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Review

1 OXYTETRACYCLINE RESIDUES IN CHICKEN BONES

2

3 **Cytotoxic effects of Oxytetracycline residues in **the** bones of broiler chickens**4 **following therapeutic oral administration of a water formulation**

5

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27 Cytotoxic effects of Oxytetracycline residues in **the** bones of broiler chickens
28 following therapeutic oral administration of a water formulation

29

30 **ABSTRACT**

31 Tetracyclines, **which** represent one of the most commonly used antibiotic **for** poultry, are
32 known to be deposited in bones, where they can **remain**, despite **the** observation of
33 appropriate withdrawal times. The aim of the study was to determine the concentration of
34 oxytretracycline (OTC) residues in **the** bone and muscle of chickens, following **the** oral
35 administration of a commercially available liquid formulation, and to test their cytotoxic
36 effects on an *in vitro* cell culture model. Seventy-two 1-day-old broiler chickens were
37 randomly allotted into two groups (control and treated animals). OTC (40 mg/kg body
38 weight) was administered via drinking water during the 1-5 and 20-25 days of life
39 **periods**. At the end of the **trial, the birds** were slaughtered and **the** OTC residues in **the**
40 target tissues were measured by means of LC-MS/MS. Cytotoxicity was assessed by
41 evaluating the pro-apoptotic effect of **the** bone residues on the **K562** erythroleukemic line
42 and **on the** peripheral blood mononuclear cells (PBMC). In all the animals, **the** OTC
43 residues in **the** muscle were far below the established MRL of 100 µg/Kg. **The OTC**
44 **levels** in **the** bones of **the** treated animals were **instead** found in the ppm range. Cell
45 cytotoxicity was assessed **by** evaluating the pro-apoptotic effect of OTC bone residues on
46 the haematopoietic cell system. **This *in vitro* system has revealed a significant pro-**
47 **apoptotic effect on both the K562 cell line and PBMC** cultures. **This result suggests**
48 potential human and animal health risks due to the entry of tetracycline residues
49 contained in **the** bones of treated livestock into the food-chain. This could be **of** concern,
50 particularly for canine and feline diets, as meat, bone meal and poultry by-products
51 represent **some of the main ingredients of pet foods**, especially in the case of dry pet

52 food. Further studies are needed to define the underlying mechanisms of cytotoxicity and
53 to evaluate the *in vivo* toxicological implications due to the observed *in vitro* effects.

54

55 **Key words:** broiler chicken, oxytetracycline, bone residue, cytotoxicity.

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INTRODUCTION

58 In intensive poultry production, environmental conditions often compromise animal health
59 and immune responses by encouraging infectious diseases to develop and spread easily. For
60 this reason, the group level therapeutic use of antibiotics is very common. The tetracycline
61 class of antimicrobial agents is one of the most commonly used antibiotics in poultry
62 production because of its low costs, efficacy and lack of side effects (Chopra and Roberts,
63 2001). Typical antimicrobial regimens used to treat gastrointestinal and respiratory diseases in
64 broiler chickens include oral medication with oxytetracycline (OTC) for 3-5 days. Although
65 OTC was one of the first tetracycline antibiotics ever produced, limited information is
66 available on its pharmacokinetics in avian species (Black, 1977; Dyer, 1989; Serrano et al.,
67 1999). It has been shown that oral administration results in low and variable systemic levels
68 of the drug and that, among edible tissues, the kidneys and liver contain the highest
69 concentrations (Black, 1977). In order to avoid the presence of drug residues in animal
70 products and to preserve consumers' health, the European Union has established maximum
71 residue levels (MRLs) for OTC in poultry products (European Union, 2010). The observance
72 of appropriate withdrawal periods for each licensed veterinary product ensures the presence of
73 OTC residues below the MRLs. However, it is well known that tetracyclines are able to
74 deposit and persist in bones because they bind directly to the bone matrix or through a more
75 complex binding that takes place between the bone matrix (especially in neonates), calcium
76 ions and the 4 rings of the basic tetracycline structure (Milch et al., 1957). Therefore, it is not
77 surprising that a high incidence of tetracycline residues has been observed in bones taken
78 from pig carcasses at slaughter houses (Kühne et al., 2000). According to the same authors,
79 even the smallest recommended sub-therapeutical dosage of OTC administered orally to
80 chickens leads to detectable residues in bones after the withdrawal of medicated feeds. As a
81 consequence, the current withdrawal times, which are based on the detectable levels of drugs

