Toxicological Implications and Inflammatory Response in Human Lymphocytes Challenged with Oxytetracycline

A. Di Cerbo,[1,*] A. T. Palatucci,[2,*] V. Rubino,[3,4] S. Centenaro,[5] A. Giovazzino,[3,4] E. Fraccaroli,[5] L. Cortese,[6] G. Ruggiero,[3] G. Guidetti,[5] S. Canello,[5] and G. Terrazzano[3,7]

[1]School of Specialization in Clinical Biochemistry, "G. d'Annunzio" University, Chieti, Italy

[2]PhD School of Science, University of Basilicata, 85100 Potenza, Italy

[3]Department of Translational Medical Sciences, University of Naples Federico II, Naples, Italy; E-mail: *terrazza@unina.it* giuseppe.terrazzano@unibas.it

[4]Research and Development Laboratory, GRAF SpA, Nonantola (MO), Italy

[5]Division of Research and Development, Sanypet SpA, 35023 Bagnoli di Sopra (PD), Italy

[6]Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Naples, Italy

[7]Department of Science, University of Basilicata, 85100 Potenza, Italy

ABSTRACT: Antibiotics are widely used in zoo technical and veterinary practices as feed supplementation to ensure wellness of farmed animals and livestock. Several evidences have been suggesting both the toxic role for tetracyclines, particularly for oxytetracycline (OTC). This potential toxicity appears of great relevance for human nutrition and for domestic animals. This study aimed to extend the evaluation of such toxicity. The biologic impact of the drug was assessed by evaluating the proinflammatory effect of OTC and their bone residues on cytokine secretion by in vitro human peripheral blood lymphocytes. Our results showed that both OTC and OTC-bone residues significantly induced the T lymphocyte and non-T cell secretion of interferon (IFN)-γ, as cytokine involved in inflammatory responses in humans as well as in animals. These results may suggest a possible implication for new potential human and animal health risks depending on the entry of tetracyclines in the food-processing chain. © 2015 The Authors Journal of Biochemical and Molecular Toxicology Published Wiley Periodicals, Inc. J. Biochem.

INTRODUCTION

The use of antibiotics in the agro-food industry is a relevant concern [1]. They have been still employed as growth promoters in livestock, aquaculture, and pesticides [1–3]. The topic is relevant considering the potential toxic risk derived by the entry/accumulation of antibiotics in animal feed and human food with consequences on health [4].

The use of antibiotics for growth promotion is prohibited in Europe, whereas the United States and Canada still allow use of antibiotics in agriculture for nontherapeutic purposes [5]. New regulations from the Food and Drug Administration (FDA) are endeavoring to reduce antibiotic contaminants in foods [6, 7]. Although allergic reactions are rarely related to antibiotics, meats and fruit induced by antibiotic residues have been reported in the literature [1, 8]. The occurrence of antibiotic toxic effects has negative consequences on the gastrointestinal tract, skin, central nervous system, and even accumulating in calcium-rich organs such as bones and teeth [9–11].

Food intolerance has described in gym subjects due to intake of meat derived from administration of animals-fed tetracycline [12]. Several models have already been proposed to demonstrate cytotoxic effects of tetracyclines [13–15]. To the best of our knowledge, the possible interplay between tetracyclines, in particular the oxytetracycline (OTC), and immune system is still lacking and not sufficiently addressed to rule out the possible toxicity to human and animal health.

In this regard, the immune system acquires a new pivotal role in modulating toxicological mechanisms that could be triggered by tetracyclines derived by ingested food. Immunity, which has the fundamental role to protect and defend the organism from the disease [16–18], is also involved in the homeostasis and health maintenance against autoimmunity diseases and tumors [17]. The exacerbation of cytotoxic CD8+ T lymphocytes [18] and CD4+ T Helper 1 (T_H1) [19] responses were associated with inflammatory diseases and autoimmunity disorders [20]. T_H1 activity is mainly based on the production of interferon-gamma (IFN)-γ, which optimizes the antimicrobial responses and fosters CD8+ T lymphocyte activity, and also appears to play a fundamental role in triggering autoimmune responses [21–29]. Natural killer (NK)-dependent secretion of IFN-γ is relevant to autoimmunity [30–33], allergy [34], and could have a pathogenetic role in gastrointestinal [35] and hematological disorders [36, 37]. T_H2 response is based on several cytokines including interleukin (IL)-4 that results in the activation of humoral immunity [21].

