

1 **Azithromycin reduces systemic inflammation and provides survival benefit in murine**
2 **model of polymicrobial sepsis**

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4 **Short title:** Azithromycin reduces systemic inflammation in sepsis

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27 **ABSTRACT**

28 Sepsis is a life threatening systemic inflammatory condition triggered as a result of excessive
29 host immune response to infection. In the past, drugs modulating immune reactions have
30 demonstrated protective effect in sepsis. Azithromycin (macrolide antibiotic) with
31 immunomodulatory activity was therefore evaluated in combination with ceftriaxone in a
32 more clinically relevant murine model of sepsis induced by caecal ligation and puncture
33 (CLP). First, mice underwent CLP and 3 h later were administered with vehicle, sub-effective
34 dose of ceftriaxone (100 mg/kg, *subcutaneous*) alone or in combination with
35 immunomodulatory dose of azithromycin (100 mg/kg, *intraperitoneal*). Survival was then
36 monitored for 5 days. Parameters like body temperature, blood glucose, total white blood cell
37 count, plasma glutathione (GSH), plasma and lung myeloperoxidase (MPO) as well as
38 cytokine (interleukin IL-6, IL-1 β , tumor necrosis factor- α) levels along with bacterial load in
39 blood, peritoneal fluid and lung homogenate were measured 18 h after CLP challenge.
40 Combination group significantly improved the survival of CLP mice. It attenuated the
41 elevated levels of inflammatory cytokines and MPO in plasma and lung tissue and increased
42 the body temperature, blood glucose and GSH which were otherwise markedly decreased in
43 CLP mice. Ceftriaxone exhibited significant reduction of bacterial count in blood, peritoneal
44 fluid and lung homogenate, while co-administration of azithromycin did not further reduce it.
45 This confirms that survival benefit by azithromycin was due to immunomodulation and not
46 by its antibacterial action. Findings of this study indicate that azithromycin in combination
47 with ceftriaxone could exhibit clinical benefit in sepsis.

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49 **Key words: Polymicrobial sepsis, cytokines, survival, bacterial count**

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53 **INTRODUCTION**

54 Sepsis is a generalized systemic inflammatory condition elicited by pro-inflammatory
55 cytokines released by host immune cell in response to exotoxin or endotoxins secreted by
56 bacteria. Overproduction of these cytokines is associated with multiple organ failure which is
57 the major cause of death in critically ill and elderly patients. Despite recent advances in our
58 understanding of the pathophysiological mechanism of sepsis and improved antimicrobial
59 therapy, the mortality rate from sepsis remains frustratingly high. Unfortunately many of the
60 therapeutic options proposed over the years for the management of sepsis and its
61 complication have either failed to meet their initial expectations or remained unproved.
62 Strategies are currently being developed to minimize the inflammatory response associated
63 with sepsis through immunotherapy (1).

64 Several reports have provided evidence of immunomodulatory activity of macrolide
65 antibiotics (2, 3, 4). As a result, macrolides have proved beneficial in chronic pulmonary
66 inflammatory conditions like diffuse panbronchiolitis, cystic fibrosis, asthma and
67 bronchiectasis (5, 6). Due to this behaviour, they have earlier demonstrated clinical benefits
68 in critically ill patients with CABP or Gram negative sepsis by reducing the disease severity,
69 length of hospital stay and mortality rates (7, 8).

70 Azithromycin, belonging to macrolide antibacterial class has been earlier reported to provide
71 survival benefit in the lipopolysaccharide (LPS) induced sepsis model (9). In the present
72 study, we aimed to extend this investigation in the caecal ligation and puncture (CLP) model
73 of polymicrobial sepsis. CLP model is widely preferred sepsis model as it closely mimics the
74 clinical condition of human sepsis (10). Azithromycin acts on Gram positive bacteria and is
75 bacteriostatic , therefore in this study it was combined with ceftriaxone, a third generation
76 cephalosporin antibiotic to control the progression of polymicrobial infection.

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79 RESULTS

80 Survival in Sepsis model

81 In the LPS induced sepsis model, azithromycin administered 1 h prior to lethal dose of LPS
82 showed dose dependent improvement in 24 h survival. Azithromycin at 100 mg/kg, *i.p.*
83 provided survival in 75 % of mice as compared to 12.5% in LPS group, which was
84 statistically significant ($p<0.05$) (**Figure 1**). Survival benefit of azithromycin was further
85 determined in CLP sepsis, a model having best correlation to human sepsis. In this model,
86 azithromycin at 100 mg/kg dose provided protection in 37.5 % of mice in contrast to 75% in
87 LPS treated mice, indicating that azithromycin might have little or no effect on the spread of
88 infection. In order to control bacterial infection, azithromycin was further combined with
89 ceftriaxone (broad spectrum antibiotic) at sub-effective dose. This dose of ceftriaxone was
90 identified from our initial survival studies in CLP mice where it provided protection in 37.5
91 % of mice (data not shown). Subsequently, sub-effective dose of ceftriaxone (100 mg/kg,
92 *s.c.*) was combined with immunomodulatory dose of azithromycin (100 mg/kg, *i.p.*) and the
93 survival was monitored for 5 days. A log-rank test analysis of the 5 day survival curve in
94 CLP sepsis showed that survival rate in combination group was significantly higher
95 compared to CLP control ($p<0.01$) (**Figure 2**).

96 Effect on blood glucose, body temperature and total WBC count

97 Blood glucose, body temperature and WBC count are affected during systemic inflammation.
98 WBC count was significantly ($p<0.001$) reduced in LPS group ($2.77\times 10^3/\mu\text{L} \pm 0.14$) compare
99 to normal group ($7.02\times 10^3/\mu\text{L} \pm 0.63$) which was not reversed by treatment with
100 azithromycin (**Figure 3A**). Blood glucose and body temperature were also significantly
101 reduced in LPS treated mice. The values of blood glucose and body temperature in normal
102 mice were 119.5 ± 2.4 mg/dL and 99.2 ± 0.7 °F which dropped to 20.67 ± 2.1 mg/dL
103 ($p<0.001$) and 92.0 ± 0.7 °F ($p<0.001$), respectively in LPS treated mice. Azithromycin at 100

104 mg/kg, *i.p.*, significantly elevated the blood glucose (51.5 ± 9.0 mg/dL, $p < 0.01$) and body
105 temperature (96.28 ± 0.4 °F, $p < 0.001$) (**Figure 3B and 3C**).

