Evaluation of the antiviral effect of chlorine dioxide (ClO_2) using a vertebrate model inoculated with avian coronavirus

Zambrano-Estrada, Xóchitl[a], Domínguez-Sánchez, Carlos[b], Banuet-Martínez, Marina[b], Guerrero de la Rosa, Fabiola[b], García-Gasca, Teresa[c], Prieto-Valiente, Luis[d], Acevedo-Whitehouse, Karina[b]

[a] Laboratory of Veterinary Diagnostic Pathology. School of Natural Sciences. Autonomous University of Queretaro. Av. De las Ciencias S/N. Juriquilla, Queretaro. Qro. C.P.76230. Mexico

[b] Unit for Basic and Applied Microbiology. School of Natural Sciences. Autonomous University of Queretaro. Anillo Vial Fray Junípero Serra, Querétaro, Qro. C.P. 76140, Mexico

[c] Laboratory of Cellular Biology. School of Natural Sciences. Autonomous University of Queretaro. Av. De las Ciencias S/N. Juriquilla, Queretaro. Qro. C.P. 76230. Mexico

[d] Bioestadística Médica y Metodología de la Investigación, Análisis Estadístico y Big Data, Universidad Católica de Murcia. Av. de los Jerónimos, 135 Guadalupe de Maciascoque Murcia, 30107. Spain

Corresponding author: Karina Acevedo-Whitehouse (karina.acevedo.whitehouse@uaq.mx)

Abstract

The need for safe and effective antiviral treatments is pressing given the number of viral infections that are prevalent in animal and human populations, often causing devastating economic losses and mortality. Informal accounts of anecdotal use of chlorine dioxide (ClO_2), a well-known disinfectant and antiseptic, in COVID-19 patients has raised concern about potential toxicity, but also raises the question that ClO_2 might elicit antiviral effects, a possibility that has never been examined *in vivo* in any animal model. Here, we challenged the hypothesis that ClO_2 decreases the viral load and virus-induced mortality in a vertebrate model. For this, we determined viral load, virus-induced lesions and mortality in 10-day old chick embryos inoculated with 10^4 mean EID_{50}/mL of attenuated Massachusetts and Connecticut avian coronavirus (IBV) strains. The ClO_2 treatment had a marked impact on IBV infection. Namely, viral titres were 2.4-fold lower and mortality was reduced by half in infected embryos that were treated with ClO_2. Infection led to developmental abnormalities regardless of treatment. Lesions typical of IBV infections were observed in all inoculated embryos, but severity tended to be significantly lower in ClO_2-treated embryos. We found no gross or microscopic evidence of toxicity caused by ClO_2 at the doses used herein. Our study shows that ClO_2 could be a safe and viable way of treating and mitigating the effects of avian coronavirus infections, and raises the possibility that similar effects could be observed in other organisms.

Keywords

Chlorine dioxide, ClO_2, coronavirus, IBV, antiviral, chick embryo

36 **Background**

37 To date, despite advances in the identification of compounds with antiviral activity, the available
38 repertoire of antiviral drugs is insufficient. Given the number of endemic viruses that impact the
39 health of wild and domestic animals as well as human populations the need for effective and safe
40 antivirals is urgent. The COVID19 pandemic has evidenced this need; since its start in January
41 2020, a steep rise in cases and associated mortality, mounting pressure on health services
42 worldwide, and a marked damage to the economy of most countries [1, 2] warrants rigorous,
43 unbiased investigation of compounds with potential antiviral action.

44 The surge in COVID19 cases and the lack of effective treatments have led to a growing number of
45 informal reports and testimonials across social networks regarding the oral and intravenous use of
46 chlorine dioxide (ClO_2) solution as an efficacious treatment of COVID19. In an unprecedented
47 move, the Senate of Bolivia authorized the extraordinary use of ClO_2 to "manufacture, sell, supply
48 and use ClO_2 to prevent and treat COVID19" (law PL N° 219/2019-2020 CS approved 15 July 2020).
49 This situation has sparked concern from health agencies of various countries regarding the
50 potential toxicity of using this disinfectant as a treatment, given that ClO_2 has strong oxidative
51 properties. Chlorine dioxide has been known since 1946 for its broad antimicrobial properties [3],
52 and it is commonly used to purify water for human consumption, decontaminate produce and
53 other food items [4], and disinfect surgical instruments [5]. According to the Food and Drug
54 Administration (FDA) of the US, consuming ClO_2 can lead to adverse effects such as 'vomiting,
55 diarrhoea, dehydration, abdominal pain, metahemaglobinemia and systemic failures that could
56 potentially lead to death'. However, an impartial review of the scientific peer-reviewed literature
57 reveals few case reports of human patients presenting adverse effects after consumption of ClO_2,
58 none of which were fatal, and reversed completely after treatment [6–9]. Published studies on

59 ClO_2 toxicity have reported null to mild effects in adult humans and other animals [10–12] and
60 have stressed that toxicity is strongly dose-dependent [12–14].

61 There is evidence of virucidal activity of ClO_2 against echovirus [15], enterovirus [16], poliovirus
62 [17], rotavirus [18], norovirus [19, 20], calicivirus [19], and coronavirus [21]. However, there are
63 few publications that explore antiviral effects *in vitro* and *in vivo*. One of the few published studies
64 found that mice exposed to influenza A virus in an environment that contained aerosolized ClO_2
65 had significantly lower mortality than mice that were solely exposed to Influenza A virus [22]. The
66 authors reported that the antiviral effect was due to denaturation of hemagglutinin and
67 neuraminidase glycoproteins, a finding that concurs with previous studies that explain the
68 virucidal mechanism of action of ClO_2 due to oxidation of amino acid residues that are key for cell
69 entry [23–25]. More recently, the antiviral mechanism of action of ClO_2 was investigated *in vitro*
70 using pig alveolar macrophages and MARC-145 cells exposed to the porcine reproductive and
71 respiratory syndrome virus (PRRSV1), finding that the chemical also inhibits the synthesis of pro-
72 inflammatory molecules that contribute to the pathogenesis of this disease [26]. The authors
73 concluded that viral synthesis of RNA and proteins was impeded by ClO_2, leading to a reduction in
74 viral replication. Despite these studies, to date there has been no published report of an *in vivo*
75 assessment of the antiviral effect of ClO_2. Here, we have investigated the effect of a ClO_2 solution
76 in 10-day old chick embryos inoculated with attenuated avian infectious bronchitis coronavirus
77 (IBV) Massachusetts and Connecticut vaccine strains, which are pathogenic for birds during their
78 embryonic stages of development [27].