82 dropping below the MRL in edible tissues, do not seem able to guarantee that bone tissues are
83 free of drug residues after the administration of tetracyclines. The toxicological implications
84 of these residues are still not fully understood. Although *in vitro* and *in vivo* tetracycline
85 cytotoxic effects have already been described, the exact underlying mechanism has not been
86 identified yet (Fife and Sledge 1998; Celik and Eke, 2011).

87 On the basis of these premises, the aim of the study was to investigate the
88 concentration of OTC residues in the bone and muscle of broiler chickens, following oral
89 administration of a commercially available liquid formulation, and to test their cytotoxic
90 effects on an *in vitro* cell culture model.

91

92

MATERIALS AND METHODS

93

Animals and housing

94 The study was supervised by the Department of Veterinary Sciences of the University of
95 Turin (Italy) and performed in the animal farm of the Department of Agriculture, Forestry and
96 Food Science of the University of Turin (Italy). The experimental protocol was designed
97 according to the guidelines of European and Italian laws pertaining to the care and use of
98 experimental animals (European directive 86/609/EEC, put into practice in Italy by
99 Legislative Decree 116/92).

100 A total of seventy two one-day-old male and female broiler chickens (Ross 708) were
101 randomly allotted into two groups (control and treated animals, n=36) and raised in floor pens
102 (three pens/group). Each pen housed 12 chickens of a homogeneous weight and sex-ratio. The
103 birds were fed a commercial organic diet, based on corn and soybean (Abello FIN-IMM s.r.l.
104 - Verzuolo, CN, Italy), which was formulated to meet or exceed the requirements
105 recommended by the National Research Council (1994) and adjusted according to the Ross
106

107 708 Broiler nutrition specification (Aviagen, 2014). The experiment was carried out in a 7m
108 wide x 50m long x 7m high poultry house, equipped with a waterproof floor and wall,
109 covered completely by tiles, and with an automatic ventilation system. The chicks were
110 distributed over 6 pens (3 pens per treatment) that were 1.0m wide x 1.50m long. Each pen
111 was furnished with mixed sawdust and rice hulls as litter. Feeds and drinking water were
112 provided *ad libitum* for the entire duration of the trial, until day 35, which corresponded to the
113 slaughter day. The lighting schedule was 23L:1D during the first 3 days, followed by 18L:6D
114 until the slaughter age. Ambient temperature was kept within the thermo neutral zone, and
115 during the first three weeks, the birds were heated by means of infrared lamps. The chicks
116 were vaccinated at hatching against Newcastle disease, Marek disease, infectious bronchitis
117 and coccidiosis. Health status and mortality were monitored daily during the entire
118 experimental period. Chicken weight and feed consumption were recorded at 1, 21 and 35
119 days of life using a high precision scale (Sartorius - Signum®), and the feed conversion ratio
120 (FCR) was calculated accordingly. Weight gain, average daily gain, daily feed consumption
121 and the feed conversion ratio were calculated for the 1-21, 21-35 and 1-35 day periods, on a
122 pen basis. Three pens were assigned to the Control group (C-group) and three pens were
123 assigned to the Oxytetracycline group (OTC-group); the mode of drug administration is
124 described hereafter.

125

126 **Treatment**

127 Therapeutic treatments were applied, in accordance with the recommendations of the
128 manufacturer, as far as the dose concentration, dosing period and withdrawal time are
129 concerned. Briefly, Oxytetracycline (Ossitetraciclina liquida 20%®, TreI, Reggio Emilia,
130 Italy) was administered via drinking water at a dosage of 40 mg/kg live weight during the
131 1-5 days of life and 20-25 days of life periods. The amount of OTC dissolved in water

132 was adjusted daily on the basis of the water intake and body weight gain. The expected
133 withdrawal time (10 days) was applied between the last OTC administration and
134 slaughtering.