106 In the CLP mice also, the total WBC count was significantly lower than the sham control
107 group ($1.13 \times 10^3/\mu\text{L} \pm 0.14$ versus $6.18 \times 10^3/\mu\text{L} \pm 0.25$; $p < 0.001$). Ceftriaxone treatment
108 increased WBC count although not statistically significant ($2.77 \times 10^3/\mu\text{L} \pm 0.73$). While,
109 azithromycin combined with ceftriaxone neither increased nor decreased the WBC count
110 ($2.58 \times 10^3/\mu\text{L} \pm 0.36$) (**Figure 4A**). In comparison to the sham group, mice with CLP caused
111 significant reduction in the blood glucose (from 99.55 ± 3.3 to 29.0 ± 13.0 mg/dL; $p < 0.001$)
112 and body temperature (from 99.33 ± 0.4 to 92.97 ± 1.1 °F; $p < 0.001$). CLP mice treated with
113 ceftriaxone (100 mg/kg, *s.c.*) significantly increased the blood glucose (54.0 ± 7.6 mg/dL;
114 $p < 0.05$) as well as body temperature (96.33 ± 0.4 °F; $p < 0.01$), and in combination with
115 azithromycin there was further increase in the blood glucose (72.7 ± 6.5 mg/dL; $p < 0.01$) and
116 body temperature (97.0 ± 0.5 °F; $p < 0.01$) (**Figure 4B and 4C**).

117 **Effect on GSH and MPO levels**

118 The levels of GSH (an endogenous thiol antioxidant) and MPO (an inflammatory enzyme)
119 are considerably altered during sepsis. There was a significant suppression in plasma GSH
120 level and significant elevation in plasma MPO levels in LPS treated mice compared to normal
121 group. GSH decreased from 2.37 ± 0.2 to 0.82 ± 0.04 mg/mL ($p < 0.001$) and MPO increased
122 from 290.2 ± 88.9 to 871.81 ± 86.2 $\mu\text{M}/\text{min}/\text{mL}$ ($p < 0.001$), while LPS treated mice
123 administered azithromycin produced dose dependent rise in GSH (0.96 ± 0.1 mg/mL at
124 50 mg/kg and 1.04 ± 0.03 mg/mL at 100 mg/kg) and decrease in MPO levels (694.03 ± 67.7
125 $\mu\text{M}/\text{min}/\text{mL}$ at 50 mg/kg and 629.08 ± 42.3 $\mu\text{M}/\text{min}/\text{mL}$ at 100 mg/kg), however the effect
126 was significant ($p < 0.05$) only at 100 mg/kg (**Figure 5A and 5B**).

127 In the CLP challenged mice, plasma GSH was significantly reduced and plasma MPO was
128 significantly increased compared to sham group (GSH: 1.02 ± 0.1 in CLP versus 2.08 ± 0.2
129 mg/mL in sham group, $p < 0.01$ and MPO: 620.64 ± 45.9 in CLP versus 105.84 ± 33.8

130 $\mu\text{M}/\text{min}/\text{mL}$ in sham group, $p<0.01$). Ceftriaxone treatment did not cause significant change
131 in the GSH and MPO. While, combination group significantly elevated GSH (1.98 ± 0.3
132 mg/mL ; $p<0.01$) and decreased MPO ($275.13 \pm 50.4 \mu\text{M}/\text{min}/\text{mL}$; $p< 0.01$) (**Figure 6A and**
133 **6B**). In lung tissue, MPO levels in CLP mice increased significantly compared to sham group
134 (60.27 Vs 8.51 U/gm of tissue). Ceftriaxone did not have any effect on lung MPO (52.04
135 U/gm), while ceftriaxone and azithromycin combination significantly reduced its value
136 (44.20 U/gm) (**Figure 6C**).

137 **Effect on circulating cytokine levels**

138 Plasma cytokine concentrations in LPS sepsis model are illustrated in **figure 7**. Pro-
139 inflammatory cytokines are the main mediators of inflammation during sepsis. As expected,
140 significant increase in IL-6, IL-1 β and TNF- α levels occurred in the LPS treated group
141 compared to control group. The values of IL-6, IL-1 β and TNF- α in normal control group
142 were 18.47 ± 3.5 , 8.28 ± 0.6 and 11.15 ± 0.5 pg/mL which significantly increased to $344.44 \pm$
143 20.8 ($p<0.001$), 983.33 ± 72.7 ($p<0.001$) and 113 ± 12.4 pg/mL ($p<0.001$), respectively in
144 LPS treated mice. Azithromycin administered to LPS challenged mice showed dose
145 dependent inhibition of these cytokines. At 100 mg/kg, azithromycin showed distinct
146 reduction in IL-6 (47.28 ± 8.4 pg/mL; $p<0.001$), IL-1 β (15 ± 1.2 pg/mL; $p<0.001$) and TNF- α
147 (34.63 ± 1.3 pg/mL; $p<0.001$) compared to LPS group.

148 Similarly, CLP challenged mice showed statistically significant rise in the plasma cytokine
149 levels compared to sham group (IL-6, TNF- α and IL-1 β : 15892 ± 1250.6 , 119.3 ± 17.4 ,
150 156.61 ± 43.9 pg/mL in CLP group versus 38.92 ± 9.0 , 7.7 ± 0.2 , 15.76 ± 2.5 pg/mL,
151 respectively in sham group). Ceftriaxone significantly reduced the IL-6 in the CLP mice from
152 15892 ± 1250.6 to 925 ± 154.5 pg/mL ($p<0.001$), TNF- α from 119.3 ± 17.4 to $41.03 \pm$
153 3.9 pg/mL ($p<0.001$) and IL-1 β from 156.61 ± 43.9 to 52.19 ± 6.2 pg/mL ($p<0.05$).
154 Combination group also demonstrated significant decrease in IL-6, TNF- α and IL-1 β

155 compared to CLP group and the values in pg/mL for each were 565 ± 25.8 ($p < 0.001$), $23.10 \pm$
156 1.2 ($p < 0.001$) and 36.23 ± 3.4 ($p < 0.01$), respectively (**Figure 8**).

157 Lung levels of IL-6, TNF- α and IL-1 β in CLP mice were 466, 251.2 and 526.6 pg/mL,
158 respectively compared to no levels in sham group (data not shown in graph). Ceftriaxone
159 treated CLP mice did not mitigate these cytokine levels while ceftriaxone and azithromycin
160 combination brought about significantly reduction (IL-6: 57.06 ± 4.07 , TNF- α : 67.17 ± 13.08
161 and IL-1 β : 316.53 ± 21.94 pg/mL) (**Figure 9**).

