A novel strategy for antimicrobial agent: Role of exogenous carbon monoxide on suppressing *Escherichia coli* vitality and toxicity

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Abstract: Sepsis is a severe systemic inflammatory response mostly caused by gram-negative bacterial infections. The rates of mortality in sepsis patients remain high. To date little is known about whether exogenous carbon monoxide can directly or indirectly inhibit or even kill gram negative bacteria. In our study, we demonstrate a critical role of CO-releasing molecules in the suppressive effects on bacterial vitality and toxicity. We found the bacterial growth and colony forming were markedly suppressed in the presence of CORM-2 with significant cell damage, decreased or disappeared pili and flagella. In contrast, qRT-PCR showed the expression of *fliA* was downregulated, while *dnaK* and *waaQ* were upregulated in *E. coli*+CORM-2. Subsequent *in vivo* experiments showed the mouse survival in the CORM-2 intervened-*E.coli* injection tended to improve with 60%-100% survival rates, and colony distribution in major organs were significantly decreased with attenuated histological damage. In parallel, cytokine levels and myeloperoxidase accumulation in livers and lungs decreased significantly compared with *E. coli* group. These data provide the first evidence and a potential strategy that exogenous carbon monoxide can significantly suppress bacterial vitality and toxicity. This may be associated with the regulatory functions of CORM-2 on the expression of essential genes (*fliA, dnaK* and *waaQ*) in *E. coli.*

Keywords: Carbon monoxide; *Escherichia coli*; vitality; toxicity; suppression.

INTRODUCTION

Sepsis is a severe systemic inflammatory response that is caused by a host's harmful inflammatory response to infection or trauma and can further develop into septic shock and multi-organ dysfunction syndrome (Tse, 2013; Sriskandan, 2007; Remick, 2007; Baillie, 2007). It is estimated that approximately 750,000 new cases of sepsis occurred annually, and 210,000 patients death attributed to sepsis with high mortality rate of 28.6% (Angus, Wax, 2001; Martin, 2012; Russell, 2006). Clinically, approximately 95% of sepsis cases are caused by bacterial infections, and up to 60% can be attributed to gram negative bacteria (Nathan, 2002; Annane, *et al.,* 2005; Hotchkiss, Nicholson, 2006). As an opportunistic pathogen, *Escherichia coli* (*E. coli*) can migrate from the gastrointestinal tract to extraintestinal sites, a phenomenon called bacterial translocation, and cause local or disseminated infections. Infection activates an inflammatory cascade in the host, which includes the influx of leukocytes (monocytes and lymphocytes), and in the case of bacterial infection, neutrophils, from the circulation into the infected microenvironment in an attempt to eliminate the inflammatory responses (Aird, 2003). The control of bacterial burden is a key event in the treatment of septic patients. Antibacterial therapy is therefore required early and effectively after diagnosis, and its delay has a clear impact on mortality (Gaieski, *et al.,* 2012; Siddiqui, *et al.,* 2009). Nevertheless, despite advancements in understanding the pathophysiology of sepsis caused by gram negative bacteria (i.e. E.coli), clinical outcomes vary and the mortality rates remain high (Ibrahim, 2012; Dombrovskiy, *et al.,* 2007; Chaudhry, *et al.,* 2008; Munford, 2006).

Endogenous carbon monoxide (CO), a bi-product of inducible heme oxygenase (HO-1) has been shown to modulate inflammation (Coburn, 2012; Ozaki, *et al.,* 2012; Ryter, Choi, 2013; Basuroy, *et al.,* 2011). Transition metal carbonyls have recently been identified as potential CO-releasing molecules (CORMs) with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs (Motterlini, *et al.,* 2003; Motterlini, *et al.,* 2002). Our earlier studies (Liu, *et al.,* 2008; Sun, *et al.,* 2007, a; Sun BW, *et al.,* 2008, a; Sun BW, *et al.,* 2007, b) first confirmed that CORM-released CO attenuated leukocytes sequestration in the liver, lung and small intestine of burned and cecal ligation and puncture (CLP)-challenged mice by interfering with nuclear factor-□B (NF-κB) activation and protein expression of intercellular adhesion molecule-1 (ICAM-1) and, therefore, suppressing the endothelial cell pro-adhesive phenotype. Others investigated the bioactivity of CO on Escherichia coli and Staphylococcus aureus, suggesting that CO cause the rapid death of the two pathogenic bacteria, and CO can be utilized as an antimicrobial agent (Nobre, *et al.,* 2007; Tavares, *et al.,* 2011). In the present study, we further observe the suppressive effects and potential mechanisms of exogenous CO on bacterial vitality and toxicity using *E. coli* ATCC25922 as a research subject and carbon monoxide-releasing molecule α (CORM-2) as an intervention.

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MATERIALS AND METHODS

Ethics Statement

The animal research was approved by the Council on Animal Care at Jiangsu University on the Protection and the Welfare of Animals and followed the National Institutes of Health guidelines for the care and use of experimental animals.

Main reagents and instruments

CO-releasing molecules II (CORM-2) was purchased from Sigma (St. Louis, USA). Inactive CORM-2 (iCORM-2) could be prepared to dissolve CORM-2 in Dimethylsulfoxide. *E. coli* ATCC25922 was kindly offered by Department of Microbiological Testing of the Affiliated Hospital of Jiangsu University. Other reagents and instruments included quantitative enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor-α (TNF-α and interleukin-6 (IL-6) (R&D Systems, Inc., USA), a reverse transcription kit, and a SYBR Premix Ex Taq^{TM} α kit (Takara, China), a nucleoprotein quantitative analyzer (Eppendorf, Inc., Germany), a high-speed and low-temperature desktop centrifuge (Beckman, Inc., USA), a VMR-type small animal anesthesia machine (Matrx, Inc., USA), a CFX96TM Real-Time System (Bio-Rad, Inc., USA), and a Transmission Electron-microscope (FEI, Inc., USA).

Growth curve measuring and colony counting

Methods using growth curve have been developed to dynamically study the growth of bacteria. The bacteria were divided into five groups (n=6), including *E. coli*, *E. coli*+200μM CORM-2, *E. coli*+400μM CORM-2, *E. coli*+200μM iCORM-2, and *E. coli*+400μM iCORM-2. *E. coli* were inoculated and cultured on the Luria-Bertani (LB) medium until they reached peak viability and activity. Single colonies were diluted at the rate of 1:100, and inoculated in 20mL of LB medium. Corresponding concentrations of CORM-2 or iCORM-2 (200μM or 400 M) were added to each group (except *E. coli* group) to intervene, and the values at OD_{600} were measured at specific time points. In addition, 100μL of the bacterial culture was appropriately diluted and evenly spread on Mueller-Hinton agar plates.