135

136 *Slaughtering procedures and sample collection*

137 At the end of the trial (35 d), six chicks per pen, (three males and three females), were
138 randomly chosen from each pen, and sacrificed by CO₂ gassing followed by neck-cutting.
139 Immediately after slaughtering, the breast and leg were deboned, muscle was ground and
140 a pool was created. Samples were stored at -20°C pending analysis. The bones obtained
141 from the breast and leg deboning (sternum, femur, tibia and fibula) were dried overnight
142 at 50°C, broken, cured in an autoclave (Alfa-10-plus) at 121°C and 1.0 bar over 30
143 minutes, dried at 50°C over 36 hours and finely ground. Samples were stored at -20°C
144 pending analysis.

145

146 *Quantification of the Oxytetracycline residues*

147 Acetonitrile, *n*-pentane, *n*-hexane, ethyl acetate and methanol HPLC grade, formic acid,
148 ethylenediaminetetraacetic acid disodium salt (Na₂EDTA), citric acid and hydrogen sodium
149 phosphate were provided by Sigma-Aldrich (Milan, Italy). The high purity water was
150 obtained from Milli-Q® purification system (Millipore, Bedford, MA, USA). A McIlvaine-
151 Na₂EDTA buffer 0.1 M was prepared by adding 12.9 g of citric acid, 10.9 g hydrogen sodium
152 phosphate and 37.18 g of EDTA Na₂ to 1 L of Milli-Q® water. The solid phase extraction
153 (SPE) C18 cartridges (3 ml, 200 mg) were from Varian (Walnut, Creek, CA, USA). The
154 analytical standards of OTC, 4-epioxytetracycline (4-epi-OTC) and
155 demethylchlortetracycline (DMCTC), used as internal standard (IS), were provided by
156 Sigma-Aldrich (Milan, Italy).

157 **Standard stock** solutions of OTC, 4-epi-OTC or DMCTC were prepared by dissolution
158 in methanol to obtain a final concentration of 1.0 mg/ml. **The standard stock** solutions were
159 stored at -20°C. **The** standard solutions were diluted in methanol to **obtain** a series of working
160 standard solutions, **which** were stored at 4°C in the dark.

161 Samples **s** were prepared according to **Oka et al.** (1998) with minor modifications.
162 Briefly, an aliquot of 1.0 g of chicken-muscle or 0.5 g **of the ground** bone pool was placed in
163 a centrifuge tube, fortified with **the** standard solution, **vortex** shaken for 30 s and left for 30
164 min at room temperature to ensure **an** appropriate distribution in the matrix. A volume of 4.0
165 mL of **the** McIlvaine-Na₂EDTA 0.1 M buffer (pH 4) was added, the mixture was **vortex**
166 shaken for 15 s and centrifuged at 2600 rpm for 5 min at 10°C. The supernatant was
167 transferred to a clean centrifuge tube, and the residue was re-extracted with 4.0 mL of **the**
168 McIlvaine-Na₂EDTA 0.1 M buffer and centrifuged. The combined supernatants were
169 degreased with 4.0 mL of *n*-pentane and centrifuged at 2600 rpm for 15 min at 10 °C. The
170 aqueous layer was loaded **into an** SPE C18 cartridge, **that had** previously **been** activated with
171 methanol and Milli-Q water. After sample loading, the SPE C18 cartridge was washed with
172 Milli-Q water. The analytes were eluted with a mixture of ethyl acetate and methanol (95:5).
173 The solvent was removed under a 40°C stream of nitrogen, and the residue was dissolved in
174 100 µL of **the** mobile phase. An aliquot (10 µL) was injected into the LC-ESI-MS/MS system.

175 Analyses were performed with a 1200 L Varian LC-MS/MS triple quadrupole
176 (Walnut, Creek, CA, USA). The mass spectrometer was equipped with an electrospray
177 interface (**ESI**), operating in the positive mode. The HPLC was equipped with two mobile
178 phase pumps (ProStar 210), a degassit on line, an autosampler (ProStar 410) and a column
179 thermostat. The ESI interface was calibrated using a **polypropylene glycol** solution (**PPG**),
180 and **the** ESI parameters were optimized for each analyte by direct infusion of **the** individual
181 standard solution into the mass spectrometer.

