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RESEARCH ARTICLE

Cytotoxic and genotoxic effects induced by enrofloxacin-based antibiotic formulation Floxagen® in two experimental models of bovine cells in vitro: peripheral lymphocytes and cumulus cells

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Abstract

The in vitro effect of enrofloxacin (EFZ) was tested on two experimental somatic bovine cells in vitro: peripheral lymphocytes (PLs) and cumulus cells (CCs). The cytotoxicity and genotoxicity of this veterinary antibiotic were assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assays, single-cell gel electrophoresis (SCGE) assay, and cytokinesis-block micronucleus cytome (CBMN cyt) assay. Cells were treated during 24 h, and three concentrations were tested (50 μg/mL, 100 μg/mL, 150 μg/mL). When EFZ was tested in PLs, the results demonstrated that the antibiotic was able to induce cell death and DNA damage with all concentrations. In addition, 50 μg/mL and 100 μg/mL EFZ increased frequencies of micronuclei (MNi). On the other hand, the highest EFZ concentration occasioned cellular cytotoxicity in CCs as evidenced by mitochondrial activity alterations. Nevertheless, EFZ was not able to induce DNA damage and MNi in CCs. These results represent the first experimental evidence of genotoxic and cytotoxic effects exerted by EFZ in bovine PLs and CCs.

Keywords Bovine cells · MTT · SCGE assay · CBMN cyt assay · Enrofloxacin

Introduction

Antibiotics are regularly used in veterinary medicine to treat and prevent diseases and for growth promotion in food animals. These compounds are commonly used in livestock activities in domestic animals and aquaculture to ensure animal welfare, guarantee product quality, and market competitiveness (Prescott et al. 2000; Kołodziejska et al. 2013).

In human and veterinary medicine, one of the most useful classes of antibiotics is the fluoroquinolones (FQs). The use of FQs is increasing, owing to their physicochemical properties and wide activity spectrum against diverse bacteria. Enrofloxacin (EFZ) is a third-generation FQ used exclusively in veterinary medicine with a small number of adverse effects. The effectiveness of EFZ has been proven during natural or experimental infections in cattle, poultry, dogs, cats, rodents, lagomorphs, and crustaceans (Brown 1996; Trouchon and Lefebvre 2016). EFZ is partially metabolized in the liver and excreted in bile and urine when active drug was at high concentrations (Otero et al. 2001). EFZ residues were detected in various tissues, such as muscle, from different animal species (Yan et al. 2008; Yu et al. 2014).

Despite its wide use in veterinary medicine, the toxic effect of EFZ in animal cells remains to be elucidated. Although genotoxic potentials of FQs have already been documented mainly using bacterium-based short-term genotoxicity tests (Hartmann et al. 1998; Kümmerer et al. 2000), there are reports

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of toxic effects in some experimental models. EFZ induces oxidative stress and apoptosis in *Ctenopharyngodon idellus* hepatic cell line (Liu et al. 2015) and causes growth inhibition on green algae and genotoxicity on *Allium cepa* roots as revealed by significant induction of micronuclei (MNi) (Magdaleno et al. 2017). In mammalian cells, genotoxic and cytotoxic properties of EFZ are poorly understood (Gorla et al. 1999). In the present study, we used two types of bovine somatic cells: peripheral lymphocytes (PLs) and cumulus cells (CCs). PL culture represents a simple practice that result in a useful tool for evaluating the exposure of genotoxic agents (Pavanello and Levis 1994). On the other hand, CC plays a key role in the reproduction process such as oocyte maturation, ovulation, and fertilization (Tanghe et al. 2002; Krisher 2004; Yuan et al. 2005). Many studies have demonstrated that CC damage produces low fertilization and blastocyst rates (Høst et al. 2002; Seino et al. 2002; Corn et al. 2005). Therefore, the aim of this study was to investigate the effect of the EFZ-based antibiotic formulation Floxagen® (5% EFZ) on PLs and CCs under in vitro conditions. For this purpose, cytokinesis-block micronucleus cytome (CBMN cyt), single-cell gel electrophoresis (SCGE), and MTT assays were used to characterize the genotoxic and cytotoxic properties of EFZ.