Ultrastructural and negative staining detection of E. coli

The bacteria were divided and cultured in the same way mentioned above. The bacteria were harvested and glutaraldehyde-fixed for ultrastructural detection. A total of 20μL of bacteria was placed on a copper net and subjected to static cultivation for 10min. Next 2% Phosphotungstic acid dye was mixed with the bacteria on each sample for 2min at room temperature. Bacterial length, lysis and cellular morphology were then detected with a transmission electron microscope after the samples were dried naturally.

Measurement of E. coli growth dynamics

After the OD_{600} value in the *E. coli* group reached 0.4, 3 μL of bacteria culture from each group was spread onto 0.3% LB plates and cultured in a CO_2 incubator at 37^{o}C for 24 h, followed by the measurement of *E. coli* growth dynamics.

Quantitative real time-PCR (qRT-PCR)

The qRT-PCR procedure was conducted to detect expression levels of *FliA*, *dnaK and WaaQ* genes. The expression ratio of three genes was evaluated relatively to the reference gene, *E.coli* 16s rRNA.

Detection of colony distribution in major mouse organs after E. coli infection

Mice were divided into six groups (n=8) and were infected with *E. coli* with CORM-2 or iCORM-2 intervention as described above. Six hours after the injection, the mice were euthanized by excessive anesthesia administration. We then obtained and homogenized the mouse livers, lungs, spleens, and kidneys, diluted the homogenate appropriately, spread it onto LB plates, cultured the samples in a CO_2 incubator at 37^{o}C for 24h, and counted the resulting colonies.

Histologic studies

The tissue morphologic characteristics of liver and lung specimens harvested from the different groups of euthanized mice were evaluated under light microscopy.

Measurement of organ function, cytokine levels and myeloperoxidase (MPO) activityin E. coli-challenged mice

Blood samples were obtained by cardiac puncture of the left ventricle. Evaluation of hepatocellular injury was performed by determining the enzymatic activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Concentrations of TNF-α and IL-6 levels in the serum and tissue homogenate were assayed using ELISA kits.

STATISTICAL ANALYSIS

Data are presented as mean ± SD. SPSS 17.0 software was used for the data handling. Repeated-measures analysis of variance (ANOVA), factorial ANOVA, one-way ANOVA, and independent sample t-test were used in the data analysis. Statistical significance was defined at $P<0.05$.

RESULTS

Effects of CORM-2 on E. coli growth curve and colony counting

E. coli were divided into five groups (n=6) mentioned in 1.2. Each group (except *E. coli* group) was added with

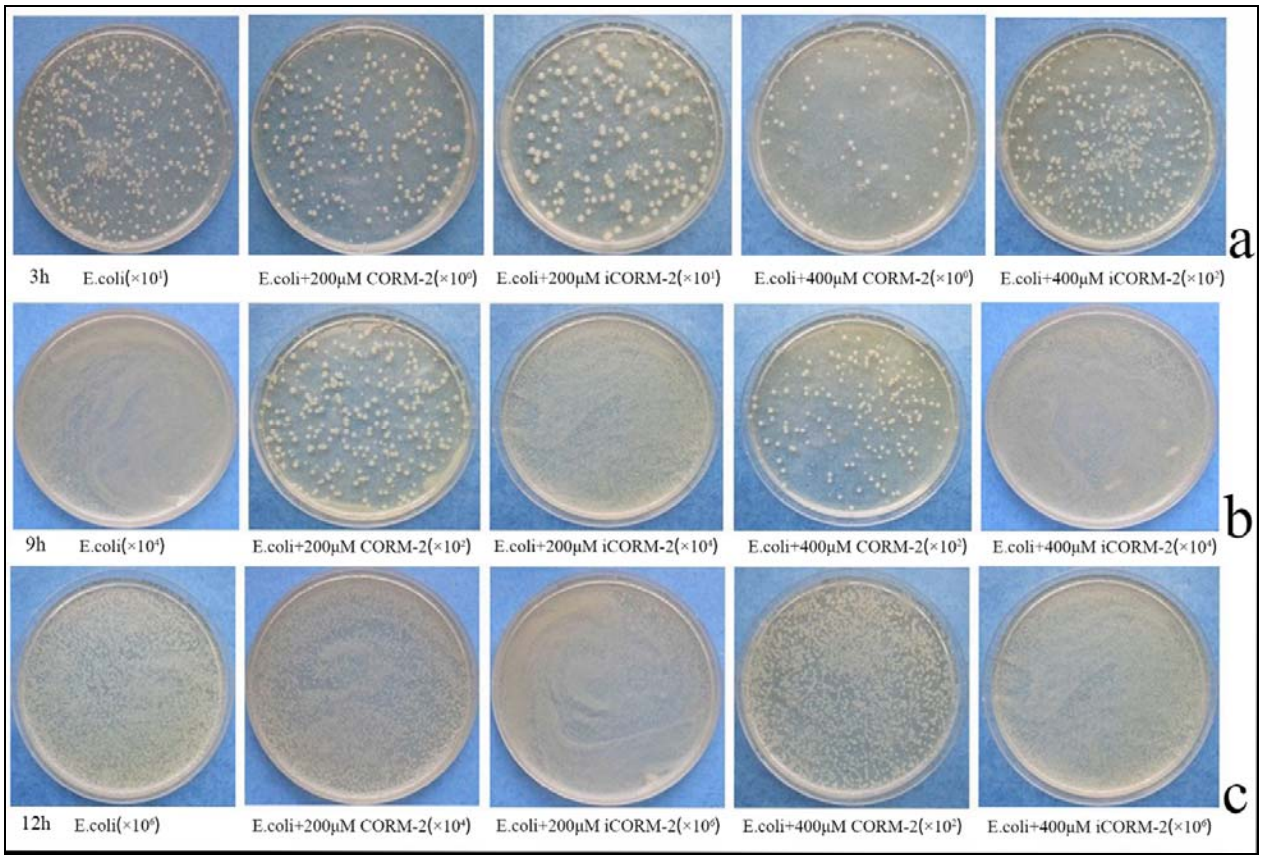

Fig. 1: Growth test of *E. coli* on Mueller-Hinton agar plates.