162 **Effect on Bacterial load in blood, peritoneal fluid and lung homogenate**

163 In order to determine whether the protective effect by combination group in the CLP model
164 was due to synergistic antimicrobial activity, bacterial colony forming units (CFU) was
165 estimated in the blood, peritoneal fluid and lung homogenate following CLP. Ceftriaxone
166 showed significant reduction in bacterial count in blood, lung and peritoneal fluid while
167 azithromycin in combination with ceftriaxone provided no further reduction compared to
168 CLP group. These suggest that azithromycin had no antibacterial effect in this polymicrobial
169 sepsis model (**Figure 10**).

170 **DISCUSSION**

171 Macrolides are reported to produce immunomodulatory action by inhibiting production of
172 reactive oxygen species, pro inflammatory cytokines and transcription factor of inflammation
173 like nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1). They are also known to
174 inhibit adhesion of molecules on endothelial cells and infiltration of neutrophils from blood
175 to tissues (11, 12). In a previous *in vitro* study, azithromycin produced dose dependent
176 reduction in cytokines and nitrate/nitrite levels in LPS stimulated J774 macrophages (13). In
177 another report, azithromycin had demonstrated protective effect from systemic damage
178 induced by lethal dose of LPS in mice by inhibiting TNF- α (9). Beneficial effect of
179 azithromycin on pulmonary inflammation has been established earlier by single oral or
180 intraperitoneal dose (9, 14, 15, 16).

181 In the present study, we have demonstrated the survival benefit of azithromycin in murine
182 model of sepsis. Clinically, sepsis is accompanied with infection and hence azithromycin was
183 evaluated in CLP model of sepsis which correlates more with human sepsis. Azithromycin
184 administered alone did not provide significant protection in CLP mice. This could be due to
185 its inability to stop the progression of infection. For survival benefit in CLP model of sepsis,
186 it is important to manage both infection and inflammation. Infection can be very well handled
187 by treatment with suitable antibacterial agents. Therefore, in the current study we have
188 combined azithromycin with ceftriaxone, a broad spectrum β -lactam antibiotic to combat
189 infection. A sub-effective dose of ceftriaxone was determined earlier in CLP mice and further
190 used in combination with immunomodulatory dose of azithromycin. Ceftriaxone plus
191 azithromycin produced beneficial protective effect in CLP challenged mice compared to
192 standalone group. This effect of combination was not associated with synergistic antibacterial
193 activity of both as azithromycin did not potentiate the antibacterial effect of ceftriaxone as
194 observed through the bacterial load in blood, lung and peritoneal fluid.

195 LPS and CLP challenge mice significant reduced the WBC count compare to normal mice
196 possibly due to immunosuppression observed during later phase of sepsis. Similar decrease in
197 leukocyte count was also noted in previous publications (17, 18). In both the models,
198 azithromycin neither elevated nor further suppressed the WBC count, thus suggesting it to
199 have no immunosuppressive property. Sepsis is reported to produce hypodynamic state by
200 reducing body temperature and blood glucose (19). Therefore these parameters were
201 measured in all the groups. As expected, mice subjected to LPS treatment or CLP challenge
202 significantly reduced the blood glucose and body temperature while azithromycin treatment
203 significantly reversed them.

204 In sepsis, reactive oxygen species and MPO production surpass antioxidant defenses and
205 leads to a state of oxidative stress that triggers inflammation and causes direct mitochondrial
206 damages, which results into sepsis-induced organ dysfunction (20). Increased free radicals

207 that caused peroxidation of membrane lipids have been associated with reduction of GSH
208 content (21). GSH an endogenous antioxidant, not only provides protection from reactive
209 oxygen species but also inhibit the production of several inflammatory cytokines and
210 chemokines (22). Unlike normal control group, LPS treated mice showed decreased in GSH
211 while azithromycin significantly increased its levels. In our study, we have demonstrated
212 elevation in plasma MPO in LPS treated mice which was significantly reduced by
213 azithromycin. In the CLP mice, as expected there was decrease in plasma GSH and increase
214 in MPO levels compared to sham group. Azithromycin in combination with ceftriaxone
215 produced significant increase in plasma GSH and suppression of MPO levels.

216 Previous studies have demonstrated that TNF- α , IL-1 β and IL-6 are the key contributors in
217 the pathogenesis of sepsis (23, 24, 25). They are primarily responsible for apoptosis, necrosis
218 of tissues and thereby multiple organ failure. TNF- α and IL-1 β are reported to initiate
219 inflammatory response in early sepsis and they stimulate the production of IL-6 which
220 dominates in the later phase of sepsis (26). IL-6 has been extensively examined in patients
221 with sepsis and its concentrations correlates more closely with severity and clinical outcome
222 (26). Present study demonstrated elevated levels of these cytokines in the LPS and CLP
223 challenged mice, while azithromycin significantly mitigated its levels. Sepsis associated lung
224 injury is considered to be a leading cause of death (27). Azithromycin in combination with
225 ceftriaxone produced statistically significant reduction in levels of inflammatory cytokines in
226 lung tissue. Azithromycin treatment also led to a significant suppression of lung MPO
227 activity, suggesting that azithromycin inhibited the neutrophil infiltration into the lung
228 parenchyma and alveolar spaces. Finding of our study is in agreement with earlier report
229 where azithromycin had demonstrated beneficial effect in combination with ceftriaxone in
230 mouse model of lethal pneumococcal pneumonia due to its immunomodulatory activity (28).
231 In the present study, we have demonstrated immunomodulatory activity of azithromycin in
232 both LPS and CLP sepsis model. Azithromycin significantly reduced the inflammatory

233 cytokines, MPO levels and increased the GSH levels which are otherwise altered to a
234 pathological level in sepsis leading to organ failure. We have further demonstrated protective
235 role of azithromycin in sepsis associated lung damage.

236 Findings of this study indicate that combination therapy of ceftriaxone and azithromycin has
237 the potentials to improve survival in critically ill patients with bacterial infection.

238 **METHODS**

239 All the experimental protocols were approved by the Institutional Animals Ethics Committee
240 (IAEC) of Wockhardt Research centre, India. Female Swiss albino mice, weighing 25-30 gm
241 were used for entire study. Mice were housed in cages with standard rodent feed and drinking
242 water provided *ad libitum*. The light cycle was controlled automatically (on at 7:00 a.m. and
243 off at 7:00 p.m.), and the room temperature was regulated to 18-22°C with 40-70% humidity.

