h at 37 ℃. Then spleen and peritoneum for pathological section (H&E) staining by Wuhan servicebio technology CO., LTD. And We use microscopy to observe.

**1.15 Statistical analysis**

Each experiment was carried out in triplicate at least. Data are presented as mean ± standard deviation (SD).

## 2. Supplementary Figures

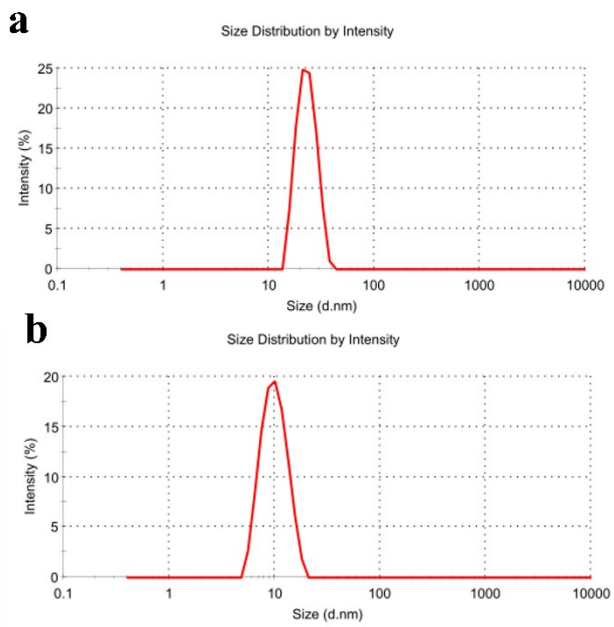


**Fig. S1.** The DLS of CoNCDs (a) and NCDs (b).

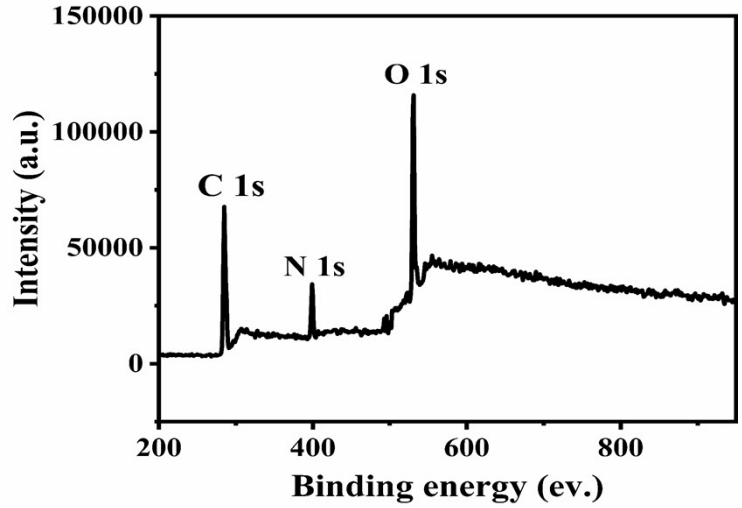