CORM-2 or iCORM-2 to coculture at corresponding concentrations (200μM or 400μM), and strain growth was monitored at hourly intervals until stationary phase was reached. Growth was monitored by both determining the optical density at 600 nm (OD_{600}) and counting the CFU. Bacterial growth was markedly suppressed in the presence of CORM-2 (figs. 1a, b, c). This was confirmed by observing growth curve of *E. coli* under intervention of CORM-2 or iCORM-2. When compared with *E. coli* group, the growth of *E. coli* in *E. coli*+200□M CORM-2 group was suppressed, and the platform phase entering time was significantly delayed, with a decrease in colony numbers (F value in figs. 2a and b were 1170.80 and 217.52 respectively, $P<0.01$). Group *E. coli*+400μM CORM-2 showed an even more obvious suppression of bacterial growth and decrease in colony numbers (F value in figs. 2c and d were 7948.34 and 14432.85 respectively, $P<0.01$).

CORM-2 was added to the bacteria culture at the concentration of 200μM or 400μM to observe its effects on the *E. coli* growth curve and resulting colonies. When compared to group *E. coli*, the bacterial growth was significantly suppressed (a, c) and colony numbers markedly decreased (b, d) in group *E. coli* + 200μM CORM-2 and in group *E. coli*+400μM CORM-2 ($P<0.01$).

Effects of CORM-2 on the ultrastructure and negative staining of E. coli

Bacteria that did not undergo CORM-2 intervention showed clear cell wall, wavy surface, and evenly distributed cytoplasm. Bacteria that were subjected to iCORM-2 intervention showed clear cell wall, wavy surface, and evenly distributed cytoplasm with no obvious vacuole or reticular structures. Bacteria subjected to CORM-2 intervention displayed blurred cell wall, decreased wavy substance, and bubbles protruding from the cell surface. Vacuoles or reticular structures, contracted cytoplasm, and decreased or disappeared pili and flagella were seen in the cells. Similar changes in the bacterial structures appeared in the negatively stained experiment (fig. 3).

The bacteria were cultured to logarithmic growth phase, each group (except *E. coli* group) was added with CORM-2 or iCORM-2 to intervene at appropriate concentrations (200μM or 400μM) for 30min Ultrastructure was observed using transmission electron micrographs (TEMs) (a, b, c at 6,600×, 8,900× and 11,500 magnification, respectively). The similar changes of bacterial structure appeared in the negative staining experiments (d, at 37,000× magnification).

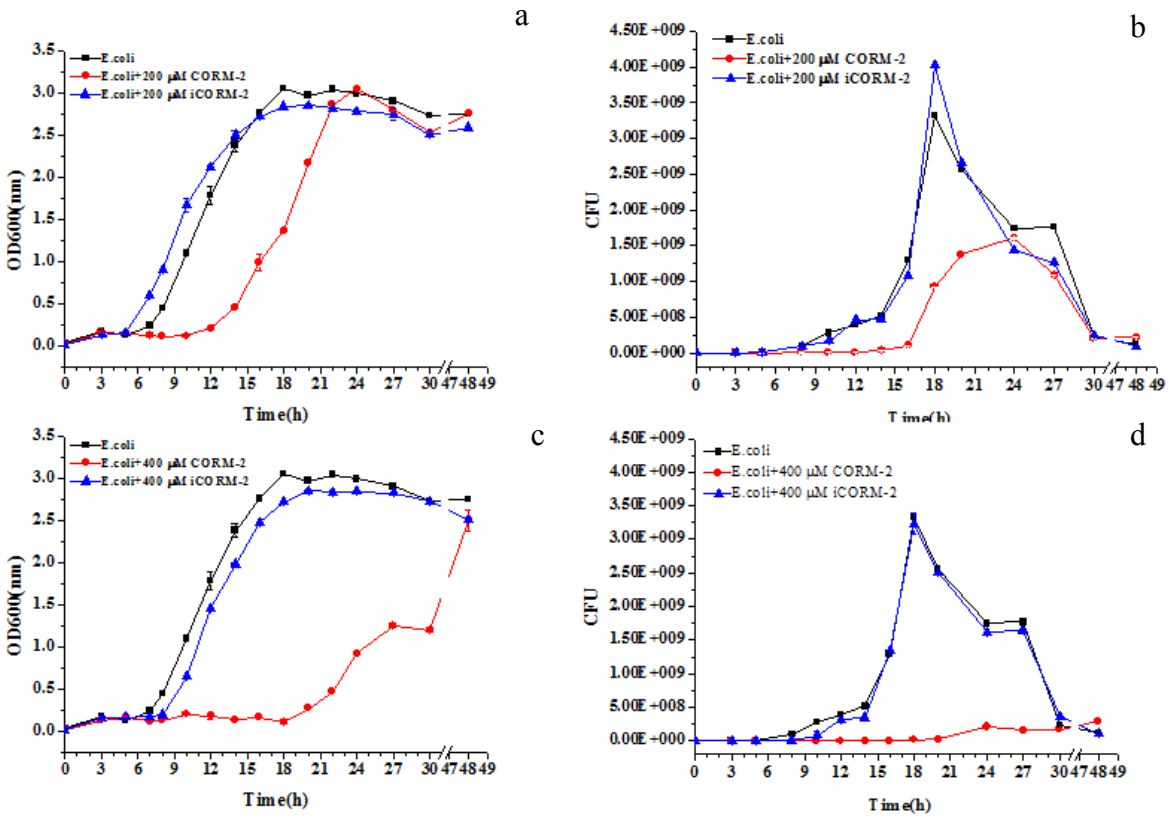

Fig. 2: Effects of CORM-2 on the growth curve and colony counting of *E. coli.*

Effects of CORM-2 on E. coli growth dynamics

To investigate whether CORM-2 can affect the growth dynamics of bacteria, *E. coli* was subjected to CORM-2 or iCORM-2 intervention and the culture were spread onto 0.3% LB plates and cultured in a CO_2 incubator at 37°C. Bacteria growth dynamics were measured up to 24h. Measurement of growth dynamics showed that, without CORM-2 intervention, *E. coli* grew well in culture with normal movement capacity. In contrast, under CORM-2 intervention, the growth ability of *E. coli* was obviously weakened and showed a markedly different pattern of growth inhibition with a dose-dependent manner in the group of *E.coli*+CORM-2 (fig. 4).