244 **LPS induced sepsis model**

245 The purpose of evaluating azithromycin in this model was to determine its
246 immunomodulatory dose in our experimental set up. LPS derived from *Escherichia coli*
247 serotype 0127:B8 (Sigma-Aldrich) was dissolved in saline and injected intraperitoneally (*i.p.*)
248 in a fix volume of 0.25 mL to induce sepsis in mice. Two different LPS doses were used; a
249 lethal dose of 1500 µg/mouse for survival study where monitoring was done up to 24 h and a
250 sub-lethal dose of 1000 µg/mouse for estimation of body temperature, blood glucose, total
251 white blood cell (WBC) count, cytokines, myeloperoxidase (MPO) and glutathione (GSH)
252 levels.

253 **Treatment groups for LPS induced sepsis model**

254 For survival experiment, 1 h prior to lethal dose of LPS, mice were administered vehicle
255 (saline) and azithromycin (Azithral; Alembic Pharmaceutical) at doses of 25, 50 and 100
256 mg/kg, intraperitoneally. From this survival study, two effective doses of azithromycin were
257 further selected to assess the different biochemical and physiological parameters in mice
258 exposed to sub-lethal dose of LPS.

259 **CLP induced sepsis model**

260 Mice were anesthetized with mixture of ketamine/xylazine (100/10 mg/kg, *i.p.*) and abdomen
261 was disinfected with iodine. A small mid-abdominal incision was made to expose the cecum.
262 A distended portion of the cecum just distal to the ileocecal valve was isolated and ligated
263 with a 3-0 silk suture in a manner not to disrupt bowel continuity. The ligated portion of
264 cecum was punctured twice with an 18-gauge needle. To ensure the patency of the punctured
265 portion, cecum was gently squeezed until small quantity of feces extruded through them. The
266 cecum was placed back in the abdomen, and the incision was closed with sutures. The mice
267 were then resuscitated with 1 mL of saline injected subcutaneously and returned to their
268 cages with free access to feed and water. Sham-operated controls were treated in an identical
269 manner, but without ligation and puncture. Survival was assessed twice a day for 5 days. In a
270 separate study, parameters like body temperature, blood glucose, total WBC count, plasma
271 GSH, cytokines and MPO levels in plasma and lung tissue along with bacterial count in
272 blood, peritoneal fluid and lung homogenate were measured after 18 h of CLP challenge.

273 **Treatment group for CLP induced sepsis model**

274 From the initial survival studies performed in CLP mice (data not shown), sub-effective dose
275 of ceftriaxone (100 mg/kg, *s.c.*) providing protection in 37.5 % of mice was determined. The
276 survival study included a sham group, CLP mice treated with vehicle (saline), ceftriaxone
277 (100 mg/kg, *s.c.*), azithromycin (100 mg/kg, *i.p.*) and both in combination. For estimation of
278 biochemical and other parameters, groups included were sham control, CLP mice treated with
279 vehicle (saline), ceftriaxone (100 mg/kg, *s.c.*) and ceftriaxone (100 mg/kg, *s.c.*) plus
280 azithromycin (100 mg/kg, *i.p.*), all administered 3 h post CLP.

281 **Experimental protocol**

282 For estimation of biochemical and other parameters, 16 mice were included in each group.
283 Mice were made septic by treatment with either LPS or by CLP technique and 18 h later body
284 temperature was measured. Half of the mice were bled through tail vein for glucose

285 estimation and followed by blood collection through retro orbital sinus in EDTA tubes for
286 total WBC count. Remaining mice were bled through retro orbital sinus in heparinised tubes
287 to obtain plasma for estimation of cytokines, MPO and GSH. Lungs were harvested from all
288 mice, rinsed with saline and weighed. Lungs from six mice were homogenized with chilled
289 saline to obtain 20% homogenate from which 100 μ l was used for bacterial count and the
290 remaining was centrifuged at 15000 rpm for 10 minutes at 4 °C for cytokine measurements.
291 Lungs from remaining mice were homogenized with 50 mM potassium phosphate buffer (pH
292 6.0) containing 0.5 % hexadecyltrimethylammonium bromide, sonicated and centrifuged to
293 obtain supernatant for MPO estimation.

294 **Measurements**

295 **Body temperature and blood glucose**

296 Rectal temperature was measured 18 h after LPS and CLP challenge using digital
297 thermometer (CareTouch[®]). Blood glucose was estimated using calibrated Bayer Contour[®]
298 TS glucometer.

299 **Estimation of Total WBC count**

300 Blood samples collected in EDTA were analyzed on automatic blood cell counter (Sysmex
301 haematology analyzer) for determining the total white blood cells.

302 **Estimation of Plasma cytokine levels**

303 Tumor necrosis factor- α (TNF- α), Interleukin 6 (IL-6), and IL-1 β were estimated using
304 commercially available enzyme-linked immunosorbent assays (ELISAs), according to
305 manufacturer's instruction (R & D Systems Inc, USA).

306 **Measurement of MPO activity in plasma and lung**

307 MPO activity was determined by O-dianisidine method with modification for 96 well plate.
308 The assay mixture consisted of 30 μ L of 0.1M phosphate buffer (pH 6), 30 μ L of 0.01M
309 hydrogen peroxide (Merck), 20 μ L plasma and lung supernatant, 170 μ L deionised water and
310 50 μ L of freshly prepared 0.02M of O-dianisidine (Sigma-Aldrich) solution. O-dianisidine

311 solution was added last and absorbance was recorded per minute at 460 nm up to duration of
312 10 minutes using spectrophotometer (SpectraMax[®] Plus 384 Microplate Reader). MPO
313 activity was expressed in $\mu\text{M}/\text{min}/\text{mL}$ of plasma and in U/gm of tissue.

314 **Determination of glutathione (GSH) content**

315 GSH was determined using Ellman's reagent [5, 5'-Dithiobis (2- nitrobenzoic acid) or
316 DTNB]. GSH (the most abundant non-protein thiol) reduces DTNB to form a stable yellow
317 product (5-mercapto-2-nitrobenzoic acid), which can be measured colorimetrically. The
318 reaction mixture consisted of 50 μL of clear plasma, 250 μL of 0.1M phosphate buffer (pH 6)
319 and 25 μL of DTNB reagent (4 mg/mL dissolved in 1% sodium citrate). This mixture was
320 then incubated for 10 minutes at 37 °C and the absorbance was read at 412 nm using
321 spectrophotometer. The GSH concentration was determined using a standard curve
322 constructed with different concentrations of reduced L- glutathione (Sigma-Aldrich).