182 The mass spectrometer parameters were: needle 5000 V, shield 600 V, housing 50 °C,
183 capillary voltage 50 V and detector voltage 1500 V. High purity nitrogen was used at 25 psi
184 as nebulizer gas and at 19 psi and 360°C as drying gas. High purity argon was used as the
185 collision gas at 2.0 mTorr. The mass spectrometer was operated at selective reaction
186 monitoring (SRM) mode to confirm the identity of the analytes in the samples by selecting
187 specific precursor-to-product ions for each analyte and by selecting the most abundant
188 transition for the quantification.

189 Separations were conducted using a Pursuit C18 (100 x 2.0 mm I.D., 5 µm) column
190 with a Polaris C18 (2.0 mm, 3 µm) guard column Varian (Walnut, Creek, CA, USA) at 25°C.
191 The A mobile phase solvent was water 0.1% formic acid, while solvent B was acetonitrile
192 0.1% formic acid. The mobile phase was delivered to the LC column at a flow rate of 0.3
193 ml/min. A gradient elution was performed: 0-3 min 10% B; 3.1-5 min 75% B; 5.1-15 min
194 10% B.

195 Calibration curves were prepared for both tissues, and good linearity was achieved
196 over the tested concentration ranges ($r^2 > 0.99$ and goodness-of-fit < 10%). The limits of
197 detection (LOD) for both OTC and 4-epi-OTC in the muscle and bones were 0.5 and 4.5
198 µg/kg, respectively. The within-day precision (R.S.D.%) and accuracy fell within the ranges
199 of -20 to +10%. The analyses were performed in triplicate.

200

201 ***Bone residue cytotoxic evaluation***

202 In order to test the potential cytotoxic role of OTC, two different conditioned cell culture
203 mediums (CCM) were used as providers of OTC residues. Briefly, to obtain CCM, 10 ml of a
204 RPMI 1640 cell culture medium was incubated and constantly shaken for 48 hours at 37°C
205 with 1 g of ground bone (sterilized by autoclaving at 121°C in a steam pressure of 2 atm for
206 10 min) from chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of

207 treatments with OTC (see the C-group and OTC-group in the Animals and housing section).
208 After incubation, the CCMs were recovered and filtered through 0.20 μ syringe filters
209 (Sartorius Stedim Biotech, Goettingen, Germany) to remove any residual ground bone
210 particles and microbial contamination. The CCMs were then diluted at 1: 1, 1: 2, 1: 4, 1: 8,
211 1:16 ratios with an absolute RPMI 1640 growth medium, and the resulting mixtures were
212 incubated with 5×10^5 cells/ml for 48 hours at 37°C and 5% CO₂ in a cell incubator (Thermo
213 Scientific Heraeus, USA). The effect of OTC alone was evaluated by incubating the drug (2
214 μ g/ml), as described above. Furthermore, 200 μ M hydrogen peroxide (H₂O₂) was used as a
215 standard positive control of apoptosis in the 5×10^5 cells/ml culture for 2 hours at 37°C, 5%
216 CO₂.

217 The used cells were the K562 erythroleukemic line (Carbone et al., 1996) or the
218 peripheral blood mononuclear cells (PBMC) from venous blood of healthy human donors
219 (Terrazzano et al., 2007). The PBMCs were obtained by centrifugation on Lymphoprep
220 (Nycomed Pharma) gradients of healthy donor buffy coats obtained from the Blood Bank of
221 the Medical School of the Federico II University of Naples, as previously described
222 (Terrazzano et al., 2007).

223 Apoptosis was assessed by staining of the cell membrane-exposed phosphatidylserine
224 with fluorescein isothiocyanate-conjugated (FITC) Annexin V, according to the
225 manufacturer's instructions (BD Pharmingen), and as previously described (De Vitis et al.,
226 2011). Samples were analyzed by means of flow cytometry, using an FACSCalibur (Beckman
227 Instruments, Fullerton, CA, USA), equipped with CellQuest Analysis Software. The FACS
228 analysis was based on Annexin V staining, and was conducted to evaluate the fluorescence
229 intensity of the staining or the percentage of cells positive to Annexin V so as to have two
230 measurements of the cells undergoing apoptosis.