E. coli was subjected to CORM-2 or iCORM-2 intervention. Bacteria growth dynamics were measured by determining their diameter up to 24h. Without CORM-2 intervention, *E. coli* grew well in culture, the movement capacity of bacteria was normal. In contrast, under CORM-2 intervention, the growth ability of *E.coli* was obviously weakened, and showed a markedly different pattern of growth inhibition with a dose-dependent manner in the of *E. coli* + CORM-2 group (a, b, c, representative bacteria growth dynamics measured at 6, 12, 24h, respectively. d, when compared to the *E. coli* group, the bacterial growth was significantly suppressed in *E. coli* +200μM and 400μM CORM-2 groups, F=117.12 and 276.74, respectively, $P<0.01$).

Effects of CORM-2 on specific gene expression in E. coli

We chose three genes (*fliA*, *dnaK* and *waaQ*) for qRT-PCR detection. Since the preliminary experiment results showed that a significant difference occurred at a smaller CORM-2 dose (200μM), we chose the CORM-2 concentration of 200μM in detecting gene expression variations. The independent sample *t*-test results indicated that, compared with group *E. coli*, the *fliA* gene expression levels in *E. coli* subjected to CORM-2 intervention decreased (t=30.28, $P<0.01$), whereas the expression levels of the *dnaK* and *waaQ* genes increased (t values of -165.54, -168.88 and -187.28 respectively $P<0.01$) (fig. 5).

When bacteria were cultured to logarithmic growth phase, each group (except *E. coli* group) was added with CORM-2 or iCORM-2 to intervene at appropriate concentration (200μM) for 30min. The bacteria were then harvested for the qRT-PCR assay. When compared with group *E. coli*, the *fliA* gene expression levels in *E. coli* with CORM-2

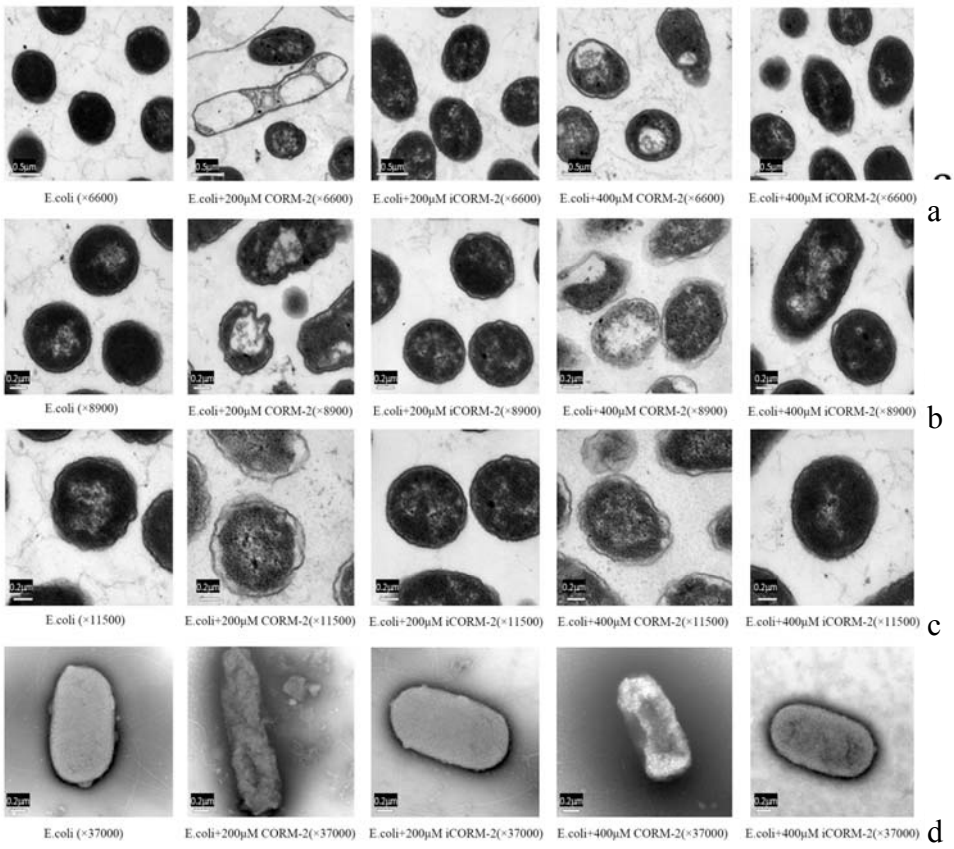

Fig. 3: Effects of CORM-2 on ultrastructure changes of *E. coli*

intervention significantly decreased (t=30.28, $P<0.01$); whereas the expression levels of *dnaK* and *waaQ* genes significantly increased (t values were -165.54 and -187.28, respectively, *, $P<0.01$).

Observation of the general mouse condition

In the *in vivo* study, the mice in the *E. coli* and *E. coli*+iCORM-2 groups showed apathetic symptoms including dull eyes, piloerection, and shortness of breath. Severe cases displayed yellow or red urine. Mice in the *E. coli*+CORM-2 group showed slight or none of the symptoms described above.

Detection of colony distribution in major mouse organs after E. coli infection

A total of 48 C_{57}BL/6 Mice were divided into six groups (n=8) and infected by *E. coli* with CORM-2 or iCORM-2 intervention. Six hours after the injection, the mice were killed by excessive anesthesia. The number of colonies in the mouse livers, lungs, spleens and kidneys of the *E. coli* and *E. coli* + iCORM-2 groups were significantly increased compared with that in the sham group ($P<0.001$), while the number of colonies in the major organs of the *E. coli* +200μM or 400μM CORM-2 were significantly decreased compared with that in the *E. coli* group (Liver: F=993.073, 313.440, respectively, $P<0.01$; Spleen: F=264.774; 1287.928, respectively, $P<0.01$; Lung: F=545.396; 2896.484, respectively, $P<0.01$; Kidney: F=2119.463; 2721.309, respectively P<0.001 fig. 6).

Mice were infected by *E. coli* with CORM-2 or iCORM-2 intervention. Six hours after the injection, mice livers, lungs, spleens and kidneys were homogenized. The colony counting in the mice liver, lung, spleen and kidney of *E. coli* and *E. coli* + iCORM-2 groups were significantly increased, while the colony counting in major organs in *E. coli*+200μM or 400μM CORM-2 groups were significantly decreased compared with that in *E. coli* group (Liver: F=993.073, Spleen: F=264.774, Lung: F=545.396, Kidney: F=2119.463, respectively, *, $P<0.01$; Liver: F=313.440, Spleen: F=1287.928, Lung: F=2896.484, Kidney: F=2721.309, respectively, **, $P<0.01$).