323 **Evaluation of Bacterial Clearance**

324 Bacterial load was determined in blood, lung homogenate and peritoneal lavage. In brief,
325 mice were anesthetized with ketamine/ xylazine after 18 h of CLP. The blood samples were
326 collected in heparinised tubes through retro orbital sinus, mixed and placed on ice bath. Mice
327 were then injected 2 mL sterile saline intraperitoneally and abdomen was gently massaged.
328 Later the skin over the abdomen was cleansed with 70% alcohol and cut open to expose the
329 peritoneal cavity to collect the peritoneal lavage fluid. Peritoneal lavage fluids, blood and
330 lung homogenate (100 μL) were placed on ice and serially diluted with sterile saline. 10 μL
331 of each diluted samples were placed on trypticase soy agar plates (BD Biosciences, San
332 Diego, CA) and incubated at 37°C for 24 h. The numbers of bacterial colonies were then
333 counted and expressed as colony-forming units (CFU) per milliliter of blood and peritoneal
334 lavage and CFU per milligram of lung tissue.

335 **Statistics**

336 Survival data were analyzed using the log rank test and Fischer exact test. Data were
337 represented as mean \pm S.E.M. Differences among groups were assessed using one-way
338 analysis of variance (ANOVA) test, followed by Dunnett's multiple comparison post hoc test.
339 Probability of $p < 0.05$ was considered to be significant. All statistical analysis was performed
340 using Graphpad prism statistical software (version 5).

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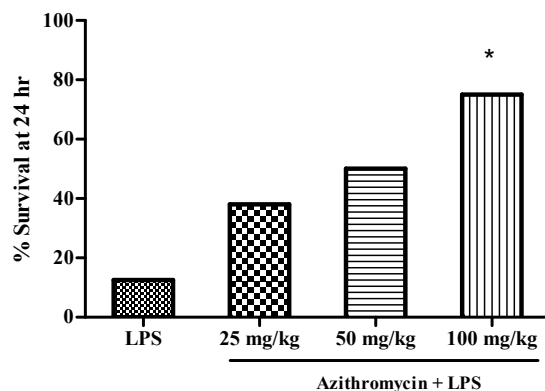
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471 **Figure 1. Survival in LPS induced mouse sepsis model:** Vehicle (saline) and azithromycin
472 (25, 50, 100 mg/kg) was administered intraperitoneally to mice (n=8), 1 h prior to LPS
473 treatment (1500 μ g/mouse) and 24 h survival was monitored. In LPS group only 12.5 % of
474 mice survived whereas LPS treated mice administered azithromycin exhibited dose
475 dependent improvement in survival with 100 mg/kg dose providing protection in 75% of
476 mice.

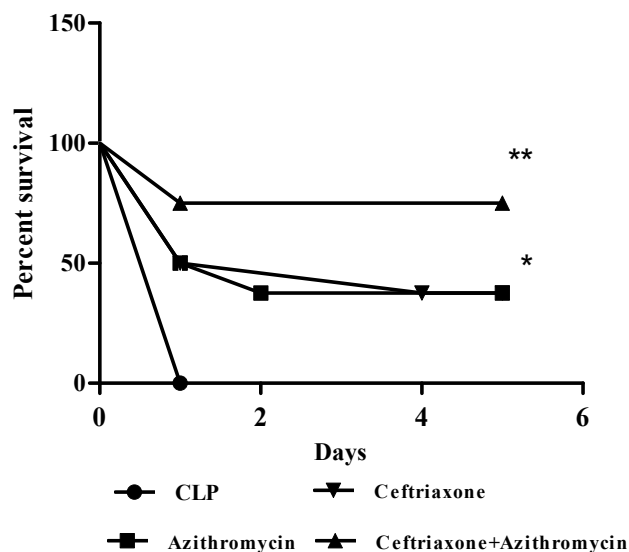
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484 **Figure 2. Survival data in CLP mice:** CLP challenged mice (n=8) were administered
485 ceftriaxone (100 mg/kg, *s.c.*), azithromycin (100 mg/kg, *i.p.*) , both in combination and
486 survival was monitored for 5 days. Ceftriaxone and azithromycin provided protection in 37.5
487 % of mice while combination provided survival in 75% of mice.

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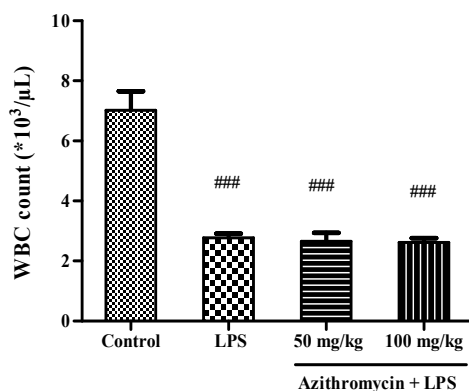
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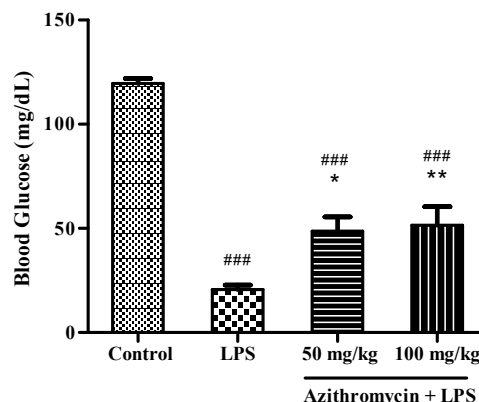
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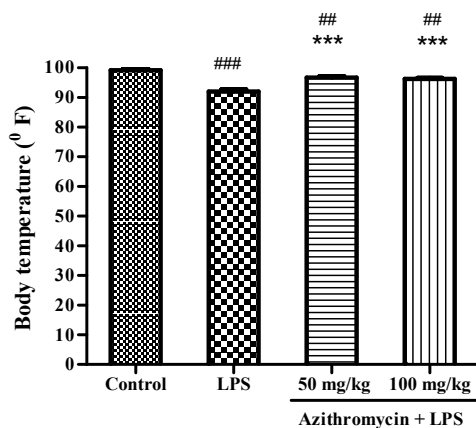


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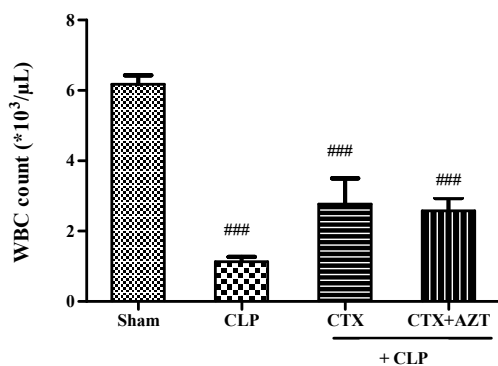