79 **Results**

80 Avian coronavirus RNA was detected in all of the embryos that were inoculated with the vaccine
81 (n=15, in all cases Cq <40). The viral load varied significantly between treatments (ANOVA;

82 $F_{2,12}$=4.421, p=0.036; Fig. 1), being 2.4 times higher in the untreated embryos. The average viral
83 load of ClO_2-treated chicks was $10^{4.3}$/mL, range: $10^{3.66}$ – $10^{5.03}$ and of untreated chicks was
84 $10^{4.83}$/mL, range: $10^{4.52}$ – $10^{5.01}$, respectively (Tukey HSD, Group E *vs*. F, p = 0.03). There were no
85 differences in the viral load between both ClO_2-treated groups (p>0.05).

86 Mortality differed significantly between virus-inoculated (46.7%) and virus-free embryos (46.7%)
87 (Fisher's exact p = 0.018), (Fig. 2). In the viral-inoculated groups, 1/5 (**20 %)** embryos treated with a
88 low dose of ClO_2, 2/5 (**40 %)** embryos treated with a high dose of ClO_2, and 4/5 (**80 %)** untreated
89 embryos died (Groups D, E and F, respectively). The odds of survival were, on average, 4.0 times
90 higher for each increasing ClO_2 dosage (Logistic regression; unilateral P-value = 0.039; see
91 supplementary material).

92 Body mass, embryonic axis length and femur length differed between the virus-inoculated and
93 virus free-groups, regardless of ClO_2 treatment. All of the virus-inoculated embryos exhibited
94 dwarfing and had, on average, 38% lower mass (t = 21.15, df = 29.40, p = 2.2 x 10^{-16}; Fig. 3A), 10%
95 shorter axis length (t = 58.43, df = 29.26, p = 2.2 x 10^{-16}; Fig. 3B), and 20% shorter femur length (t =
96 8.49, df = 23.34, p = 1.4 x 10^{-8}; Fig 3C) than the virus-free groups. When analysing growth in the
97 virus-inoculated groups, mass was significantly higher in embryos that were treated with ClO_2 (t = -
98 2.74, df = 12.98, p = 0.017). Body length of virus-inoculated chicks did not vary according to ClO_2
99 treatment (p > 0.1). See supplementary material for photographs of the embryos.

100 Lesions previously described in embryos infected with avian coronavirus were observed at post
101 mortem examination. Namely, curling, the presence of white caseous material (urates), thickened
102 amnion and allantoic membranes that adhered to the embryos, oedematous serous membranes,
103 epidermal congestion, and subcutaneous haemorrhage (Table 2). Virus-inoculated embryos
104 treated with ClO_2 had a lower risk of epidermal congestion (RR = 0.4; Wald 95% CI: 0.187- 0.855; p

105 = 0.04), haemorrhage (RR = 0.1; Wald 95% CI: 0.016 - 0.642; p = 0.002), curling (RR = 0.019; Wald
106 95% CI: 0.125 - 0.844; p = 0.017) and thickened membranes (RR = 0; p = 0.003) than untreated
107 infected embryos. All the embryos had a pale liver and mildly congested lungs, regardless of their
108 experimental group. Pale enlarged kidneys were observed in the virus-inoculated groups but not in
109 the virus-free groups, regardless of ClO_2-treatment (see Table 2).

110 Microscopic lesions compatible with avian IBV infection were observed in various organs in all
111 virus-inoculated groups (Fig. 4). The severity of the lesions was either similar or slightly lower in
112 the embryos that had been treated with ClO_2 than in the embryos that did not receive any
113 treatment (Table 3). Two exceptions were the kidneys and the duodenum. In the kidneys, swelling
114 and degeneration of renal tubular epithelium was more common and more severe in the infected
115 chicks that were administered ClO_2 than in the infected embryos that did not receive ClO_2,
116 although the former presented mitotic cells. The duodenal villi of the embryos in the IBV-infected
117 groups were longer (ANOVA; $F_{5,18} = 5.62$, p = 0.003), and their base was wider (ANOVA; $F_{5,18} =$
118 13.65, $p = 1.39 \times 10^{-05}$) than embryos from the none-infected groups, and they were moderately
119 congested. Duodenal villous atrophy varied amongst groups (ANOVA; $F_{5,18} = 5.71$, p = 0.003) and
120 *post-hoc* comparisons revealed that the significant differences were E *vs.* B (p = 0.021) and E *vs.* C
121 (p = 0.001). The percent of bursal lymphoid tissue decreased markedly in the virus-inoculated
122 embryos (ANOVA; $F_{5,12} = 3.58$, p = 0.033; see Fig. 4). Virus-inoculated embryos showed mild
123 apoptosis in the thymus and heterophilic infiltration. Amongst the six experimental groups, all of
124 the embryos examined presented subacute heterophilic bursitis, and pulmonary interstitial
125 multifocal heterophilic foci with congestion (Table 3).

126 There were no observable alterations to the architecture and integrity of the tissues of non-
127 infected embryos that were administered ClO_2 (Experimental groups B and C), nor was any
128 difference in the area (μm^2) of the thyroid (p>0.1). ClO_2-treated groups showed a slightly higher

129 myeloid to erythroid ratio in the bone marrow than the experimental control group and the viral
130 control group, although the difference was not statistically significant (ANOVA; $F_{5,15} = 2.33$, $p =$
131 0.094).

132 **Discussion**

133 We have investigated the antiviral effect of chlorine dioxide (ClO_2) using avian infectious bronchitis
134 coronavirus (IBV)-infected chick embryos as models. At the concentrations used (30 ppm and 300
135 ppm), both below the reported NOAEL [13], we observed a reduction in viral titre in infected
136 embryos that were treated with ClO_2. Furthermore, mortality decreased substantially in the ClO_2-
137 treated embryos, although alterations to chick development were prevalent regardless of
138 treatment.

139 Virulent avian IBV strains typically have a burst size of 10 to 100 infective units per cell [28], which
140 can reach peak virus titres of $10^{6.5}$–$10^{8.5}$ $TCID_{50}$ after 36 hours [29]. The vaccine strains used here
141 have lower replication efficiencies as they are attenuated [30], but they are capable of replicating
142 and causing damage in chick embryos [27]. With 2,000 infective units (200 µl of 10^4 infective
143 units/mL) inoculated into each embryo, the ClO_2 treatments decreased viral load 2.4-fold
144 compared to the infected non-treated embryos, representing an average difference of 42,711
145 infective units. This result could be explained by two non-mutually exclusive mechanisms. Firstly,
146 direct destruction or neutralization of the virions exposed to ClO_2 could have occurred by
147 denaturing of envelope glycoproteins following oxidation of amino acid residues [22, 23, 25].
148 Secondly, viral replication efficiency could have decreased due to ClO_2–induced biochemical
149 changes in the extra- or intracellular milieu, impeding the synthesis of viral RNA and proteins [26,
150 31].