231

232 *Statistical analysis*

233 The statistical analysis for the growth performance parameters was performed with SPSS 17
234 for Windows (SPSS, Inc., Chicago, IL, USA). The experimental unit was the pen. Before
235 testing for group differences, normality of data distribution and homogeneity of variances
236 were assessed using the Shapiro-Wilk test and the Levene test, respectively. Growth
237 performance data from the C-group and OTC-group were compared by means of the
238 Student's *t*-test. Results were considered statistically significant for $P < 0.05$. A statistical
239 trend was considered for $P < 0.20$. The results are presented as mean values \pm SD.

240 The analysis pertaining to the pro-apoptotic effect was performed using the Mann-
241 Whitney test, and the results were considered significant when $P < 0.05$.

242

243

RESULTS

244 The birds remained healthy for the whole period, no signs of illness were observed and the
245 mortality rate was zero for both groups. Growth performance was not influenced by the
246 treatment (Table 1), and a positive numerical trend was observed for the OTC group for the
247 final individual body weight (day 35), average daily gain (1-35 d), weight gain (1-35 d) and
248 feed consumption over the 21-35d period.

249 As shown in Table 2, the concentrations of OTC, expressed as the sum of the parent
250 drug and its 4-epimer, were far below the established MRL (100 $\mu\text{g}/\text{kg}$) in the muscle of the
251 treated birds. On the other hand, about 100-fold higher levels were measured in the bones.
252 Neither the muscle nor the bone samples showed measurable concentrations of the drug in the
253 control birds.

254 Figure 1 reports data from one representative experiment for Apoptosis detection in
255 the K562 cell line, in terms of peak overlay (panel A) and individual peaks (panel B). Forty-
256 eight hours of incubation with OTC-CCM (ratio 1:2) (peak 3) induced a significant increase

257 in Annexin V staining, compared to the cell culture condition in the medium alone (peak 2),
258 which represents the basal level of the apoptosis that occurs in the K562 cell line without any
259 incubation of CCM. Therefore, OTC-CCM seems able to induce apoptosis in the K562 cell
260 line. C-CCM, instead, only induced slight Annexin V staining (peak 4) at the same ratio.
261 These results point out that C-CCM did not affect cell viability to any great extent.
262 Interestingly, similar OTC-CCM and C-CCM effects were observed when PBMC was used
263 instead of the K562 cell lines (Figure 2). The overall analysis of all the experiments suggest
264 that a 48-hour incubation with OTC-CCM induces significant increases in the percentage of
265 cells undergoing apoptosis in both K562 and PBMC cell cultures (Figure 2, panels A and B,
266 respectively). The effect was observed to be significant at ratios of 1:2, 1:4 and 1:8 ($P < 0.05$),
267 but not at the ratio of 1:16. The increase was also statistically significant after 24 hours at the
268 ratios of 1:2 and 1:4, but was only slightly detectable after 8 and 12 hours of incubation (data
269 not shown). It should be noted that the incubation with 2 $\mu\text{g}/\text{ml}$ of pure OTC elicited similar
270 effects on apoptosis to those obtained with OTC-CCM. Furthermore, the OTC-CCM and
271 pure-OTC effects were quite similar to those elicited through the use of H_2O_2 , which was used
272 as a standard control of apoptosis induction. Despite the fact that apoptosis induction was
273 evident, even after culture incubation with C-CCM at a ratio of 1:2 ($P < 0.05$), the increase
274 was significantly lower than that obtained with OTC-CCM 1:2 ($P < 0.05$). It should be
275 pointed out that CCM was used instead of direct incubation with ground bone, since the latter
276 showed an extensive cytotoxic effect, which was probably due to direct contact with cells and
277 oxygen subtraction from the system ascribable to the volume occupancy of the same ground
278 particles in the culture medium (data not shown).