It is worth noting that several extrinsic factors, such as drugs and chemicals, can induce the development of autoimmune conditions [38–43].

Moreover, the use of these drugs to treat inflammatory conditions is still not conclusive and, in some way, controversial as well as the abuse of some veterinary drugs, including tetracyclines, which have a global impact on the environment that could be of great relevance [[44]–[50]].

OTC represents the main drug used to control gastrointestinal and respiratory diseases in broiler chickens [51], although its accumulation has been described in chicken edible tissues [52]. As a consequence, the European Union established the maximum residue level of OTC in poultry meat [53] to limit drug residues and to preserve health of final consumers that are mainly represented by domestic animals and humans.

Based on a previous study on the toxicity of OTC [54], we investigated the potential toxic effect of OTC in an in vitro human lymphocyte model. In particular, we addressed the potential induction of IFN-γ production caused by the in vitro exposure of human T and non-T lymphocytes to OTC or to chicken bone-derived residues.

MATERIALS AND METHODS

Cells

Peripheral blood samples from healthy donor volunteers were collected by vein puncture according to standard procedures and used within the 3 h from the collection. Informed consent was obtained in accordance with the Declaration of Helsinki, as approved within the study protocol by the Institutional Review Board at the Federico II University of Naples. Peripheral blood mononuclear cells (PBMC) were used as mixed population of T (CD3+) and non-T (CD3–) (the latter are mainly represented by NK cells) lymphocytes [17, 18]. Identification of cell subpopulations was performed by immune-fluorescence and flow cytometry (see paragraph 2.4, Monoclonal Antibodies, Flow Cytometry, Detection of Intracellular IFN-γ, and IL-4 Productions). PBMC were isolated by centrifugation on Lymphoprep (Nycomed Pharma) gradients, as described [35].

OTC and the Conditioned Cell Medium

To test the potential toxic role of OTC (Oxytetracycline 20%®, TreI, Reggio Emilia, Italy) and OTC bone residues, two different conditioned cell culture mediums (CCM) were used, as previously described [54]. Briefly, to obtain CCM, 10 mL of a RPMI 1640 cell culture medium was incubated and constantly shaken for 48 h at 37°C with 1 g of ground bone (sterilized by autoclaving at 121°C in a steam pressure of 2 atm for 10 min) from chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of treatments with OTC [54]. After incubation, the CCMs were recovered and filtered through 0.20 μm syringe filters (Sartorius Stedim Biotech, Goettingen, Germany) to remove any residual ground bone particles and microbial contamination.

Apoptosis Detection

Apoptosis detection was performed as previously described [54]. Briefly, OTC- CCM and C-CCM were used at the dilution of 1:4 with an absolute RPMI 1640 growth medium, and the resulting mixtures were incubated with 5×10^5 PBMC/mL for 10 or 48 h at 37°C and 5% CO_2 in a cell incubator (Thermo Scientific Heraeus). The effect of OTC alone was evaluated by incubating the drug (1 μg/ mL), as described above.

Apoptosis was assessed by staining of the cell membrane-exposed phosphatidylserine with

fluorescein isothiocyanate-conjugated (FITC) Annexin V, according to the manufacturer's instructions (Becton Dickinson PharMingen, San Jose, CA) and as previously described [55]. Samples were analyzed by means of flow cytometry, using a two laser-equipped FAC-SCalibur (Becton Dickinson PharMingen, San Jose, CA), and the CellQuest Analysis Software. The FACS analysis was based on the percentage of Annexin V-positive cells to have a measurement of the cells undergoing apoptosis.

Monoclonal Antibodies, Flow Cytometry, Detection of Intracellular IFN-γ, and IL-4 Productions

FITC, PE, Cychrome, and APC labeled mAbs against CD3, CD8, CD4, IFN-γ, IL-4, and isotype-matched controls (Becton Dickinson PharMingen, San Jose, CA) were used to identify the CD8+ T cytotoxic, CD4+ T_H lymphocytes, or CD3– non-T cells.

To analyze the production of IFN-γ and IL-4, purified PBMC were cultured overnight (10–12 h) in the presence of *phorbol*-12-myristate-13-acetate (PMA) and ionomycin (Sigma). To avoid extracellular cytokine export, the cultures were performed in the presence of 5 μg/mL of Brefeldin-A (Sigma-Aldrich) as described [56].