Histological examination of the major mouse organs after E. coli infection

The organ sections of the animals in each group were evaluated microscopically using H+E staining. Histological analysis showed that the liver and lung sections from mice in the sham group demonstrated a

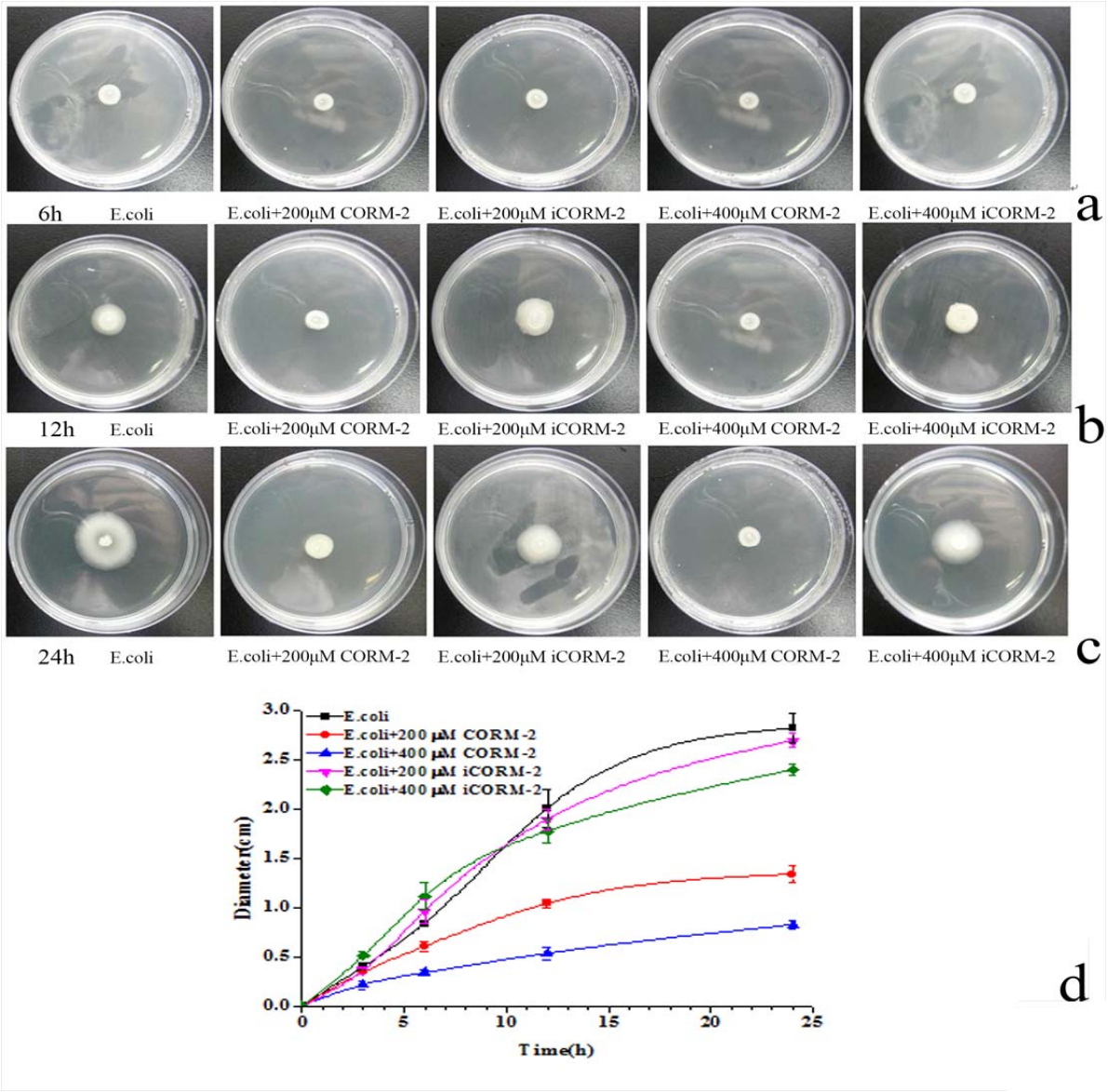

Fig. 4: Effects of CORM-2 on *E. coli* growth dynamics.

normal architecture. During the early phase of *E. coli* infection (6h), there was an increase in the number of neutrophils and significantly increased number of granulocyte infiltrations in the livers and lungs at 12h into the *E. coli* infection. These morphological changes in both lung and liver suggested the presence of systemic inflammation and multiple organ damage after *E. coli* challenge. In contrast, histological damage in and infiltration of the inflammatory cells to the lung and liver tissues were significantly attenuated in mice with 200μM CORM-2-invented *E. coli* injection, providing evidence that CORM-2 may be involved in the suppressive effects against *E. coli* vitality and toxicity (fig. 7).

Mice were infected by *E. coli* with 200μM CORM-2 or 200μM iCORM-2 intervention. After 12h of infection, the liver and lung specimens harvested. The tissues were embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Tissue morphologic characteristics were evaluated under light microscope. **a, e**: Sections from sham mice had normal architecture of the liver and lung; **b, f**: Sections from bacteria infected mice showed granulocyte infiltrations were significantly increased in liver and lung at 12h of *E. coli* infection; **c, g**: Section from mice with 200μM CORM-2 intervened *E. coli* infection showed that histological damage in lung and liver tissue and infiltration of the inflammatory cells to the tissue were significantly attenuated. The figure is

representative of at least three experiments performed on different days.