279

280

DISCUSSION

281 Growth performance resulted to be within the range described in the Ross 708 Broiler
282 Performance Objectives (Aviagen, 2014), thus confirming that appropriate animal care and
283 welfare conditions had been maintained throughout the study. The lack of influence of OTC
284 on the growth performance parameters was an expected result as the drug had been
285 administered at a therapeutic dosage regimen rather than for growth promoting purposes.
286 Nevertheless, it cannot be excluded that an antibacterial action contributed to the positive
287 trend of the body weight and weight gain (Butaye et al., 2003).

288 Our findings confirm that the bones of broiler chickens treated with therapeutic
289 dosages of oral OTC contain considerable amounts of OTC residues. The finding agrees with
290 those of previous studies, thus demonstrating that bone represents a target tissue for
291 tetracycline localization (Milch et al., 1957; Buyske et al., 1960; Kühne et al., 2000). The
292 incidence of tetracycline antibiotic residues in the bones of slaughtered animals seems to vary
293 from 18.8% to 100%, depending on the species. In a study performed by Kühne et al. (2000),
294 the bones of chickens treated for 10 days with low oral dosages of OTC still contained
295 detectable levels of the drug after a withdrawal time of 15 days. Interestingly, in the present
296 study, the concentrations of OTC were below the MRLs set by the European Community (100
297 µg/kg) in all the muscle samples. Similar results have been obtained in turkeys, in which the
298 existence of a correlation between the percentage of positive results in the kidneys and liver
299 and that in the bones has been pointed out (Kühne and Mitzscherling, 2003).

300 In the present study, CCM containing OTC residues induced a significant pro-
301 apoptotic effect in both the K562 and PBMC cell cultures. It is worth noting that if one
302 considers the CCM as a source of OTC, this *in vitro* system represents a useful model to test
303 the cytotoxic effects of this drug.

304 A great deal of evidence supports the *in vitro* and *in vivo* cytotoxicity of tetracyclines
305 (van den Bogert et al., 1981; Shao and Feng, 2013; Chi et al., 2014). Several possible

306 mechanisms have been suggested, including the inhibition of mitochondrial protein synthesis
307 and of the antioxidant defense system (van den Bogert et al., 1981; Chi et al., 2014).
308 Moreover, the *in vitro* effects of tetracyclines have been related to the inhibition of
309 lymphocyte proliferation and the negative modulation of neutrophil phagocytic functions
310 (Thong and Ferrante, 1980).

311 As far as PBMC cell cultures are concerned, the effects of OTC-CCM appear to be
312 dose-dependent, and comparable with that of hydrogen peroxide at a dilution ratio of 1:2. The
313 effect of pure OTC on inducing apoptosis also appears to be rather relevant, since there has
314 been no definitive observation on the toxic effects of this compound on mammalian cells and,
315 in particular, on humans.

316 Although a threshold for OTC *in vitro* toxicity could not be established, the most
317 interesting result is that OTC residues in the bones of slaughtered animals maintain cytotoxic
318 effects, in spite of mechanical and thermal treatments. In fact, the possible toxicological risks
319 depend on the degree of biological activity maintained by the drug residues in fresh raw
320 materials or following feed processing. Meat, bone meal and poultry by-products are the main
321 ingredients of pet foods, especially in the case of dry pet food. For the latter, the most
322 frequently used processing technology to produce canine and feline diets or dinner ingredients
323 is extrusion. The extrusion process usually involves the application of both relatively high
324 temperatures (80-200°C) and short residence times (10-250 seconds) (Serrano and Agroturia,
325 1996). The thermal stability of tetracycline antibiotics in animal food products has been the
326 subject of different studies (Ibrahim and Moats, 1994; Hassani et al., 2008). The variety of
327 methodologies employed in the heat inactivation experiments and in the detection of the
328 antibiotic residual concentrations could explain the differences in the results. However,
329 according to Hassani et al. (2008), low-temperature-long-time treatments (conventional
330 sterilization) could destroy >98% of the initial concentration of tetracycline residues, but