151 The effects of reduced viral titres in the infected embryos were evident, with a 50 to 75%
152 reduction in mortality in the treated embryos treated with 300 ppm and 30 ppm of ClO_2,
153 respectively. However, developmental abnormalities were observed in the majority of the infected
154 embryos, including those that received ClO_2 treatment. Namely, dwarfing, assessed by body mass,
155 axis length, and femur length were significantly lower in all infected embryos, as expected to occur
156 in IBV infections [32]. In contrast, curling [33, 34] was virtually absent in the embryos that received
157 ClO_2 after infection. Lesions associated con IBV infection [35, 36] were observed in most of the
158 infected embryos, including those that were administered ClO_2. However, for most of these
159 lesions, severity was lower or similar in the ClO_2-treated groups. One exception was in the kidneys,
160 where nephrosis was more severe and frequent in the IBV-infected embryos treated with ClO_2.
161 Nephrosis is unlikely to have occurred due to ClO_2 treatment *per se*, given that none of the
162 uninfected embryos that were administered ClO_2 showed kidney abnormalities. Similarly, atrophy
163 of the duodenal villi was highest in the inoculated group that was administered a high dose of
164 ClO_2, but absent in the non-infected groups that only received ClO_2. If duodenal atrophy and
165 nephropathogenicity in IBV-infected embryos are mitigated by inflammatory responses [37], it is
166 possible that the observed damage is due to ClO_2-driven downregulation of acute inflammation
167 that allowed virion replication in the tubular epithelium and in the duodenal villi. This scenario
168 could be plausible if we consider that ClO_2-treated pig alveolar macrophages and African green
169 monkey kidney cells infected with the porcine reproductive and respiratory syndrome virus
170 (PRRSV1) downregulate pro-inflammatory cytokines IL-1, IL-6 and TNF-α [26]. There are two drugs,
171 based on chloride, whose describe mechanism of action might be similar to that elicited by ClO_2.
172 One is the drug NP001 (a pH-adjusted stabilized form of $NaClO_2$) exerts strong anti-inflammatory
173 responses by inhibiting macrophage activation in humans, even after a single dose [38]. In turn,
174 WF10 (the commercial name for tetrachlorodecaoxyde, $Cl_4H_2O_{11}$), an oxidizing agent that contains

175 63.0 mmol/L chlorite and 56.4 mmol/L chloride [39] induces apoptosis of inflammatory cells and
176 downregulates pro-inflammatory genes [40, 41]. More studies will be needed to establish whether
177 the effect of ClO_2 on IBV-infected chicks is due to similar mechanisms. Interestingly, the affected
178 renal tubules of the chick embryos had mitotic cells, suggestive of regeneration as a reparative
179 response to damage of the renal tubular epithelium [42–44]. This possibility will need to be
180 explored further in the animal model used in our study. Unfortunately, knowledge of IBV
181 pathogenesis, immune responses and tissue reparation in the embryonated egg is limited. In
182 hatched birds, IBV can impact lymphocyte populations by inducing apoptosis, thus impeding virus
183 clearance [45]. We found some evidence of this effect, as the virus-inoculated embryos had a
184 reduced percent of bursal lymphoid tissue.

185 It is plausible that even though ClO_2 limited viral replication, given that the embryos were only
186 administered a single dose of ClO_2 solution, not all virions were eliminated. The viruses that
187 remained viable after administration of the ClO_2 were able to replicate, yielding lower viral titres,
188 and the damage that they caused to the embryos was less severe and ultimately led to less
189 mortality than in the untreated infected embryos. Repeated administrations of ClO_2 solution might
190 have reduced the viral load further, plausibly leading to even less virus-induced damage, a
191 possibility that we could not explore in this model. However, an important result of our study was
192 the lack of evidence of tissue damage caused by ClO_2 itself. Only one (20%) of the uninfected
193 embryos treated with the high dose of ClO_2 died, and this difference was not statistically
194 significant to the experimental control. It is possible that the death was due to other causes, as a
195 20% embryo mortality rate is considered normal in aviculture [46, 47].

196 **Conclusions**

197 Chlorine dioxide had an unequivocal antiviral effect in the model system used in this study, with a
198 reduction in viral RNA, virus-induced lesions and mortality and no evidence of toxicity at the doses
199 used herein. Further studies should aim to test the antiviral effect of ClO_2 in hatched chicks, which
200 have a more mature immune system, and where repeated administrations are easier to procure.
201 However, it is promising that we found an evident effect against IBV without any significant
202 adverse effect or evidence of toxicity to the chick embryos. Much research needs to be done
203 before it is possible to generalize and extrapolate our findings to other viruses and animal hosts.

204 **Methods**

205 We aimed to examine whether chlorine dioxide elicits a measurable antiviral effect *in vivo*, and
206 investigate toxicity associated with the administration of this chemical compound. For this, we
207 selected the chicken embryo as a vertebrate model and avian infectious bronchitis coronavirus
208 (IBV) as a virus model. The protocol that is described below was registered at the School of Natural
209 Sciences of the Autonomous University of Queretaro (Mexico). The study was carried out in
210 compliance with the American Veterinary Medical Association (AVMA) guide for humane
211 treatment of chick embryos and approved by the Autonomous University of Queretaro Research
212 Ethics Committee

213 We used 30 10-day old SPF RossxRoss chick embryos (Pilgrims, Mexico) incubated at 38°C and 65-
214 70% humidity [48]. Five chick embryos were assigned to each of the six experimental groups at
215 random and treated according to their group (see Table 1). Embryos were also placed at random in
216 the incubator to avoid potential variations in temperature and humidity between the edges and
217 the centre of the incubator. All embryos were inoculated via the chorioallanotic sac, as
218 recommended for avian coronavirus replication [49, 50]. Details on inoculation can be seen as
219 Supplementary Materials.

220 Seven days after inoculation, embryos were sacrificed according to guidelines on humane
221 treatment and euthanasia of chick embryos over 13 days of age (AVMA 2020). The embryos were
222 placed at 4 °C for 4 h to ensure blood coagulation and avoid contamination of the allantoic fluid
223 [48]. The eggshell over the air chamber was disinfected with 70% ethanol and 3.5% iodine [48] and
224 removed together with the chorioallantoic membrane. Allanotic fluid (750 µl) was collected and
225 stored at −70 °C.

226 Each embryo was cleaned with distilled water to remove any remaining albumin, vitellus and
227 amniotic fluid. Body mass was determined with a precision scale (±0.1 mg). Embryonic axis length
228 was measured with digital callipers (± 0.1 cm). The body was examined for macroscopic lesions
229 typically caused by avian coronavirus, such as cutaneous haemorrhages, stunting, curving, urate
230 deposits in the kidney and feather alterations [49, 51]. Chick embryos were considered to have
231 died during the experiment when there was evidence of disconnected or detached blood vessels
232 of the chorioallantoic membrane or their organs exhibited imbibition of haemoglobin. Further
233 evidence of mortality was determined by examining the presence of autolysis at microscopic
234 examination.