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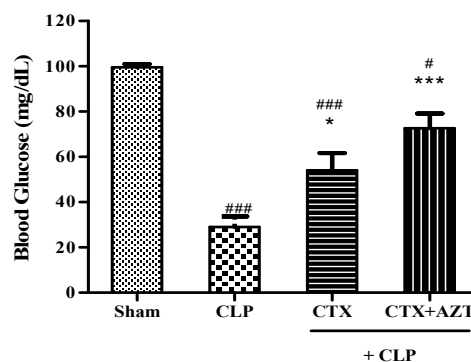
503 **Figure 3. Effect on WBC (A), blood glucose (B) and body temperature (C) in LPS**
504 **treated mice:** Mice (n=8) were administered azithromycin (50 and 100 mg/kg, *i.p.*) or
505 vehicle (saline), 1 h prior to LPS treatment (1000 $\mu\text{g}/\text{mouse}$) and the body temperature, blood
506 glucose and WBC count were determined at 18 h after LPS injection in 6 mice. LPS group
507 showed significantly lower WBC, blood glucose and body temperature compared to normal
508 control group. In LPS treated mice, azithromycin exhibited no effect on the WBC count;
509 while blood glucose and body temperature increased significantly at both the doses compare
510 to LPS group. Values represent means \pm SEM. $\text{###}p < 0.01$, $\text{####}p < 0.001$ indicated value versus
511 control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in LPS plus azithromycin versus LPS group

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4A

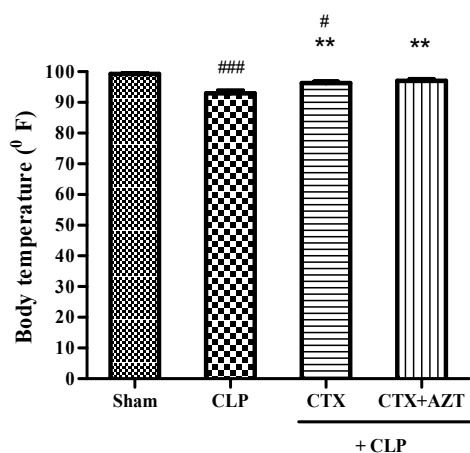


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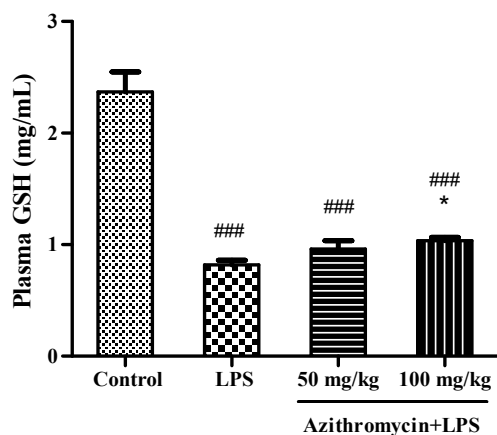


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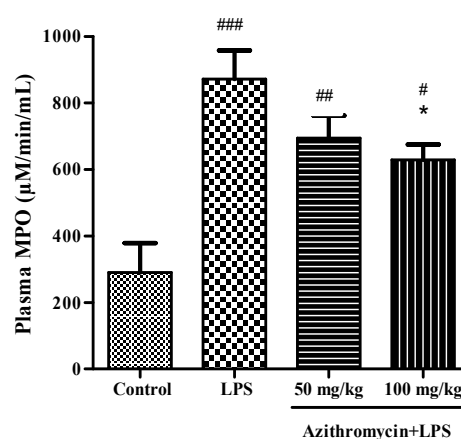
516 **Figure 4. Effect of ceftriaxone (CTX) and ceftriaxone (CTX) + azithromycin (AZT) on**
517 **WBC (A), blood glucose (B) and body temperature (C) in CLP mice:** After 3 h of CLP
518 challenge, mice (n=8) were administered vehicle (saline), ceftriaxone (100 mg/kg, *s.c.*) and
519 ceftriaxone (100 mg/kg, *s.c.*) + azithromycin (100 mg/kg, *i.p.*) and 18 h later WBC, blood
520 glucose and body temperature were measured. CLP mice significantly reduced WBC, blood
521 glucose and body temperature versus sham group. An increase in WBC was observed in
522 ceftriaxone and combination group; however it was not statistically significant versus CLP
523 control. There was a significant increase in blood glucose and body temperature in
524 ceftriaxone and combination group; however the improvement in blood glucose and body

525 temperature was better in combination. Values represent means \pm SEM. # p <0.05, ### p <0.001
526 indicated value versus control; * p <0.05, ** p <0.01 and *** p < 0.001 indicates CLP plus
527 ceftriaxone or CLP plus (ceftriaxone + azithromycin) versus CLP group.

528 **5A**



5B



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530 **Figure 5. Effect on GSH (A) and MPO (B) in the LPS treated mice:** Mice (n=8) were
531 administered vehicle (saline) and azithromycin (50 and 100 mg/kg, *i.p.*), 1 h prior to LPS
532 challenge and the plasma GSH and MPO were measured at 18 h post LPS injection in 6 mice.
533 LPS group showed significant decrease in GSH and increase in MPO levels compared to
534 control group. While, LPS treated mice administered azithromycin (100 mg/kg),
535 demonstrated significant increase in GSH and decrease in MPO. Values represent means
536 \pm SEM. # p <0.05, ## p < 0.01, ### p <0.001 indicated value versus control; * p < 0.05 indicates
537 LPS plus azithromycin versus LPS group.

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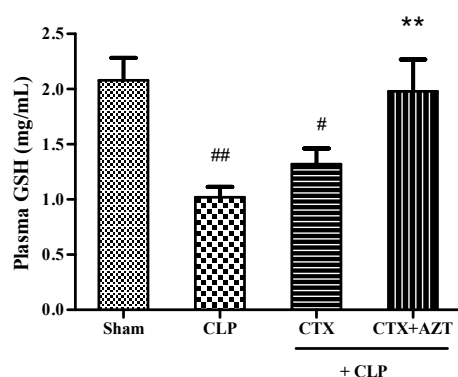
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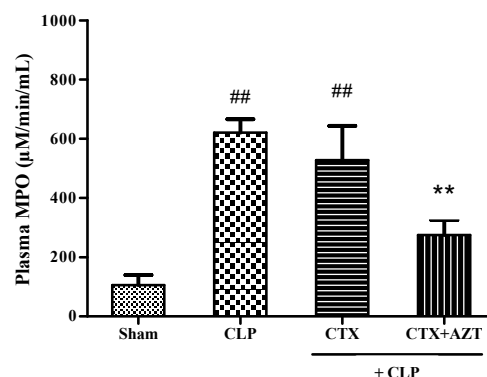
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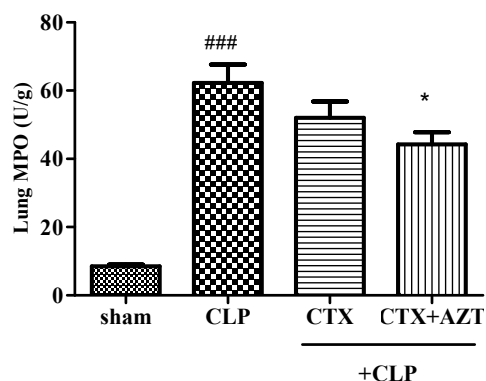