**Fig. S2.** Full XPS spectrum of CoNCDs.

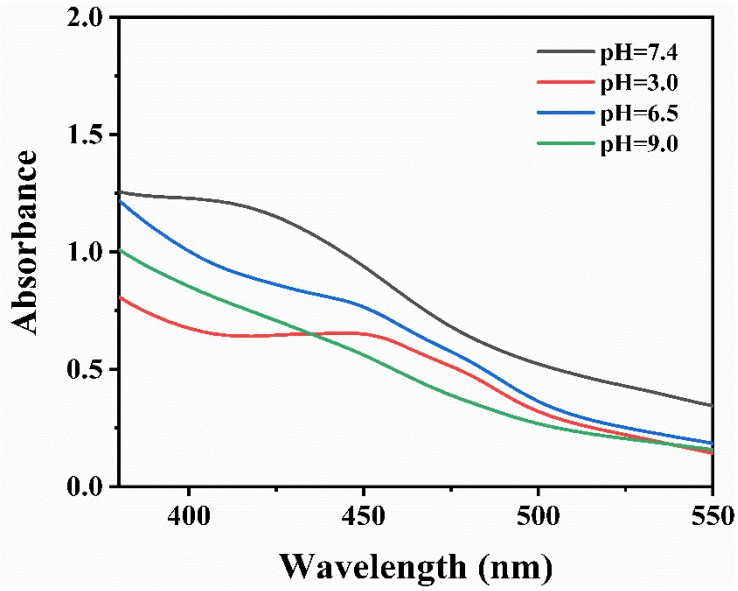


**Fig. S3.** The absorbance value in different pH.

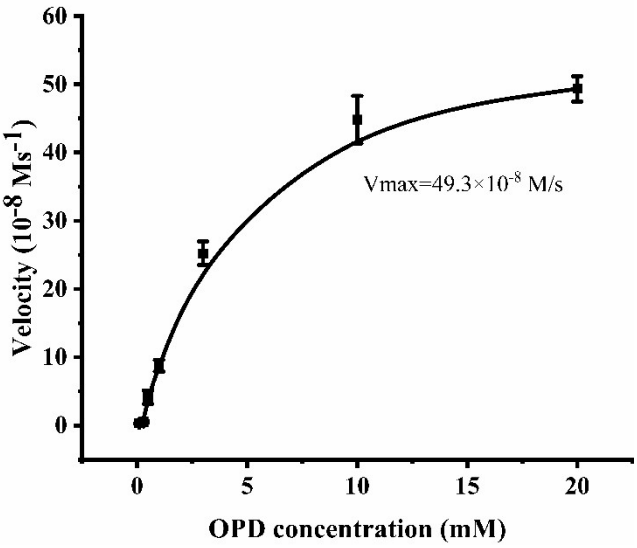


**Fig. S4.** Steady-state kinetic of CoNCDs when OPD is used as substrate.

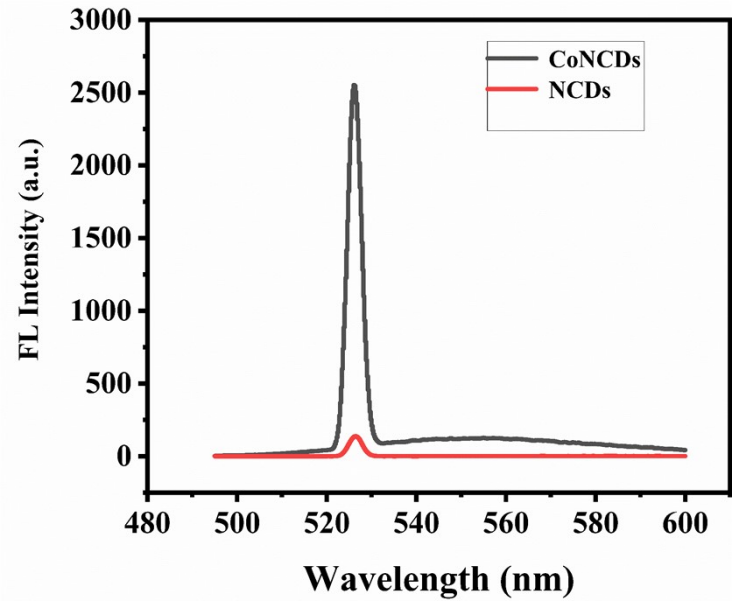


**Fig. S5.** ROS generation of CoNCDs and NCDs

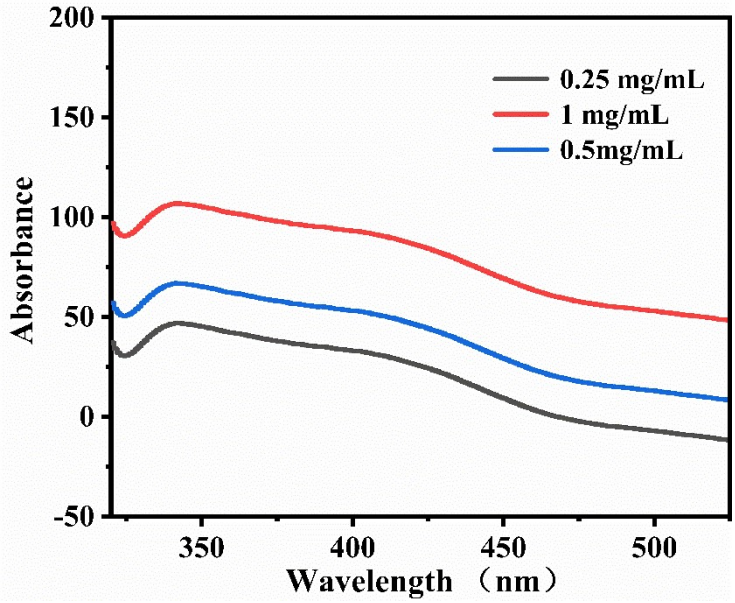


**Fig. S6.** Fluorescence spectra of the different concentrations of CoNCDs with TA.

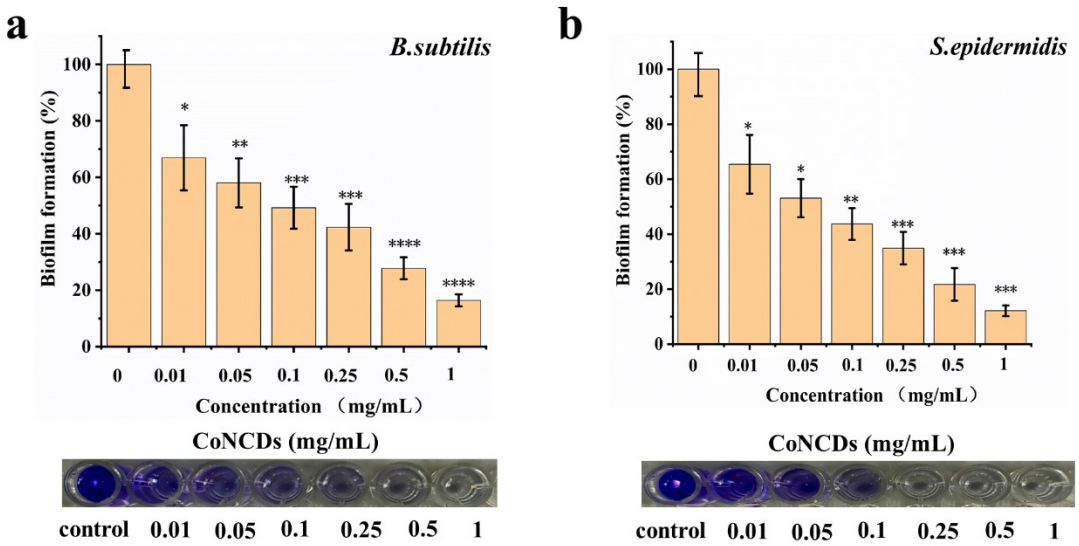