235 Both femurs were dissected and their length was determined and averaged with a digital calliper
236 as a surrogate measure of chick development. Samples of all organs and tissues were collected,
237 including those known to be affected by the Massachusetts and Connecticut strains of avian
238 coronavirus, namely trachea, lung, proventricle, duodenum, liver, kidneys and the Bursa of
239 Fabricius [27, 49, 52, 53], and those reportedly affected by exposure to ClO_2 [13, 54, 55].

240 Samples were cut longitudinally, immersed in 10% buffered formalin (pH 7.4), paraffin-embedded
241 and sectioned at 3 µm, stained with haematoxylin-eosin and observed under the microscope at
242 40X and 100X. To assess haematopoietic status, bone marrow preparations were used for a 200-

243 cell differential count to classify the marrow precursors and to determine the myeloid:erythroid
244 (M/E) ratio for each embryo. This ratio was obtained by dividing the sum of all the granulocytic
245 cells by the sum of all the erythrocytic cells [56].

246 The virus was quantified in the allantoid fluid with an RT-qPCR protocol that targeted a 100 bp
247 fragment of the avian coronavirus N gene with primers IBV-pan_FW-1 (5'-CAG TCC CDG ATG CNT
248 GGT A) and IBV-pan_RV (5'-CC TTW SCA GMA ACM CAC ACT)[57]. Details on the protocol can be
249 seen as Supplementary Materials. We used a cDNA sample extracted from 500 µL of the vaccine
250 as a positive control (the vaccine contained 10^4 mean embryo infective dose (EID_{50})/mL of
251 coronavirus strains Massachusetts and Connecticut). A linear regression was used to calculate the
252 correlation coefficient (R^2 = 0.99) and the slope value (b = -5.062) of the RNA copy number and Cq
253 values using a 10-fold dilution of the vaccine (10^4 to 10^{-1}; see standard curve in Supplementary
254 material). The number of viral RNA copies (hereafter viral load) was determined by comparing the
255 Cq against this standard curve [57].

256 Contingency tables were built to investigate differences in the proportion of dead *vs*. live chick
257 embryos between experimental groups, and patterns were examined by logistic regression
258 (Armitage et al., 2001). The unilateral p-value was considered pertinent because of the strong
259 prior probability that ClO_2 should not decrease the survival rate of embryos. The risk of developing
260 virus-related lesions between treated and un-treated embryos was calculated using Fisher exact
261 tests. Body mass, morphometrics, viral load, and lesion severity were compared among groups by
262 one-way ANOVA and post-hoc Tukey HSD tests. All analyses were performed in R version 3.6.3
263 [58].

264 **Statement of conflict of interest**

265 The authors declare they do not have any conflict of interest regarding this submission.

Acknowledgments

We thank Luis A. Soto-García and Karla Zamora y Cuevas for their help with photographic and video documentation of the experiment.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

K.A-W. and T.G-G. conceived the idea. K.A-W. supervised the experiments and wrote the manuscript. X.Z-E. performed all gross examinations and histopathology analyses. C.D-S. conducted molecular assays and artwork. M.B-M. and F.G-D performed the inoculation experiments and assisted during necropsies. K.A-W. and L.P-V conducted the statistical analyses. All authors participated in the discussion of results.

References

1. Duan H, Wang S, Yang C. Coronavirus: limit short-term economic damage. Nature. 2020.

2. Nicola M, Alsafi Z, Sohrabi C, Kerwan A, Al-Jabir A, Iosifidis C, et al. The socio-economic implications of the coronavirus pandemic (COVID-19): A review. International Journal of Surgery. 2020.

3. Sigstam T, Rohatschek A, Zhong Q, Brennecke M, Kohn T. On the cause of the tailing phenomenon during virus disinfection by chlorine dioxide. Water Res. 2014;48:82–9.

4. López-Gálvez F, Randazzo W, Vásquez A, Sánchez G, Decol LT, Aznar R, et al. Irrigating Lettuce with Wastewater Effluent: Does Disinfection with Chlorine Dioxide Inactivate Viruses? J Environ

287 Qual. 2018;47:1139–45.

288 5. Doona CJ, Feeherry FE, Setlow P, Malkin AJ, Leighton TJ. The Portable Chemical Sterilizer (PCS),
289 D-FENS, and D-FEND ALL: novel chlorine dioxide decontamination technologies for the military. J
290 Vis Exp. 2014;:e4354.

291 6. Loh JMR, Shafi H. Kikuchi-Fujimoto disease presenting after consumption of "Miracle Mineral
292 Solution" (sodium chlorite). BMJ Case Rep. 2014;2014.

293 7. Bathina G, Yadla M, Burri S, Enganti R, Prasad Ch R, Deshpande P, et al. An unusual case of
294 reversible acute kidney injury due to chlorine dioxide poisoning. Ren Fail. 2013;35:1176–8.

295 8. Romanovsky A, Djogovic D, Chin D. A case of sodium chlorite toxicity managed with concurrent
296 renal replacement therapy and red cell exchange. J Med Toxicol Off J Am Coll Med Toxicol.
297 2013;9:67–70.

298 9. Kishan H. Chlorine dioxide-induced acute hemolysis. Journal of medical toxicology : official
299 journal of the American College of Medical Toxicology. 2009;5:177.

300 10. Lubbers JR, Chauan S, Bianchine JR. Controlled clinical evaluations of chlorine dioxide, chlorite
301 and chlorate in man. Environ Health Perspect. 1982;46:57–62.

302 11. Akamatsu A, Lee C, Morino H, Miura T, Ogata N, Shibata T. Six-month low level chlorine dioxide
303 gas inhalation toxicity study with two-week recovery period in rats. J Occup Med Toxicol.
304 2012;7:2.

305 12. Ma J-W, Huang B-S, Hsu C-W, Peng C-W, Cheng M-L, Kao J-Y, et al. Efficacy and Safety
306 Evaluation of a Chlorine Dioxide Solution. Int J Environ Res Public Health. 2017;14.

307 13. Bercz JP, Jones L, Garner L, Murray D, Ludwig DA, Boston J. Subchronic toxicity of chlorine
308 dioxide and related compounds in drinking water in the nonhuman primate. Environ Health

309 Perspect. 1982;46:47–55.

310 14. Hose JE, Di Fiore D, Parker HS, Sciarrotta T. Toxicity of chlorine dioxide to early life stages of
311 marine organisms. Bull Environ Contam Toxicol. 1989;42:315–9.

312 15. Zhong Q, Carratalà A, Ossola R, Bachmann V, Kohn T. Cross-Resistance of UV- or Chlorine
313 Dioxide-Resistant Echovirus 11 to Other Disinfectants. Front Microbiol. 2017;8:1928.

314 16. Jin M, Shan J, Chen Z, Guo X, Shen Z, Qiu Z, et al. Chlorine dioxide inactivation of enterovirus 71
315 in water and its impact on genomic targets. Environ Sci Technol. 2013;47:4590–7.

316 17. Simonet J, Gantzer C. Degradation of the Poliovirus 1 genome by chlorine dioxide. J Appl
317 Microbiol. 2006;100:862–70.