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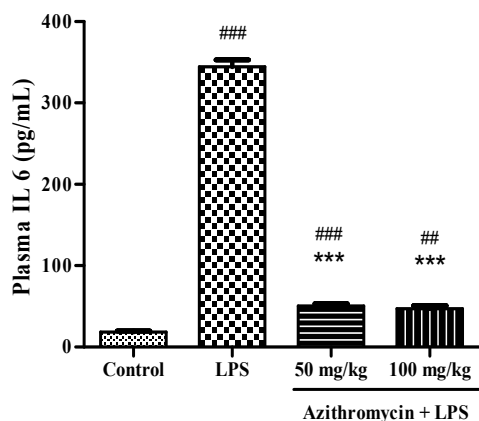


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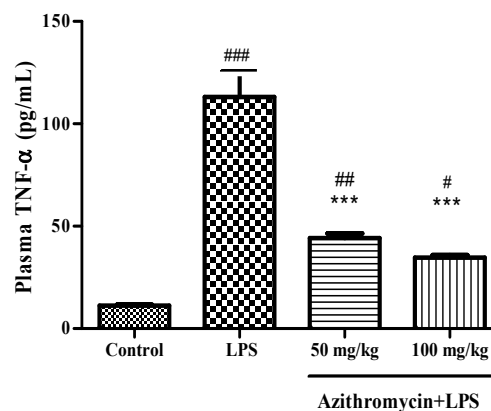
548 **Figure 6. Effect of ceftriaxone (CTX) and ceftriaxone (CTX) + azithromycin (AZT) on**
549 **GSH (A) and MPO (B) in CLP mice:** Vehicle (saline), ceftriaxone (100 mg/kg, *s.c.*) and
550 ceftriaxone (100 mg/kg, *s.c.*) + azithromycin (100 mg/kg, *i.p.*) were administered 3 h after
551 CLP (n=8). Plasma GSH, plasma and lung MPO were measured after 18 h of CLP. CLP mice
552 significantly reduced GSH and increased MPO versus sham group. CLP mice treated with
553 ceftriaxone had no effect on GSH and MPO but the combination group significantly elevated
554 plasma GSH and diminished plasma and lung MPO levels versus CLP group. Values
555 represent means \pm SEM. # p <0.05, ## p <0.01 indicated value versus sham group; * p < 0.05 and
556 ** p <0.01 indicates CLP plus (ceftriaxone + azithromycin) versus CLP group

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558 7A

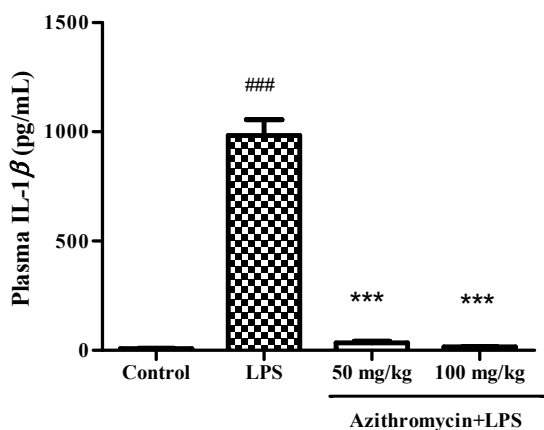


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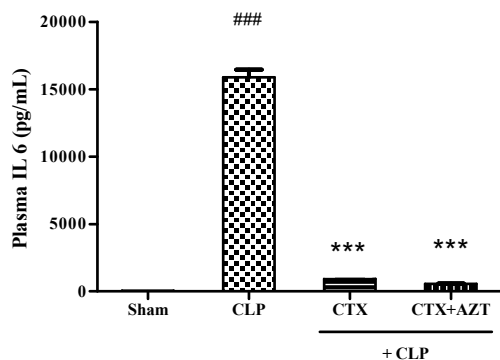


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562 **Figure 7. Effect on plasma cytokines IL-6 (A), TNF-α (B) and IL-1β (C) in the LPS**
563 **treated mice:** Mice (n=8) were administered vehicle (saline) and azithromycin (50 and 100
564 mg/kg, *i.p.*), 1 h prior to LPS challenge and the plasma cytokines were measured at 18 h after
565 LPS injection in 6 mice. LPS group showed significant increase in these cytokines compared
566 to normal control. While, LPS treated mice administered azithromycin (50 and 100 mg/kg),
567 demonstrated significant decrease in IL-6, IL-1β and TNF-α. Values represent means ±SEM.
568 #p<0.05, ##p< 0.01, ###p<0.001 indicated value versus normal control; ***p< 0.001
569 indicates LPS plus azithromycin versus LPS group.

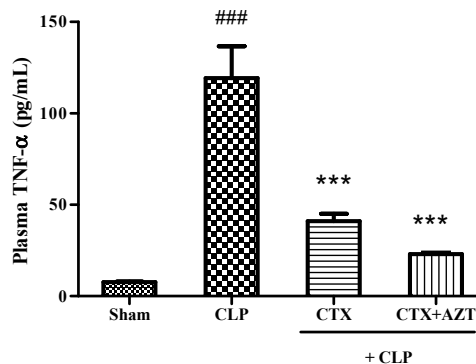
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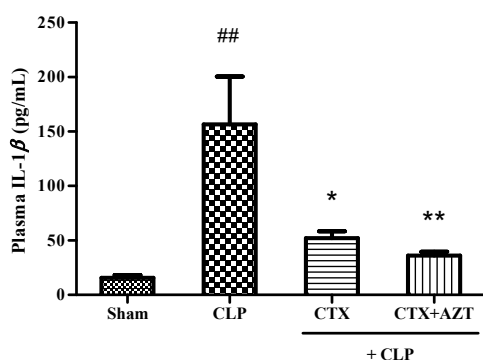