Intracellular IFN-γ and IL-4 production was detected by using a triple staining technique and flow cytometry analysis. Briefly, after the incubation the culture was harvested, the cells were fixed and permeabilized by using a cytokine staining kit, following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Samples were analyzed by flow cytometry (see description in the *Apoptosis Detection* section).

Statistical Analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). All data are presented as the means ± standard error of the mean and were first checked for normality using the D'Agostino-Pearson normality test. The analysis pertaining to the proinflammatory effect was analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. $p < 0.05$ was considered significant. Statistical analysis was specifically performed to evidence differences within the pairs of comparison (OTC vs. ctr, OTC-CCM vs. ctr, C-CCM vs. ctr, as indicated in the Figures 1 and 3).

RESULTS

The Toxic Effect of OTC and Their Residues from Bone as Induction of Apoptotic Phenomenon in PBMC

According to previous data [56], the OTC was able to induce the apoptosis in PBMC after 48 h of incubation (Figure 1, panel A). A similar effect was obtained using the OTC-CCM, whereas no results were obtained with C-CCM (Figure 1).

Note that the incubation with OTC or with OTC-CCM did not exert significant apoptosis phenomenon in PBMC after an incubation of approximately 10–12 h (Figure 2).

These results significantly confirmed the toxicity of OTC and of their residues in bone (OTC-CCM) and also evidenced the difference between the early (10-12 h) and late (48 h) exposure to this drug. Such data allowed to identify the range of time (10-12 h), in which the cells do not undergo OTC-dependent apoptosis, to perform experiments of cytokine secretion (see the Materials and Methods section).

The Proinflammatory Toxic Effect of OTC and Their Residues from Bone as Significantly Increasing IFN-γ Production in T Lymphocytes as well as in Non-T Cells

In the light of the described observation (see the preceding paragraph), we evaluated the other possible alterations caused in human lymphocytes after the exposure to OTC [54]. With this aim, we focused on the IFN-γ production, as the main proinflammatory cytokine is able to foster the T_H1 and T cytotoxicity immune-responses as well as to be involved in several etiopathogenetic mechanisms on the basis of inflammatory-mediated disease [19].

Since the evaluation in vitro of IFN-γ production by T and non-T lymphocytes is usually performed in short time (8–18 h) to obtain an optimal functional cytokine secretion [56] and we demonstrated that after 10 h the apoptosis was not induced (Figure 1, panel B), we incubated human PBMC with OTC and OTC-CCM for 10 h to avoid this phenomenon. Indeed, the good viability of lymphocytes is crucial for the induction of cytokine production functions.

As shown, the incubation with OTC and OTC-CCM was able to significantly increase the IFN-γ production in CD4+ T_H cells (R1 in Figure 2 and panel A in Figure 3) and CD8+ lymphocytes (R2 in Figure 2 and panel B in Figure 3) as well as in non-T cells (R3 in Figure 2 and panel C in Figure 3). The cytokine was slightly detectable at the dilution 1:8 and 1:16 of OTC-CCM (data not shown), whereas
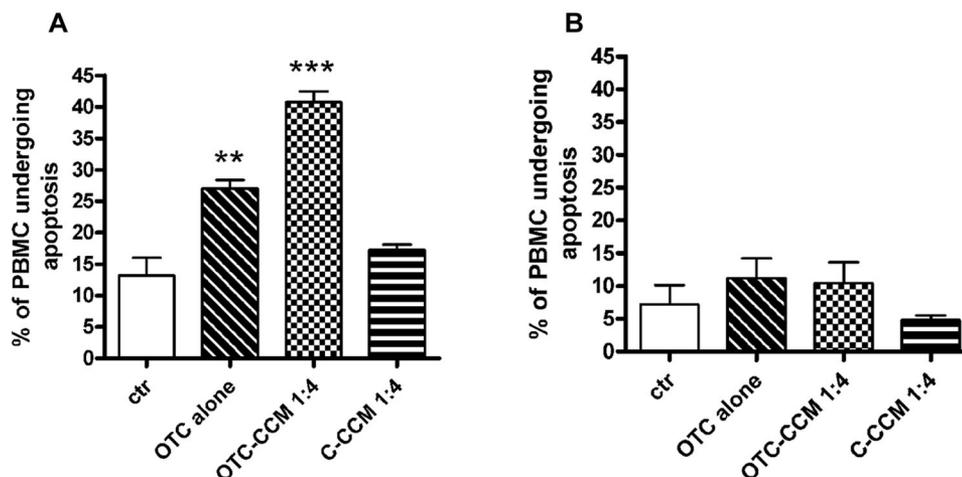
FIGURE 1. Apoptosis induction measured as a percentage of PBMC positive for the FITC-Annexin binding. The graph bar columns represent the mean values of the percentage of PBMC undergoing apoptosis in the performed experiments ($n = 4$). The different cell incubations and conditioned cell culture medium dilutions are indicated on the x-axis. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of a treatment with OTC, a growth medium with the addition of 1 μg/ mL of OTC alone. The bar column depicted as "ctr" indicates the incubation in the growth medium with Annexin V staining, which has been used as a control of the apoptosis that occurs in the cells when in a culture without any other incubation is maintained. The statistical significance is indicated with asterisk(s): *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the dilution of 1:2 appeared to induce high level of apoptosis [56].