331 high-temperature-short-time treatments (e.g. UHT) would leave **the** residues in the 50–90%
332 range **unaltered**. Finally, heat stability depends on the type of matrix. Although it is assumed
333 that tetracyclines are not very heat resistant, residues in bones seem to be more stable
334 (Honikel et al., 1978). When considering an intermediate product from a rendering plant
335 mixed with bone splinters containing bound tetracycline residues, a complete destruction
336 during a heat treatment at 133°C for up to 45 min could not be demonstrated (**Kühne** et al.,
337 2001). It should be noted that most heat stability studies **have** evaluated the degradation of
338 parent drugs without considering **the** possible formation of breakdown products endowed with
339 toxic effects. Little is known about the breakdown products formed from tetracycline and
340 OTC during heat treatments (**Gratacós-Cubarsí et al., 2007; Kühne et al., 2001**). Therefore, **it**
341 cannot **be excluded** that the cytotoxic effects observed on the K562 and PBM cells can be
342 ascribed to **different** compounds **from** OTC.

343 Besides the fact that **it is necessary to** be cautious in drawing conclusions about *in vivo*
344 toxicological implications due to the observed *in vitro* cytotoxic effects, it cannot be excluded
345 that OTC residues in **the** bones of treated chickens can induce biological responses in pets and
346 human consumers. Interestingly, **higher** OTC and doxycycline serum levels than the safety
347 limits **have been** observed **in gym-trained subjects with food intolerance symptoms** (Di Cerbo
348 et al., 2014). The authors speculated that tetracycline antibiotic residues transferred to **the**
349 final consumers **could** act as haptens **and induce** specific intolerance to a wide variety of food.
350 **Further studies are needed to confirm this hypothesis.**

351 Following oral treatment with therapeutic doses of OTC, residues in the ppm range
352 accumulate in **the** bones of treated animals. Although little is known about the biological
353 activity of **the** residues contained in target organs of slaughtered animals, **the present** findings
354 **suggest** *in vitro* pro-apoptotic effects on normal and cancer cells **of humans**. Further studies
355 are needed to define the mechanisms **responsible for** the cytotoxic effect. Potential human and

356 animal health risks, due to the entry of tetracycline residues contained in **the** bones of treated
357 livestock into the food-chain, should not be underestimated.

358

359

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362

363

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442 **Table 1.** Growth performance parameters in control animals and in broiler chickens treated
 443 with Oxytetracycline (OTC) (mean values \pm SD, Student's t test, n= 3).

	Control group	OTC group	P-value
Growth performance			
Mortality rate (%)	-	-	-
Individual body weight (g)			
day 1	42.3 \pm 1.2	42.1 \pm 0.9	0.805
day 21	834.5 \pm 15.7	867.1 \pm 57.0	0.395
day 35	1729.2 \pm 11.2	1771,89 \pm 11.2	0.155
Average daily gain (g)			
1-21days	37.7 \pm 0.7	39.3 \pm 2.8	0.395
21-35days	63.9 \pm 1.5	64.6 \pm 2.0	0.646
1-35days	48.2 \pm 0.3	49.4 \pm 1.2	0.160
Weight gain (g)			
1-21days	792.3 \pm 14.6	825.0 \pm 57.8	0.395
21-35days	894,7 \pm 20.9	904,8 \pm 28.6	0.646
1-35days	1686.9 \pm 11.6	1729.8 \pm 41.5	0.160
Daily feed consumption (g)			
1-21days	59.8 \pm 3.5	60.3 \pm 5.3	0.898
21-35days	112.1 \pm 2.0	115.8 \pm 1.6	0.068
1-35days	80.7 \pm 2.8	82.5 \pm 3.1	0.503
Feed conversion ratio			
1-21days	1.58 \pm 0.06	1.53 \pm 0.04	0.324

21-35 days	1.76 ± 0.06	1.79 ± 0.05	0.461
1-35days	1.67 ± 0.06	1.67 ± 0.04	0.889

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451 **Table 2.** Oxytetracycline (OTC) concentrations ($\mu\text{g}/\text{kg}$), expressed as the sum of the parent
452 drug and 4-epimer, in the bone and muscle of control and treated broiler chickens ($\mu\text{g}/\text{kg}$ on
453 dry matter basis, mean values \pm SD, $n=3$).