318 18. Chen YS, Vaughn JM. Inactivation of human and simian rotaviruses by chlorine dioxide. Appl
319 Environ Microbiol. 1990;56:1363–6.

320 19. Montazeri N, Manuel C, Moorman E, Khatiwada JR, Williams LL, Jaykus L-A. Virucidal Activity of
321 Fogged Chlorine Dioxide- and Hydrogen Peroxide-Based Disinfectants against Human Norovirus
322 and Its Surrogate, Feline Calicivirus, on Hard-to-Reach Surfaces. Front Microbiol. 2017;8:1031.

323 20. Lim MY, Kim J-M, Ko G. Disinfection kinetics of murine norovirus using chlorine and chlorine
324 dioxide. Water Res. 2010;44:3243–51.

325 21. Wang X-W, Li J-S, Jin M, Zhen B, Kong Q-X, Song N, et al. Study on the resistance of severe
326 acute respiratory syndrome-associated coronavirus. J Virol Methods. 2005;126:171–7.

327 22. Ogata N, Shibata T. Protective effect of low-concentration chlorine dioxide gas against
328 influenza A virus infection. J Gen Virol. 2008;89 Pt 1:60–7.

329 23. Ison A, Odeh IN, Margerum DW. Kinetics and mechanisms of chlorine dioxide and chlorite

330 oxidations of cysteine and glutathione. Inorg Chem. 2006;45:8768–75.

331 24. Stewart DJ, Napolitano MJ, Bakhmutova-Albert E V, Margerum DW. Kinetics and mechanisms
332 of chlorine dioxide oxidation of tryptophan. Inorg Chem. 2008;47:1639–47.

333 25. Noss CI, Olivieri VP. Disinfecting capabilities of oxychlorine compounds. Appl Environ
334 Microbiol. 1985;50:1162–4.

335 26. Zhu Z, Guo Y, Yu P, Wang X, Zhang X, Dong W, et al. Chlorine dioxide inhibits the replication of
336 porcine reproductive and respiratory syndrome virus by blocking viral attachment. Infect Genet
337 Evol J Mol Epidemiol Evol Genet Infect Dis. 2019;67:78–87.

338 27. Tsai C-T, Tsai H-F, Wang C-H. Detection of infectious bronchitis virus strains similar to Japan in
339 Taiwan. J Vet Med Sci. 2016;78:867–71.

340 28. Robb JA, Bond CW. Coronaviridae. In: Fraenkel-Conrat H, Wagner RR, editors. Comprehensive
341 Virology. Boston: Springer; 1979.

342 29. Otsuki K, Noro K, Yamamoto H, Tsubokura M. Studies on avian infectious bronchitis virus (IBV).
343 II. Propagation of IBV in several cultured cells. Arch Virol. 1979;60:115–22.

344 30. Tsai C-T, Wu H-Y, Wang C-H. Genetic sequence changes related to the attenuation of avian
345 infectious bronchitis virus strain TW2575/98. Virus Genes. 2020;56:369–79.

346 31. Enjuanes L, Almazán F, Sola I, Zuñiga S. Biochemical aspects of coronavirus replication and
347 virus-host interaction. Annu Rev Microbiol. 2006;60:211–30.

348 32. Balasubramaniam A, Sukumar K, Suresh P, Puvarajan B. Molecular characterisation of
349 membrane glycoprotein and 5b protein of nephropathogenic infectious bronchitis virus. Vet
350 World. 2013.

351 33. Wickramasinghe INA, de Vries RP, Gröne A, de Haan CAM, Verheije MH. Binding of avian
352 coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. J Virol.
353 2011;85:8903–12.

354 34. Mork A-K, Hesse M, Abd El Rahman S, Rautenschlein S, Herrler G, Winter C. Differences in the
355 tissue tropism to chicken oviduct epithelial cells between avian coronavirus IBV strains QX and
356 B1648 are not related to the sialic acid binding properties of their spike proteins. Vet Res.
357 2014;45:67.

358 35. Butcher GD, Winterfield RW, Shapiro DP. Pathogenesis of H13 nephropathogenic infectious
359 bronchitis virus. Avian Dis. 1990;34:916–21.

360 36. Cook JKA, Jackwood M, Jones RC. The long view: 40 years of infectious bronchitis research.
361 Avian Pathol. 2012;41:239–50.

362 37. Chhabra R, Ball C, Chantrey J, Ganapathy K. Differential innate immune responses induced by
363 classical and variant infectious bronchitis viruses in specific pathogen free chicks. Dev Comp
364 Immunol. 2018;87:16–23.

365 38. Miller RG, Zhang R, Block G, Katz J, Barohn R, Kasarskis E, et al. NP001 regulation of
366 macrophage activation markers in ALS: a phase I clinical and biomarker study. Amyotroph Lateral
367 Scler Frontotemporal Degener. 2014;15:601–9.

368 39. Veerasarn V, Khorprasert C, Lorvidhaya V, Sangruchi S, Tantivatana T, Narkwong L, et al.
369 Reduced recurrence of late hemorrhagic radiation cystitis by WF10 therapy in cervical cancer
370 patients: a multicenter, randomized, two-arm, open-label trial. Radiother Oncol J Eur Soc Ther
371 Radiol Oncol. 2004;73:179–85.

372 40. Giese T, McGrath MS, Stumm S, Schempp H, Elstner E, Meuer SC. Differential effects on innate

373 versus adaptive immune responses by WF10. Cell Immunol. 2004;229:149–58.

374 41. Yingsakmongkol N, Maraprygsavan P, Sukosit P. Effect of WF10 (immunokine) on diabetic foot
375 ulcer therapy: a double-blind, randomized, placebo-controlled trial. J foot ankle Surg Off Publ Am
376 Coll Foot Ankle Surg. 2011;50:635–40.

377 42. Fujigaki Y. Different modes of renal proximal tubule regeneration in health and disease. World
378 J Nephrol. 2012;1:92–9.

379 43. Lombardi D, Becherucci F, Romagnani P. How much can the tubule regenerate and who does
380 it? An open question. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc.
381 2016;31:1243–50.

382 44. Toback FG. Regeneration after acute tubular necrosis. Kidney Int. 1992;41:226–46.

383 45. Caron LF. Etiology and immunology of infectious bronchitis virus. Rev Bras Cienc Avic. 2010.

384 46. Romanoff AL. Spontaneous malformations. In: Romanoff AL, editor. Pathogenesis of the avian
385 embryo: an analysis of causes of malformations and prenatal death. New York: Wiley Interscience;
386 1972.

387 47. Fasenko GM, O'Dea EE. Evaluating broiler growth and mortality in chicks with minor navel
388 conditions at hatching. Poult Sci. 2008.

389 48. Guy JS. Isolation and propagation of coronaviruses in embryonated eggs. Methods Mol Biol.
390 2015.