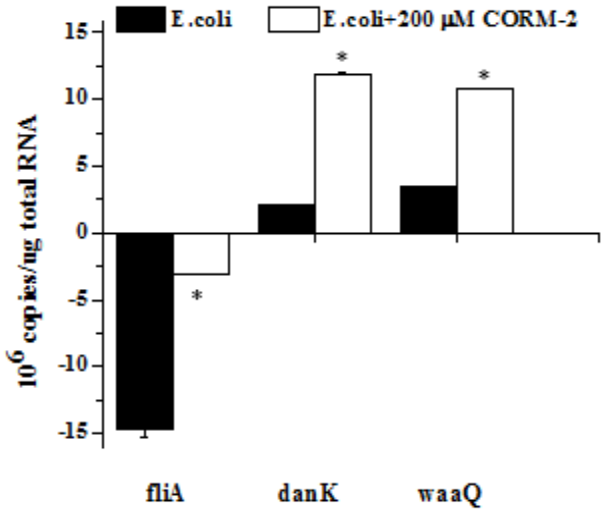

Fig. 5: Effects of CORM-2 on specific gene expression in *E. coli*

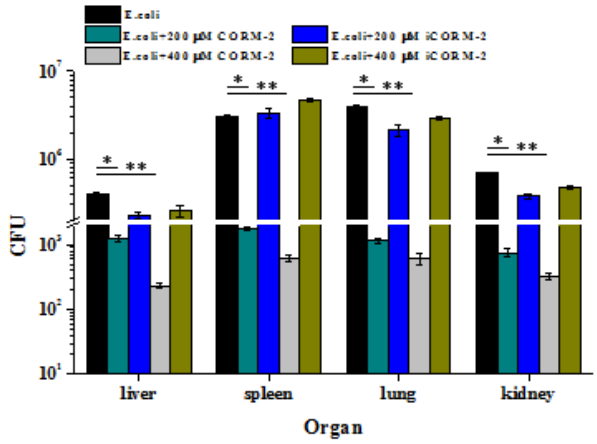

Fig. 6: Effect of CORM-2 on colony distribution in major mouse organs after *E. coli* infection.

Effects of CORM-2 on TNF-α and IL-6 levels in E. coli-infected mice

CORM-2- or iCORM-2-intervened *E. coli* were intraperitoneally injected into the mice. According to the results of factorial ANOVA done using the least significant difference method, compared with the *E. coli* and *E. coli*+iCORM-2 groups, the expression levels of TNF-α and IL-6 in the serum and liver and lung homogenates significantly decreased in the *E. coli* + CORM-2 group (F=10,948.24 and 317.72, respectively, *P*<0.01). Further analysis indicated that at 6h after the injection, expression levels of TNF-α and IL-6 in the *E. coli* + 200μM CORM-2 group decreased significantly compared with the *E. coli* group (figs. 8 a, b, F=482.386, 720.738, 408.219 and 46.352, 62.420, 126.869, respectively, P<0.01); the decrease of TNF-α and IL-6 expression levels in the *E. coli* +400μM CORM-2 group were even more obvious compared with those of the *E. coli* group (figs. 8 a, b, F=557.901, 806.49, 379.621 and 54.721, 99.319, 141.541, respectively *P*<0.01). Twelve hours after the injection, the down regulating effects of CORM-2 on TNF-α and IL-6 levels were maintained in the *E. coli* +400μM CORM-2 group (figs. 8c, d, F=175.598, 598.528, 523.884 and 214.861, 550.349, 2646.107, respectively *P*<0.01).

Effects of CORM-2 on organ function and MPO activity in E. coli infected mice

Hepatocyte injury was evaluated by determining the serum concentrations of ALT and AST. Twelve hours after *E. coli* or iCORM-2–intervened *E. coli* injection, serum hepatic transaminases were found to be markedly increased, whereas the values were significantly decreased in mice given the 200μM CORM-2-invented *E. coli* injection (compared with *E. coli* group, F=185.540 and 107.314, respectively, *P*<0.01). This decrease was found more obvious in mice given the 400□M CORM-2-invented *E. coli* injection (compared with *E. coli* group, F=282.476 and 249.222, respectively, *P*<0.01) (fig. 8e).

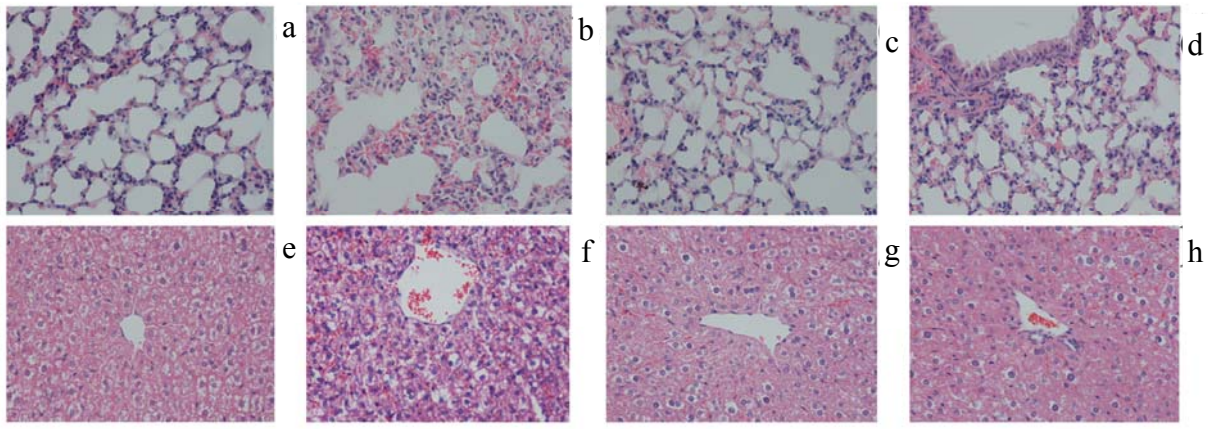

Fig. 7: Effects of CORM-2 on organs injury after *E. coli* infection

To determine whether *E. coli* -induced increases in polymorphonuclear leukocyte (PMN) accumulation in the livers and lungs were effectively prevented by CORM-2, the activity of MPO, an enzyme within the azurophilic granules of neutrophils, was assessed. MPO activity increased in the *E. coli* and *E. coli* + iCORM-2 group. When compared with group *E. coli*, the activity of MPO in the *E. coli* +200μM CORM-2 group was significantly suppressed in the mouse livers and lungs (F=235.477 and 473.192, respectively, $P<0.01$); the suppression was even more obvious in the *E. coli* +400μM CORM-2 group (F=130.048 and 283.646, respectively, $P<0.01$) (fig. 8f).