	Control group	OTC group
Muscle	<LOD	12.3 \pm 6.9
Bone	<LOD	1286.3 \pm 256.6

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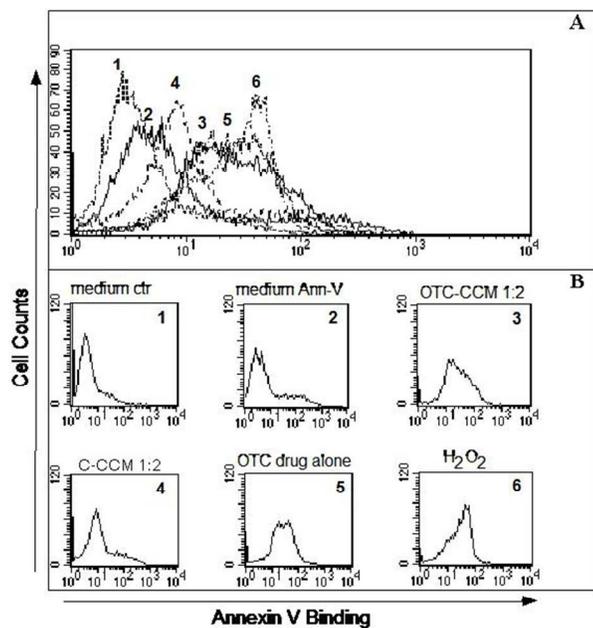
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456 **Figure 1.** Apoptosis induction evaluated as fluorescence intensity of FITC-Annexin V-
457 staining in one representative experiment. The upper panel refers to the overlay of all the
458 fluorescence peaks in the different conditions for the K562 cell line cultures. The lower
459 panels represent the fluorescence peaks for each cell condition. The x-axis shows the
460 fluorescence intensity of Annexin V binding on a logarithmic scale. The amplitude of the
461 apoptosis induction is proportional to the right sliding of the peak on the x axis towards higher
462 values of fluorescence for Annexin-staining (to facilitate the reader's interpretation: peak 1 is
463 the one that shows the lowest intensity, while 6 represents the peak at the highest intensity in
464 the figure). In all the panels, the peaks correspond to the following different K562 cell culture
465 conditions: 1= in a growth medium alone without Annexin V staining, as a control of the cell
466 natural fluorescence background; 2= in a growth medium with Annexin V staining, as a
467 control of the apoptosis that occurs in the K562 cell line maintained in a culture without any
468 other incubation; 3 and 4= are the peaks that represent the growth medium with the addition
469 of a conditioned cell culture medium obtained from the ground bone of chickens reared in the
470 presence (3= OTC-CCM) or in the absence (4= C-CCM) of a treatment with oxytetracycline,
471 at a dilution of 1:2, stained with Annexin V; 4= in a growth medium with the addition of C-
472 CCM, at a dilution of 1:2, stained with Annexin V; 5= in a growth medium with the addition
473 of 2 µg/ml of oxytetracycline (OTC), stained with Annexin V; 6= in a medium with the
474 addition of 100 µM H₂O₂, stained with Annexin V. The medium volumes for the different
475 cell cultures were the same. See the Material and methods section for the Annexin V staining.

476 **Figure 2.** Apoptosis induction measured as a percentage of cells positive for the FITC-
477 Annexin binding in the K562 cell line culture (panel A) and of PBMC (panel B). The graph
478 bar-columns represent the mean values of the percentage of cells undergoing apoptosis in all
479 the performed experiments. The different cell incubations and conditioned cell culture
480 medium dilutions are indicated on the x axis. The abbreviations indicate the growth medium
481 with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone
482 of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of a treatment
483 with oxytetracycline, a growth medium with the addition of 2 µg/ml of oxytetracycline (OTC)
484 or with 100 µM H₂O₂. All the cell cultures were stained with Annexin V (see the Material and
485 Methods section). It should be noted that the bar-column of the medium alone indicates
486 incubation in a growth medium with Annexin V staining, which has been used as a control of
487 the apoptosis that occurs in the cells when in a culture without any other incubation is
488 maintained. The statistical significance is indicated with an asterisk for each of the pairs of
489 columns placed under the horizontal square brackets for the coupled-comparison.

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Review