**Fig. S7.** Biofilm formation (%) and the remaining biofilms were quantified by crystal violet staining in the presence of CoNCDs at different concentration. (a) *B.subtilis*, (b) *S.epidermidis*, ****P＜0.001，*** P＜0.005, ** P＜0.01, * P＜0.05.

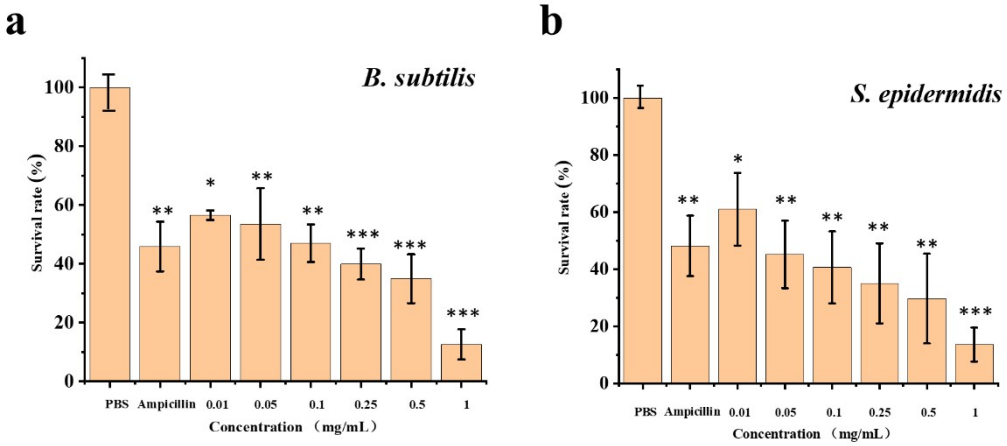


**Fig. S8.** Relative bacteria viabilities of CoNCDs at different concentration. (a) *B.subtilis*, (b) *S.epidermidis*, ****P＜0.001，*** P＜0.005, ** P＜0.01, * P＜0.05.