In the same cytokine production test, the incubation of PBMC from 12 to 18–24 h resulted in a very poor cell viability (data not shown) likely dependent on the here described proapoptotic effect of OTC and on Brefeldin A exposure used to allow the cytokine intracellular retention for the measurement (see the Materials and Methods section)

It is worth noting that C-CCM incubation did not produce a cytokine increase, while appeared to reduce IFN-γ production. Although we did not investigated this phenomenon, we do not exclude that some substances present in the bone (i.e., cytokines derived from osteoblasts or fibroblasts) may have an inhibitory role on the cytokine secretion. However, the difference in effects between OTC-CCM and C-CCM highlights the specificity of OTC action since the used chickens were of the same type and the only difference was the OTC administration [54].

The basal IL-4 production was only slightly detectable in T and non-T lymphocytes, as expected in PBMC from healthy donors after exposure to PMA and ionomycin [56] and was not modulated after 10 h of OTC or CCM incubations (data not shown).

DISCUSSION

In this article, we suggest that an antibiotic, the OTC, is able to determine the in vitro toxic effects. Data

acquire great relevance especially in light of the wide use of OTC in animal breeding.

The OTC or the OTC-conditioned culture medium, obtained with the incubation of ground bone from OTC-treated chickens, appeared to generate the in vitro toxic effect of cell death by apoptosis in human cells [56].

Here, we suggest that the toxicity of OTC may also be extended to the induction of a proinflammatory microenvironment potentially responsible for initiation of tissue inflammatory spreading [21] or of autoimmune diseases [59].

In this regard, besides the ability to induce mortality of both the T lymphocytes and non-T cells in an in vitro system for incubation with OTC for 48 h, the drug potently promotes the production of proinflammatory cytokines in the first 10–12 h of cell exposure. Specifically, human lymphocytes increase their production of IFN-γ when exposed to the OTC or to the conditioned culture media with the bone of chickens treated with such drug as usually happens in livestock common breeding [51]. In our in vitro model, both the innate (non-T cells that are mainly represented by NK lymphocytes) and acquired (CD8+ and CD4+ lymphocytes) immunity [17, 18] appeared to be involved in this process and to suffer the OTC-dependent toxicity.