Mice were infected by *E. coli* with 200μM or 400μM CORM-2 intervention. At 6h after the injection, expression levels of TNF-α and IL-6 in the *E. coli*+200μM CORM-2 group decreased significantly compared with the *E. coli* group; the decrease of TNF-α and IL-6 expression levels in the *E. coli* +400μM CORM-2 group were even more obvious compared with the *E. coli* group. After 12h of injection, the downregulating effects of CORM-2 on TNF-α and IL-6 levels were maintained in the *E. coli* +400μM CORM-2 group. Mice were infected by *E. coli* with CORM-2 or iCORM-2 intervention. Twelve hours after *E. coli* or iCORM-2–intervened *E. coli* injection, serum hepatic transaminases were found to be markedly increased, whereas the values were significantly decreased in mice with 200μM CORM-2-invented *E. coli* injection. This decrease was found more obvious in mice with 400μM CORM-2-invented *E. coli* injection. The activity of MPO increased in the *E. coli* and *E. coli* +iCORM-2 group. When compared with the *E. coli* group, the activity of MPO in the *E. coli* +200μM CORM-2 group was significantly suppressed in mice livers and lungs; and the suppression was even more obvious in the *E. coli* +400μM CORM-2 group.

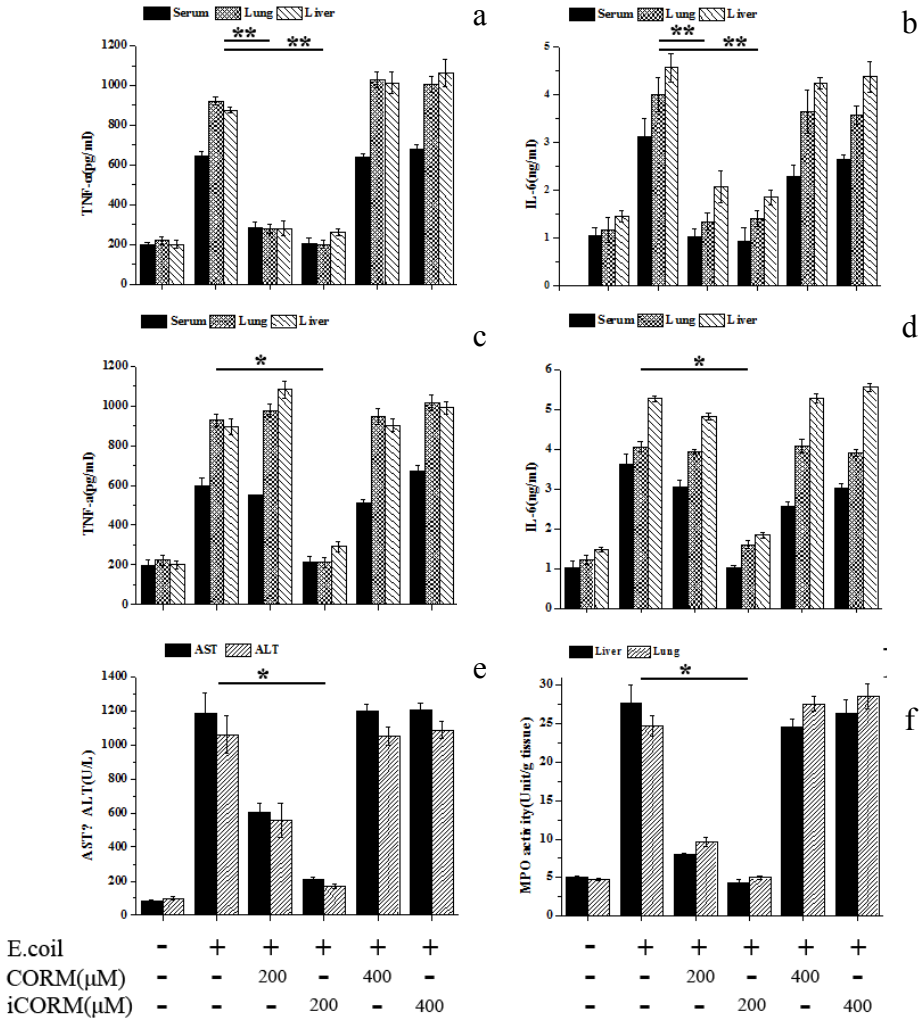

Fig. 8: Effects of CORM-2 on cytokines levels, organ function and MPO activity in *E. coli* infected mice.

DISCUSSION

The fundamental pathogenesis of sepsis has not yet been fully elucidated, and it is thought to involve numerous aspects including complex systemic inflammatory responses, gene polymorphism, immune dysfunction, coagulation abnormalities, tissue injury, and host abnormal reactions to pathogenic microorganisms and their toxins (Harjai *et al.,* 2013; Condotta *et al.,* 2013; Hernández *et al.,* 2013). Sepsis, which continues to be a clinical challenge in clinic as a leading cause of morbidity and mortality in severely ill patients, is closely related to changes in multi-system and multi-organ pathophysiology (Albayrak *et al.,* 2013; Dellinger *et al.,* 2013). Our previous data indicated that the intestine is one of the tissues that is most sensitive to the ischemia and reperfusion (I/R) induced by thermal injury or CLP (Xu, *et al.,* 2012) and that the translocation of intestinal bacteria or endotoxin is one of the most important pathogenic mechanisms.

E. coli belongs to a category of enteric bacilli that is commonly found in the human large intestine (Kabanov, Prokhorenko 2013; Villa *et al.,* 2013; Hernandez *et al.,* 2013). Most *E. coli* strains are harmless and keep a symbiotic relationship and benefit human by preventing the establishment of other pathogenic bacteria within the gut and producing vitamin K2 (Beyer *et al.,* 2013). However, during severe infection or sepsis, decreased immunity and a long-term lack of intestinal stimulation occur. As an opportunistic pathogen, *E. coli* can migrate from the gastrointestinal tract to extraintestinal sites, a phenomenon called bacterial translocation (Hansom *et al.,* 2012; Gouache *et al.,* 2013; Sözen *et al.,* 2012; Çitak *et al.,* 2013) and cause local or disseminated infections by its virulence factors including endotoxin, capsular, type III secretion systems, adhesins and exotoxins (Splichalova, Splichal, 2012; Pham *et al.,* 2012). Therefore, gram-negative *E. coli* ATCC25922 was employed as a research subject to observe the suppressive effects of exogenous CO on bacterial vitality and toxicity.

As an important chemical gas messenger, CO participates in various pathological processes including oxidative stress-induced inflammatory response, ischemia-reperfusion injury (Wei *et al.,* 2010) and endotoxin shock (Tsoyi *et al.,* 2010), and plays an essential role in anti-inflammation, anti-apoptosis, cell protection and antimicrobial effects (Davidge *et al.,* 2009; Desmard *et al.,* 2009). Studies have confirmed that exogenous CO can significantly improve the functions of the heart, brain, liver, lungs and kidneys and suppress inflammatory responses during sepsis (Sun *et al.,* 2008b; Sun *et al.,* 2007c). Although there are various bacterial intervention methods, no studies aimed at exploring the effects of exogenous CO on bacterial vitality and toxicity has been published.