391 49. Escorcia M, Fortoul TI, Petrone VM, Galindo F, López C, Téllez G. Gastric gross and microscopic
392 lesions caused by the UNAM-97 variant strain of infectious bronchitis virus after the eighth
393 passage in specific pathogen-free chicken embryos. Poult Sci. 2002;81:1647–52.

394 50. Jordan FT, Nassar TJ. The combined influence of age of embryo and temperature and duration
395 of incubation on the replication and yield of avian infectious bronchitis (IB) virus in the developing
396 chick embryo. Avian Pathol. 1973;2:279–94.

397 51. Alexander DJ, Senne DA. A Laboratory Manual for the Isolation, Identification and
398 Characterization of Avian Pathogens. 2008.

399 52. Cavanagh D. Severe acute respiratory syndrome vaccine development: experiences of
400 vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 2003;32:567–82.

401 53. Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res. 2007;38:281–97.

402 54. Harrington RM, Shertzer HG, Bercz JP. Effects of chlorine dioxide on thyroid function in the
403 African green monkey and the rat. J Toxicol Environ Health. 1986;19:235–42.

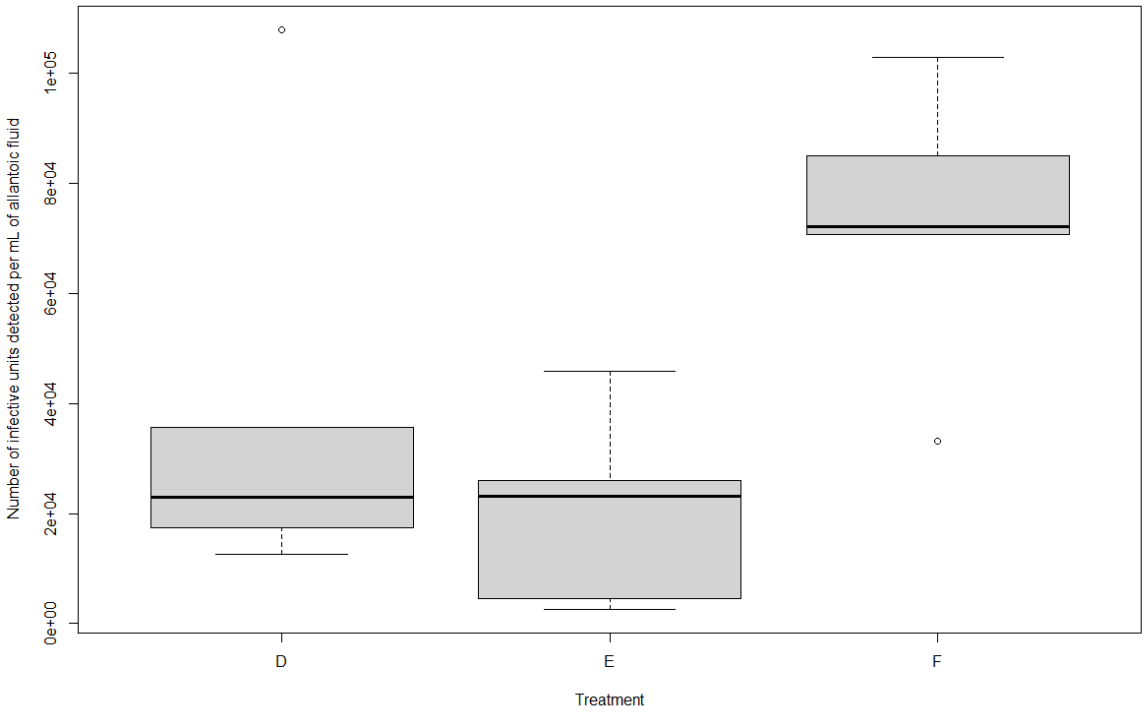
404 55. Environmental Protection Agency. Toxicological Review of Chlorine dioxide and Chlorite.
405 Washington, D.C.; 2000. http://www.epa.gov/iris.

406 56. Schalm OW, Jain NC. Schalm's veterinary hematology. 4th editio. Philadelphia: Lea & Febiger;
407 1986.

408 57. Naguib MM, El-Kady MF, Lüschow D, Hassan KE, Arafa A-S, El-Zanaty A, et al. New real time
409 and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection
410 (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and
411 Newcastle Disease viruses. J Virol Methods. 2017;245:19–27.

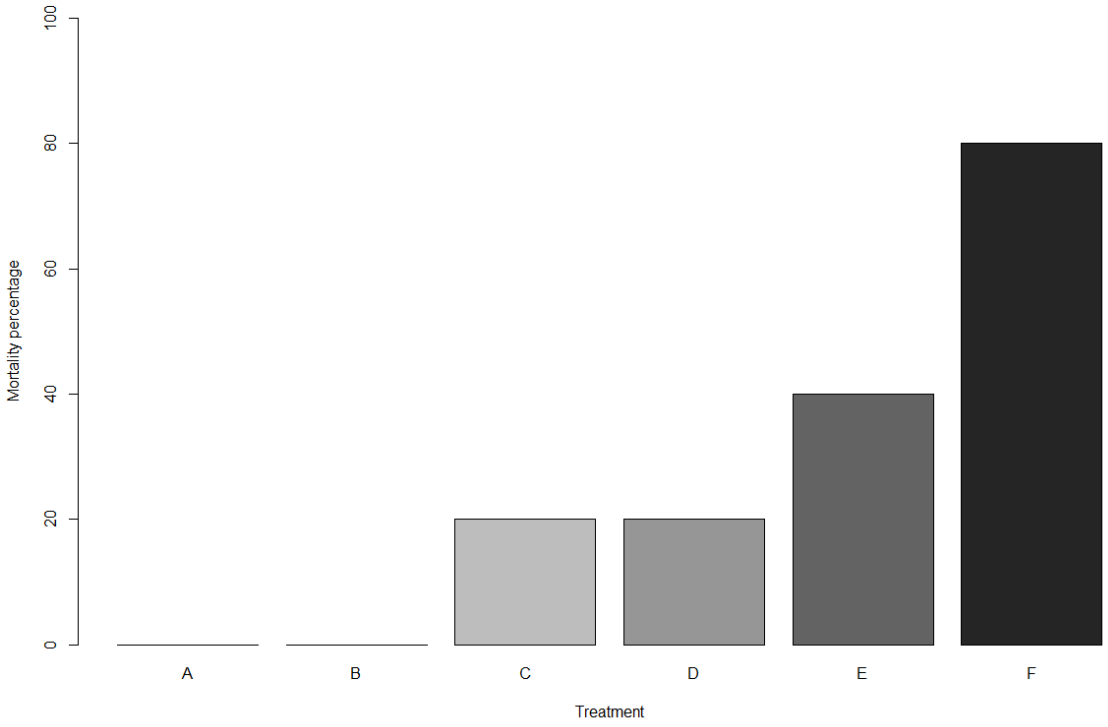
412 58. R Core Team. R Development Core Team. R A Lang Environ Stat Comput. 2016.