In this context, it is known that IFN-γ represents the main cytokine involved in the immune response [19], as well as a crucial element in the onset of impaired tissue homeostasis conditions, typically related to autoimmunity or chronic inflammation [23–31].
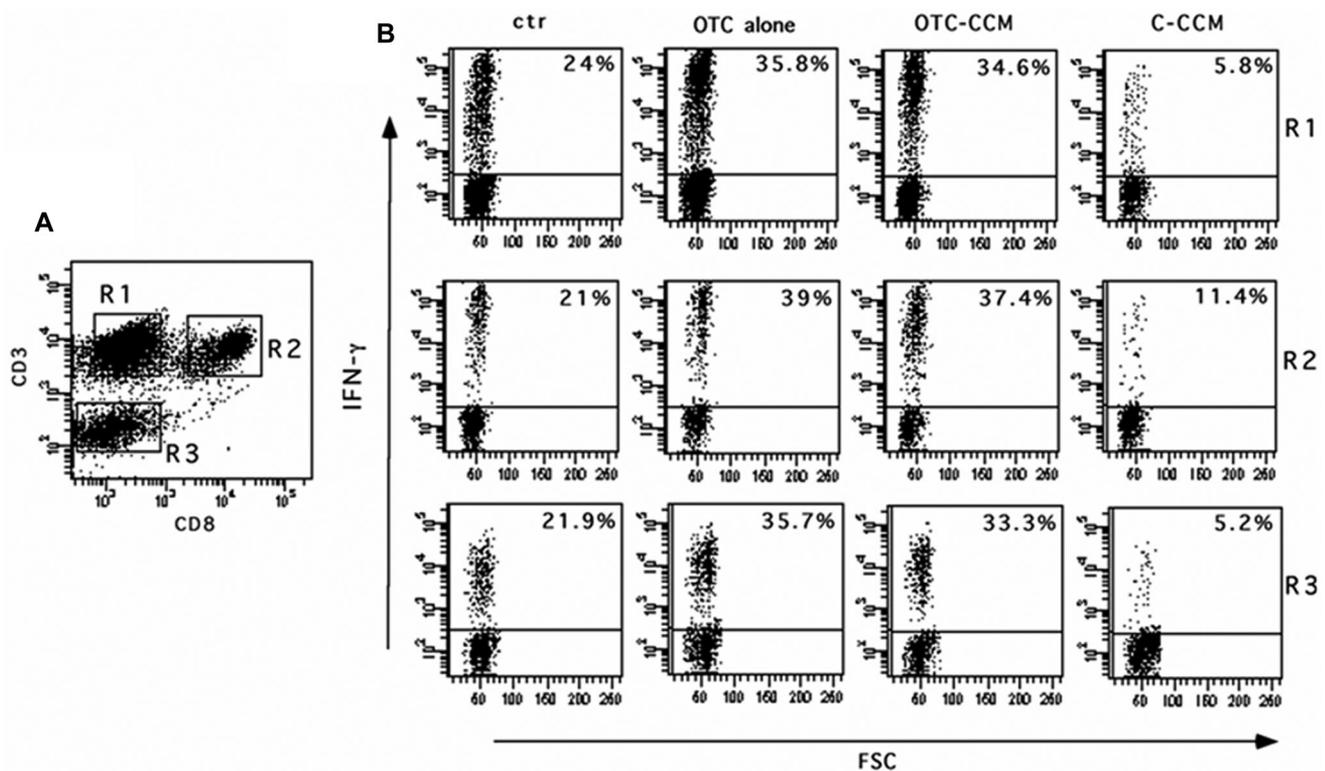
FIGURE 2. One representative experiment showing the IFN-γ production in CD4+ and CD8+ T lymphocytes and in non-T cells. Cytokine production was evaluated as the percentage of IFN-γ producing cells. *Panel A* refers to fluorescence gating strategy to identify the CD4+ T lymphocytes (CD3+ CD8–, cells in R1), CD8+ T lymphocytes (CD3+ CD8+ cells in R2) and the non-T cells (CD3– cells in R3). *B panels* represent the percentage of IFN-γ producing CD4 T (R1), CD8 T (R2), and non-T (R3) cells. The different cell incubations and conditioned cell culture medium dilutions are indicated on the top. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of an OTC treatment, a growth medium with the addition of 1 μg/ mL of OTC alone. The condition indicates as "ctr" refers to basal IFN-γ production. All the cell cultures (ctr, OTC alone, OTC-CCM and C-CCM) were maintained in a growth medium added with PMA and ionomycin to induce cytokine production (see the Materials and Methods section).

A number of workers [46–52] have suggested that OTC would likely to represent a toxic compound and could be harmful to human health and animals that can eat meat derived from chikens by intensive livestock.

In addition, the induction of cell mortality could generate an altered tissue condition, as well as a relevant impact on tissue homeostasis [57, 58] and the emergence of autoimmune reactions [59–63].

Both pets and humans could take this antibiotic as a residue from meat or in meat-derived products and might likely suffer the OTC-dependent toxicity. In this respect, it is interesting that, over the past 20 years, there has been an exacerbation of the emergence of immune-mediated diseases (such as allergies, autoimmune reactions, and disorders of the gastrointestinal tract and the skin) in domestic animals [64–67] and humans [68, 69]. Moreover, it is surprising that the drastic increase of antibiotics resistance phenomena is partly due to the widespread and uncontrolled use of drugs in breeding [7, 70–74]. We previously correlated the use

of specific meats to the occurrence of these pathologies in humans [12].