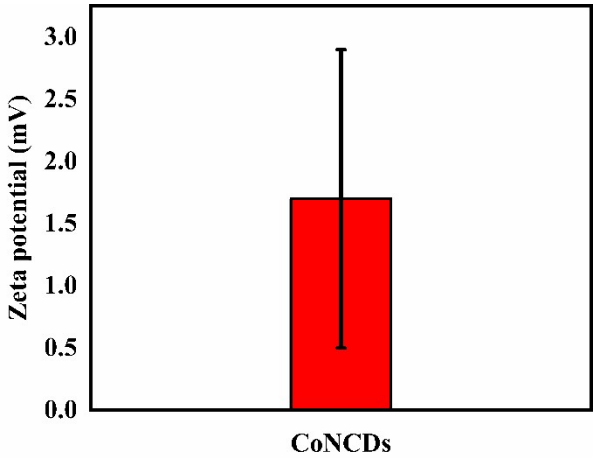


**Fig. S9**. The Zeta potential of CoNCDs.

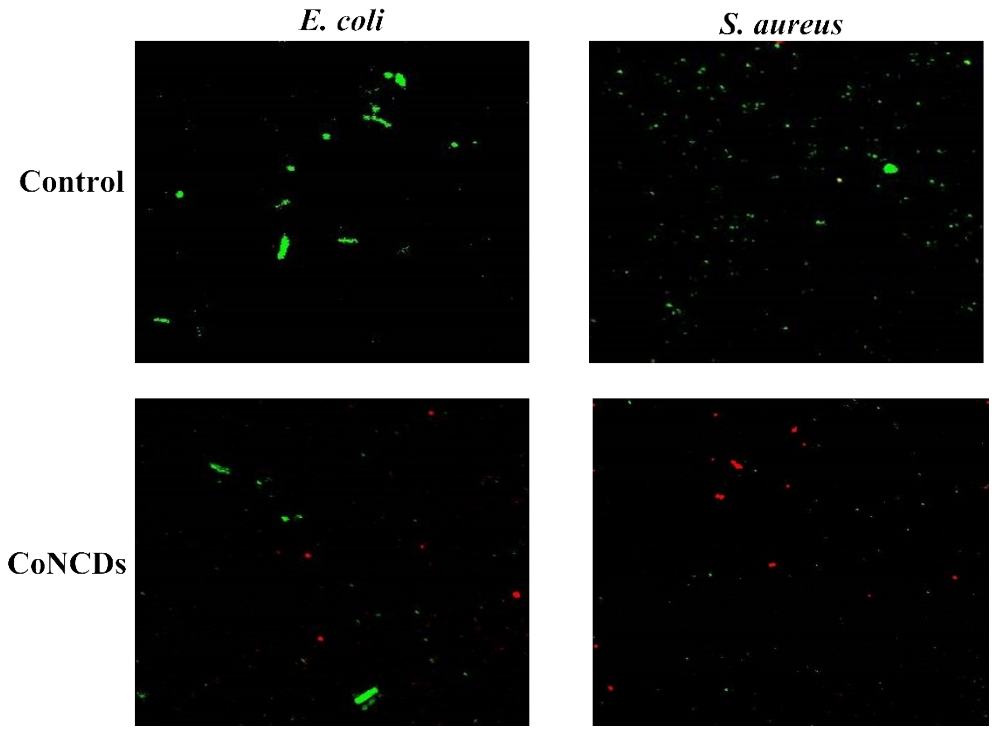


**Fig. S10.** Typical fluorescence images of *E. coli* (left) and *S. aureus* (right) by CoNCDs.

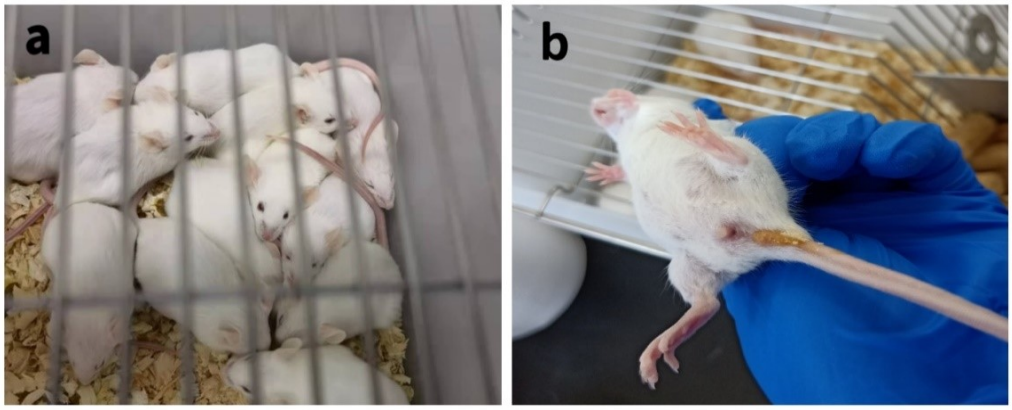


**Fig. S11.** The picture of mice in acute peritoneal infection model.