413



414
415

416 **Figure 1.** Virus RNA copy number in the allantoic fluid of the infected embryos per treatment group. Virus
417 copy number was calculated by the geometric mean of the triplicate Cq value referred to the standard curve
418 (see methods). Experimental groups D to F contained virus-inoculated embryos (D: Low dose of ClO_2, E: High
419 dose of ClO_2, F. Viral control). For details on treatments, see Table 1.



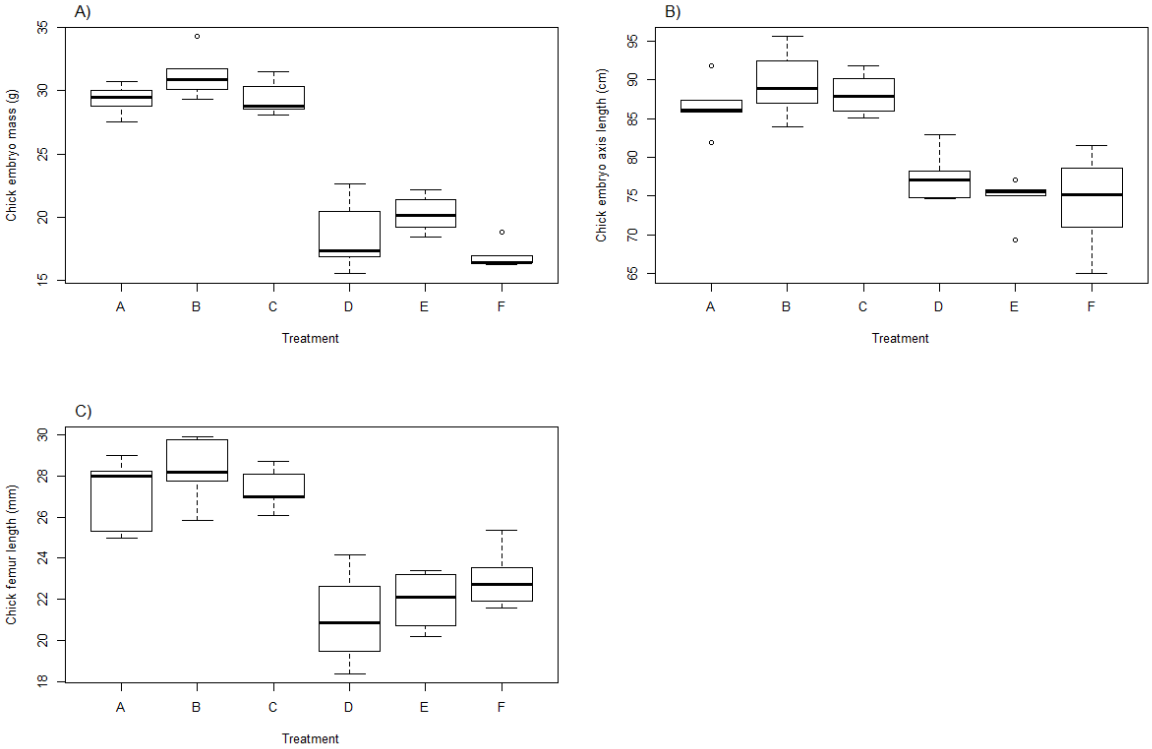
420

421 **Figure 2.** Chick embryo mortality during the experiment. Experimental groups A to C contained virus-free

422 embryos (A: Experimental control, B: Low dose of ClO_2, C: High dose of ClO_2); experimental groups D to F

423 contained virus-inoculated embryos (D: Low dose of ClO_2, E: High dose of ClO_2, F. Viral control). For details

424 on treatments, see Table 1.



425

426 **Figure 3.** Chick embryo development at the end of the experiment (day 17 of incubation). A) mass, B) body

427 axis, C) femur length. Experimental groups A to C contained virus-free embryos (A: Experimental control,

428 B: Low dose of ClO_2, C: High dose of ClO_2); experimental groups D to F contained virus-inoculated embryos

429 (D: Low dose of ClO_2, E: High dose of ClO_2, F. Viral control). For details on treatments, see Table 1.