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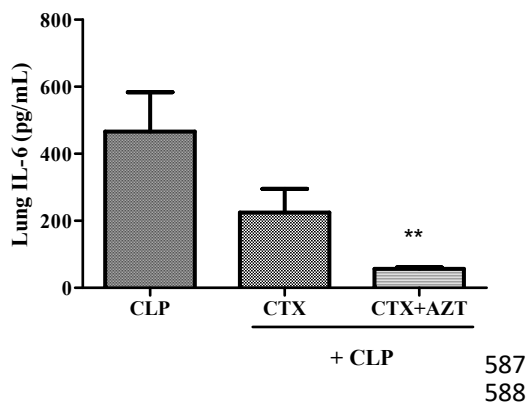
575 **Figure 8. Effect of ceftriaxone (CTX) and ceftriaxone (CTX) + azithromycin (AZT) on**
576 **Plasma IL-6 (A), TNF-α (B) and IL-1β (C) in the CLP mice:** After 3 h of CLP challenge,
577 mice were treated with vehicle, ceftriaxone (100 mg/kg, *s.c.*) and ceftriaxone (100 mg/kg,
578 *s.c.*) + azithromycin (100 mg/kg, *i.p.*) and the plasma cytokine were measured 18 h after CLP
579 (n=6). There was a significant increase in cytokine levels in the CLP mice versus sham
580 group. Ceftriaxone showed significant reduction in elevated cytokine levels, which was
581 further reduced in the combination group. Values represent means ±SEM. ##p<0.01,
582 ###p<0.001 indicated value versus sham group; *p<0.05, **p<0.01 and ***p< 0.001
583 indicates CLP plus ceftriaxone or CLP plus (ceftriaxone + azithromycin) versus CLP group

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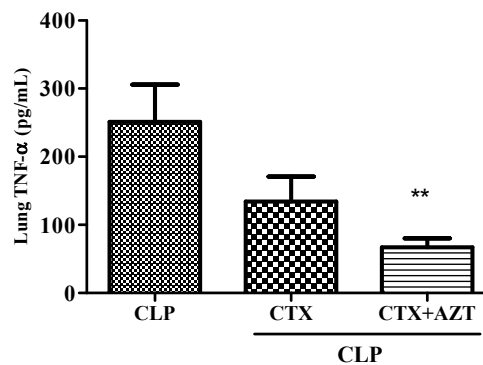
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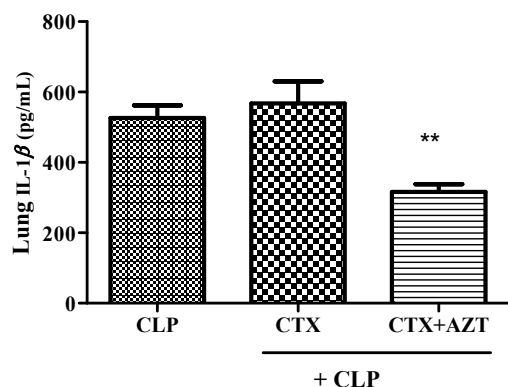


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591 **Figure 9. Effect of ceftriaxone (CTX) and ceftriaxone (CTX) + azithromycin (AZT) on**

592 **Lung IL-6 (A), TNF- α (B) and IL-1 β (C) in the CLP mice:** After 3 h of CLP challenge,

593 mice were treated with vehicle, ceftriaxone (100 mg/kg, *s.c.*) and ceftriaxone (100 mg/kg,

594 *s.c.*) + azithromycin (100 mg/kg, *i.p.*) and the plasma cytokine were measured 18 h after CLP

595 (n=6). There was a significant increase in cytokine levels in the CLP mice versus

596 undetectable levels in sham group (data not shown). Ceftriaxone did not reduce the elevated

597 cytokine levels in CLP mice while combination group significantly suppressed it. Values

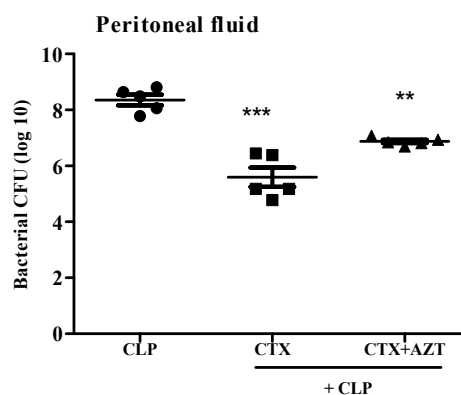
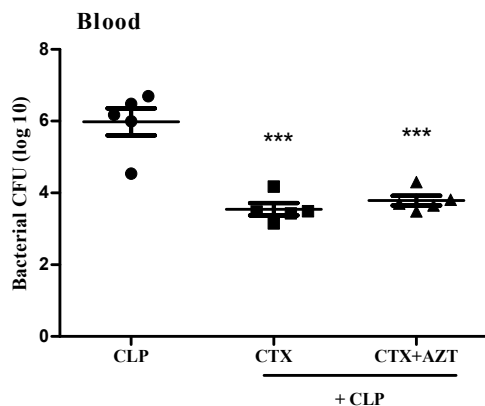
598 represent means \pm SEM. **p<0.01 indicates CLP plus (ceftriaxone + azithromycin) versus

599 CLP group

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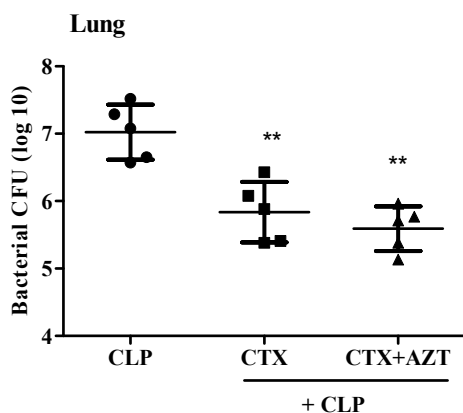
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605 **Figure 10: Effect of ceftriaxone (CTX) and ceftriaxone (CTX) + azithromycin (AZT) on**
606 **bacterial count in blood (A) , peritoneal fluid (B) and Lung tissue (C) in CLP mice:** Mice
607 (n=6) were treated with ceftriaxone (100 mg/kg, *s.c.*) and ceftriaxone (100 mg/kg, *s.c.*) +
608 azithromycin (100 mg/kg, *i.p.*), 3 h after CLP and the bacterial counting was done at 18 h
609 post CLP challenge in 5 mice. CLP group showed positive bacterial culture while ceftriaxone
610 treatment significantly reduced CFU count which was not further reduced in combination
611 with azithromycin. Values represent means \pm SEM. ** $p < 0.01$, *** $p < 0.001$ indicates CLP
612 plus ceftriaxone or CLP plus (ceftriaxone + azithromycin) versus CLP.