This unusual increase is probably dependent on a complex set of multifactor events related to new life habits of humans and pets, as well as to the increasingly introduction of industrialized diets. Hence, the needing to change the approach to livestock by promoting sustainable breeding avoiding overcrowding and by reducing antibiotics favoring the use of alternative treatments. Unfortunately, the use of several drugs can promote development of autoimmunity [38–43].

In conclusion, a special attention is probably needed on nutrition for large mass since it might expose humans and pets to increased risk of disease.

STUDY LIMITATIONS

Notably, this research has some study limitations. In this regard, the absence of in vivo experiments, able
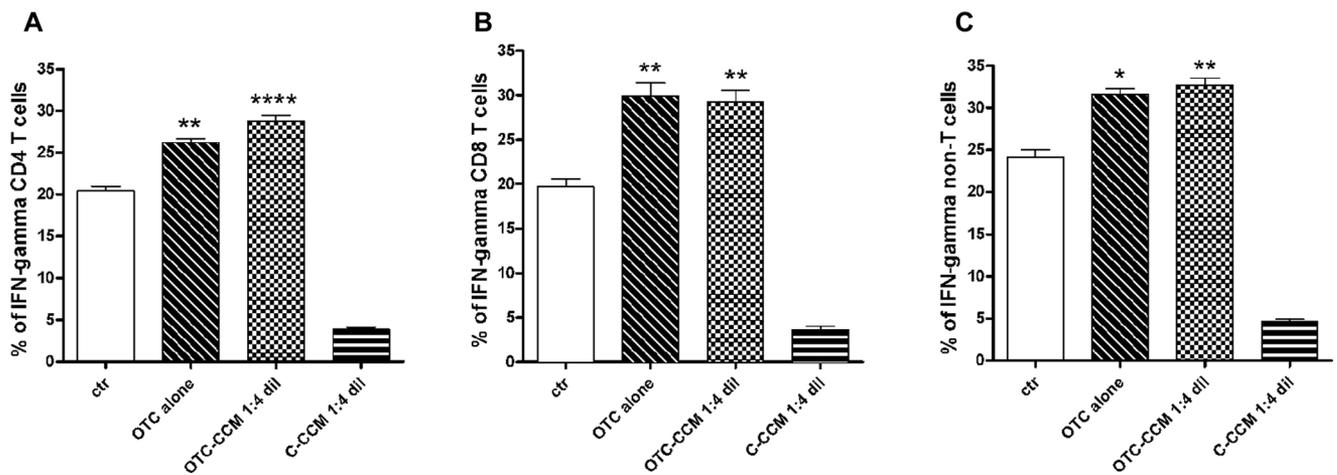
FIGURE 3. Statistical analysis of the all experiments ($n = 10$) showing the IFN-γ production in CD4+ and CD8+ T lymphocytes and in non-T cells. Cytokine production was evaluated as the percentage of IFN-γ producing cells. The bar column graphs represent the mean values of the percentage of IFN-γ producing cells. The different cell incubations and conditioned cell culture medium dilutions are indicated on the x axis. The abbreviations indicate the growth medium with the addition of a conditioned cell culture medium (CCM) obtained from the ground bone of chickens reared in the presence (OTC-CCM) or in the absence (C-CCM) of an OTC treatment, a growth medium with the addition of 1 μg/ mL of OTC alone. The condition indicates as "ctr" refers to basal IFN-γ production. All the cell cultures (ctr, OTC alone, OTC-CCM and C-CCM) were maintained in a growth medium added with PMA and Ionomycin to induce cytokine production (see materials and methods). *Panels A, B,* and *C* show IFN-γ production in CD4+ T lymphocytes, CD8+ T lymphocytes and in non-T cells, respectively. The statistical significance is indicated with asterisk(s): *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

to verify the in vitro observed OTC toxicity, represents the main relevant limitation. Therefore, clinical studies are required to ascertain the in vivo effect of the drug in inducing the inflammatory status in animals and/or in humans.

In addition, the use of CCM obtained by incubation with bones from chickens reared in the presence of OTC did not directly demonstrate that the cell toxicity is due to bone's drug residues and did not ruled out that other substances could have a role.

CONFLICT OF INTERESTS

None of authors have financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper. This research was performed in collaboration with some scientists from the Division of Research and Development of Sanypet SpA and of GRAF S.p.A (as indicated in the author's affiliation) according to scientific and ethical principles of the scientific community. None financial funding was obtained from Sanypet Industry nor from GRAF Lab for this research study

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