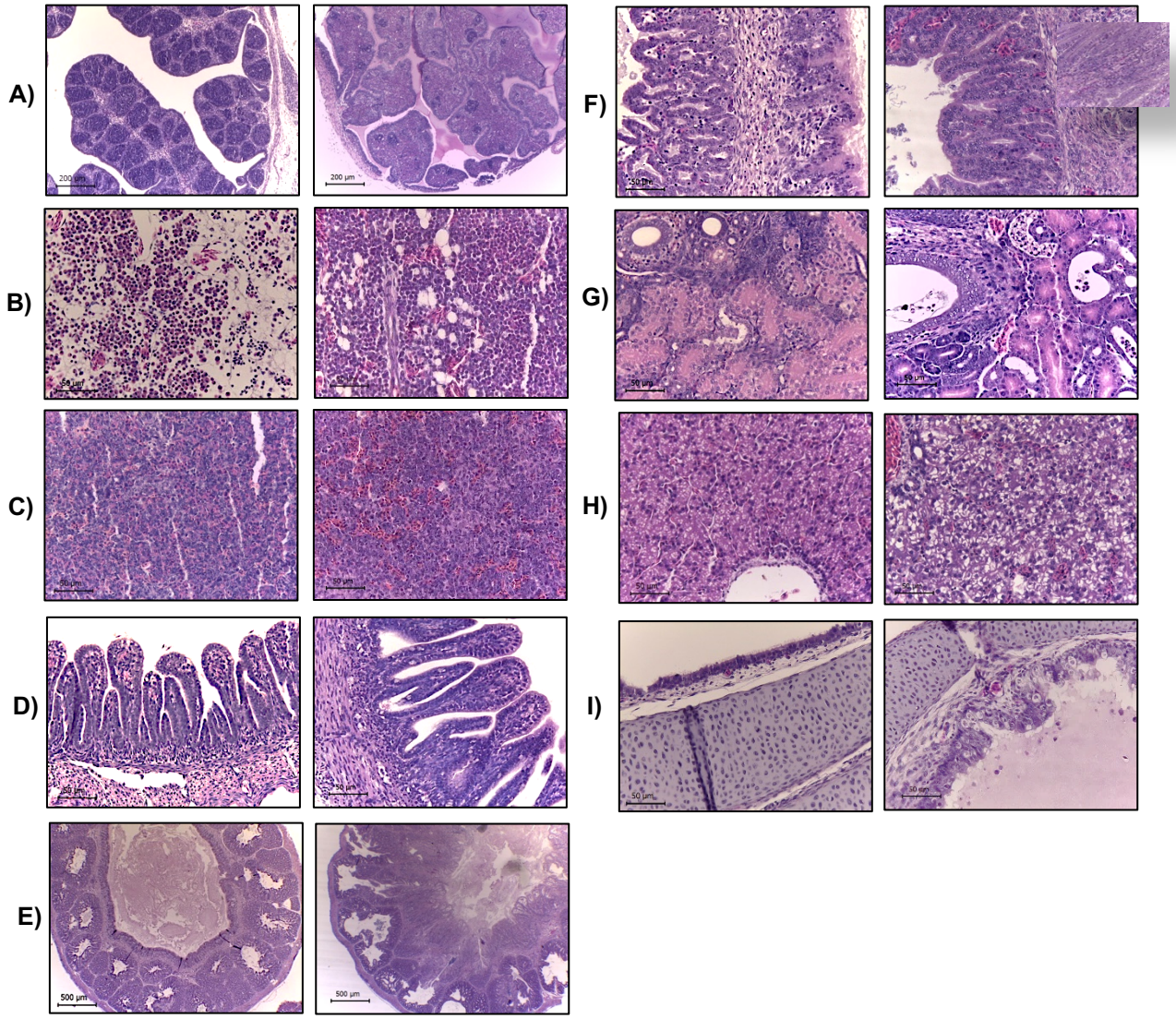


Figure 4. Histology of selected tissues from 17-day-old chick embryos. In each row, microphotograph pairs show H&E-stained representative tissues of non-infected (left) and infected embryos (right), A) Bursa of Fabricio (100X). Infected embryos showed severe lymphoid depletion, with approximately 10% of active lymphoid tissue and abundant heterophils. B) Bone marrow (400 X). Infected embryos showed an increase in erythroid cellularity ne, C) Spleen (400 X), Infected embryos showed reticuloendothelial hyperplasia, D) Duodenum (400 X). Infected embryos showed mild villous atrophy, E) Proventricle (40X). Infected embryos showed epithelial hyperplasia with desquamation and hyaline material, F) Proventricle (400X). Image shows the increased length of the proventricular folds and mitotic cells indicative of epithelial

452 regeneration, G) Kidney (400X). Infected embryos showed swelling, cell detritus and protein within the
453 tubules, and basophilic material compatible with urate crystals, H) Liver (400X). Infected embryos showed
454 glucogenic degeneration, I) Trachea (400X). Infected embryos showed hyperplasia and epithelial
455 degeneration. Scale = 50 µm in all panels except for E (scale = 500 µm).

Table 1. Experimental groups used to assess the *in vivo* antiviral effect of high and low concentrations of ClO2 solution in chick embryos. The table shows the number of embryos included in each group. (LD: Low dose, HD: High dose)

Group	N	Treatment
Group A (Experimental control)	5	200 µl of sterile 0.9% saline solution
Group B (ClO_2 LD)	5	100 µl of sterile ClO_2 solution (30 ppm[1]) and 100 µl of sterile 0.9% saline solution
Group C (ClO_2 HD)	5	100 µl of sterile ClO_2 solution (300 ppm[2]) and 100 µl of sterile 0.9% saline solution
Group D (Virus + ClO_2 LD)	5	100 µl of resuspended avian coronavirus vaccine and 100 µl of sterile ClO_2 solution (30 ppm)
Group E (Virus + ClO_2 HD)	5	100 µl of resuspended avian coronavirus vaccine and 100 µl of sterile ClO_2 solution (300 ppm)
Group F (Viral control)	5	100 µl of resuspended live attenuated avian coronavirus vaccine (Bron Blen® Merial, containing 10^4 of mean embryo infective dose (EID_{50})/mL of coronavirus strains Massachusetts and Connecticut) and 100 µl of sterile 0.9% saline solution

456 [1] This concentration is 10 times below the no-observed-adverse-effect level (NOAEL; 3.5 mg/kg per day)
457 determined for ClO_2 (Bercz et al. 1982), considering an embryonic mass of 10 g at the time of inoculation.
458 [2] This concentration is equal to the NOAEL considering an embryonic mass of 10 g at the time of inoculation.

Table 2. Macroscopic abnormalities observed at necropsy in the chick embryos. The table shows the number of embryos in each group that presented each lesion. (LD: Low dose, HD: High dose)

	Dwarfing	Curling	White caseous material	Thickened membranes	Oedema	Epidermal congestion	Pale and enlarged kidneys
Group A (Experimental control)	0/5[f]	0/5[f]	0/5[f]	0/5[f]	0/5	0/5[f]	0/5
Group B (ClO_2 LD)	0/5[f]	0/5[f]	0/5[f]	0/5[f]	0/5	0/5[f]	0/5
Group C (ClO_2 HD)	0/5[f]	0/5[f]	1/5[f]	0/5[f]	0/5	2/5	0/5
Group D (Virus + ClO_2 LD)	5/5[a]	1/5	1/5[f]	0/5[f]	0/5	2/5	5/5[f,a]
Group E (Virus + ClO_2 HD)	5/5[a]	0/5[f]	1/5[f]	0/5[f]	0/5	2/5	3/5[a]
Group F (Viral control)	5/5[a]	4/5[a]	4/5[a]	4/5[a]	1/5	5/5[a]	1/5

459

[a]: Indicates a significant difference to the experimental control

[f]: Indicates a significant difference to the viral control

Table 3. Microscopic abnormalities related to avian coronavirus infection in the chick embryos. (LD: Low dose, HD: High dose)

	A (Exp. control)	B (ClO_2 LD)	C (ClO_2 HD)	D (Virus + ClO_2 LD)	E (Virus + ClO_2 HD)	F (Viral control)
Trachea	-	Mild hyperplasia	Mild hyperplasia	Karyorexis, focal hyperplasia	Moderate congestion	Mild hyperplasia
Lung	Mild to moderate congestion	Moderate congestion, interstitial oedema	Mild to moderate congestion, interstitial oedema	Moderate to severe congestion, karyolysis	Mild to moderate congestion, moderate oedema	Mild to moderate congestion, severe to moderate oedema
Proventricle	-	-	-	Moderate hyperplasia	Mild hyperplasia	Moderate hyperplasia, mild congestion
Duodenum	-	-	-	Moderate congestion		Mild diffuse congestion, atrophy of intestinal villi
Liver	-	-	-	Gluogenic degeneration, mild congestion	Gluogenic degeneration, mild congestion	Gluogenic degeneration, moderate congestion, periportal eosinophilic material
Kidneys	-	-	-	Degeneration w/ regeneration, congestion, mild heterophilic infiltration, urates	Degeneration w/ regeneration, congestion, mild heterophilic infiltration, urates	Haemorrhage, multifocal heterophilic infiltration, urates
Spleen	-	-	-	Reticuloendothelial hyperplasia	Reticuloendothelial hyperplasia	Reticuloendothelial hyperplasia
Bursa of Fabricius	Subacute heterophilic bursitis	Subacute heterophilic bursitis	Subacute heterophilic bursitis	Subacute heterophilic bursitis	Subacute heterophilic bursitis	Subacute heterophilic bursitis