Growth curve and colony counting reflect bacterial growth, by which we can realize real-time monitoring of bacterial growth in any time period (Bren *et al.,* 2013; Yamamotoya *et al.,* 2012). Our experiment showed that bacterial vitality and toxicity were significantly suppressed after CORM-2 intervention and that, compared with the *E. coli* and *E. coli* +iCORM-2 groups, the ultrastructure of the cells in the *E. coli* +CORM-2 group showed blurred cell walls, decreased amounts of wavy substances, and bubbles protruding from the cell surfaces. Vacuoles or reticular structure as well as contracted cytoplasm and decreased or disappeared pili and flagella were also seen. Similar changes in the bacterial structures appeared in the negatively stained experiment. In parallel, measurement of *E. coli* growth dynamics showed that, compared with the *E. coli* group, the movement capacity of bacteria was obviously weakened in the *E.coli*+CORM-2 group. These finding indicate that CORM-2 can cause significant damage to bacterial structure, which might be one of the most important mechanisms of bacterial vitality and toxicity suppression.

In the present study, qRT-PCR was preformed on three genes, *fliA, dnaK* and *waaQ*, which have significant different expression after CORM-2 intervention in *E. coli*. *FliA* is the σ 28 factor that interacts with the coding region of flhA and can recognize promoter, cause DNA hybrid melting, and suppress non-specific transcription. The *dnaK* gene encodes molecular chaperone DnaK (HSP70), which belongs to the heat shock protein super family and plays an essential role in stressed conditions (Savitt *et al.,* 2012). DnaK is highly conserved during evolution, protecting cell survival and maintaining normal intracellular life activities. The chaperone complex DnaK-DnaJ-GrpE has essential functions in stabilizing post-transcriptional protein expression and maintaining protein structure and function. WaaQ, which is encoded by the *waaQ* gene, is an operon in the biosynthesis of the LPS core (Wang and Quinn 2010). Mutation in the *waaQ* gene leads to disappearance of the LPS core band, while *waaQ* inactivity can improve cell adsorption under both aerobic and anaerobic conditions. The qRT-PCR results showed that the expression of these three essential genes (*fliA, dnaK* and *waaQ*) was significantly influenced after 200μM CORM-2 intervention. The observed down regulation in *fliA* gene expression indicated that CORM-2 can influence flagellum formation and metabolism, thus affecting the motor ability of *E. coli*. Expression levels of *dnaK* and *waaQ* were upregulated, which affected cell stress status and LPS synthesis. This might be another important mechanism by which CORM-2 suppresses bacterial vitality and toxicity. For the first time, we report here that exogenous CO intervenes in the expression of *fliA, dnaK* and *waaQ* genes in *E. coli*.

To further investigate whether the vitality and toxicity of CORM-2 intervened *E. coli* were suppressed and CORM-2 intervened *E. coli*-induced inflammation was attenuated *in vivo*, we intraperitoneally injected CORM-2- or iCORM-2-intervened *E. coli* into C57BL/6 mice. The mouse survival rate, numbers of colonies counted in the mouse livers, lungs, spleens and kidneys, and tissue MPO as well as cytokine levels was measured. We found that the mice in the *E. coli* and *E. coli* +iCORM-2 groups showed symptoms such as apathy and shortness of breath. However, the mice in the *E. coli* +CORM-2 group showed slight or none of the symptoms described above. The survival rates of mice in the *E. coli* +CORM-2 group tended to improve in a dose-dependent fashion, with 60%-100% survival rate in the *E. coli* +CORM-2 group treated with 200μM and 400μM CORM-2.

Furthermore, we found that 6h after injection with *E. coli* or iCORM-2 intervened *E. coli,* the colony counts in the mouse livers, lungs, spleens and kidneys were significantly increased. However, the colony counts in the above organs were markedly decreased in the *E. coli* +CORM-2 group. This finding indicates that CORM-2 can effectively inhibit bacterial vitality, thus decreasing the bacterial growth and colony formation.

MPO is an enzyme that is found predominantly in the azurophilic granules of PMN. Tissue MPO activity is frequently utilized to estimate leukocyte infiltration and correlates significantly with PMN counts determined histochemically within tissues (Palani *et al.,* 2012; Zhan *et al.,* 2011). In the present study, we found that the MPO activities in the livers and lungs were markedly enhanced after the injection of *E. coli* or *E. coli* +iCORM-2, indicated that *E. coli* injection induced tissue PMN accumulation and lead to organ inflammation and tissues damage. However, obvious decrease occurred in tissue MPO activity in the livers and lungs of mice injected with CORM-2 intervened *E. coli*, which strongly supports the potential mechanisms of CORM-2 suppressing the vitality and toxicity of *E. coli*

TNF-α is a pleiotropic cytokine with strong pro-inflammatory and immunomodulatory properties that plays a critical role in inflammation and inflammatory disease (Peña *et al.,* 2010; Gül *et al.,* 2011). Thus, we further investigated whether CORM-2 intervened *E. coli* injection can downregulate the tissue homogenate and serum levels of TNF- α and IL-6. The results showed that in mice injected with *E. coli* or *E. coli* +iCORM-2, tissue homogenate and serum TNF-α and IL-6 levels increased significantly, whereas obvious decrease occurred in tissue homogenate and serum TNF-α and IL-6 levels in mice injected with CORM-2-intervened *E. coli*. Our findings strongly indicated that CORM-2 appears to suppress the vitality and toxicity of *E. coli*, therefore inhibiting upregulation of inflammatory production induced by *E. coli*, and effectively alleviating inflammatory response in the livers and lungs.

CONCLUSION

These data of the present study consisting of measured growth conditions, colony formation, and important gene expression of *E. coli in vitro*, detected survival rate, cytokine expression level, and MPO activity in bacteria-injected mice indicated that exogenous CO can significantly suppress bacterial vitality and toxicity which leads to a decrease in *E. coli* ability to induce inflammation, resulting in a higher rate of mice survival. This may be associated with the regulatory functions of CORM-2 on the expression of essential genes (*fliA, dnaK* and *waaQ*) in *E. coli*.

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