Materials and methods

Test compounds

Floxagen® (5% EFZ) was purchased from Vetanco S.A. (Buenos Aires, Argentina). Floxagen® was added to culture media to obtain 50 μg/mL, 100 μg/mL, and 150 μg/mL EFZ. Concentrations selected were based on previous studies conducted by Chen et al. (2011).

Experimental design

Genotoxicity and cytotoxicity assays of PLs and CCs were performed at the end of treatments. Bleomycin (BLM) (1 μg/mL) (Gador Laboratories Nippon Kayaku Co., Japan) was used as a positive control for SCGE and CBMN cyt assays, and cells grown in medium without any other component were used as a negative control. BLM was dissolved in distilled water before treatment. Ethanol (ETOH, 10%; Merck KGaA, Darmstadt, Germany) was used as a positive control for MTT assay.

Bovine peripheral lymphocytes

Jugular venous blood samples (5 mL) from three healthy animals were collected and drawn into heparinized tubes (BD, Heidelberg, Germany). Blood samples were cultivated under standard conditions (37 °C, humidified atmosphere, 5% CO_2) in RPMI 1640 medium supplemented with 10% FCS, containing 1% phytohemagglutinin (Gibco, Invitrogen), 100 IU penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) for 48 h until treatment with EFZ for another 24 h.

Bovine cumulus cells

Bovine ovaries obtained from an abattoir were transported to a laboratory in sterile NaCl solution (9 g/L), with antibiotics at 37 °C within 3 h of slaughter. The ovaries were pooled regardless of the estrous cycle stage of donors. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3–8 mm) using an 18-G needle. We selected intact COCs with an evenly granulated cytoplasm using a low-power (× 20–× 30) stereo microscope. Cumulus cells were isolated from oocytes by repeated pipetting with a narrow-bore glass pipette in the TCM medium 199 and cultured at 39 °C in 5% CO_2 in air with saturated humidity for 4 days until the cells were nearly confluent. Then, CCs were treated with EFZ for another 24 h.

MTT assay

The MTT assay is used to determine the respiratory activity of mitochondrial succinate–tetrazolium reductase system, which converts the yellow tetrazolium salt into a blue formazan dye (Robb et al. 1990). PLs (5×10^5 cells/mL) or CCs (1×10^5 cells/mL) were seeded in culture medium and treated with EFZ during 24 h. The MTT stock solution (Sigma, St. Louis, MO, USA) was added at a final concentration of 0.25 mg/mL (Wu et al. 2013) and 5 mg/mL (Bautista Garfias et al. 2000) for CCs and PLs, respectively, and incubated at 37 °C for 3 h. Afterwards, 100 μL DMSO per well was added to dissolve the formazan blue crystals. Measurement of absorbance was performed at 550 nm with a microplate spectrophotometer (Multiskan™ Go, Thermo Fisher Scientific). To analyze the mitochondrial activity of the PLs, red cells were removed after EFZ treatment using ammonium–chloride–potassium lysis buffer (Sigma, St. Louis, MO, USA) followed by two washes with PBS. Results were expressed as the mean ± SEM of absorbance (arbitrary units, AU) from three independent experiments.

Assay for single-cell gel electrophoresis

SCGE was performed using the alkaline version described by Singh et al. (1988) with slight modifications (Tice and Strauss 1995). This technique version detects DNA migration caused by strand breaks, alkaline labile sites, and transient repair sites. Briefly, slides were covered with a first layer of 180 μL of 0.5% normal agarose (Carlsbad, CA, USA). An amount of 75 μL of 0.5% low-melting point agarose (Carlsbad, CA, USA) was mixed with approximately 15,000 CCs or 15 μL

of blood samples and then was suspended and layered onto slides, which were immediately covered with cover slips. After agarose solidification at 4 °C for 10 min, cover slips were removed and slides were immersed overnight at 4 °C in fresh lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, and 10% dimethyl sulfoxide) (Sigma, St. Louis, MO, USA). The slides were equilibrated in alkaline solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) (Sigma, St. Louis, MO, USA) for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, slides were neutralized by washing them three times with 0.4 M Tris buffer (pH 7.5) (Sigma, St. Louis, MO, USA) every 5 min and subsequently washed in distilled water. Slides were stained with 1/1000 SYBR Green I (Molecular Probes, Eugene, OR, USA) solution (Olive 1999). Two hundred randomly selected comet images were analyzed per treatment.

Scoring was performed at × 400 magnification using a fluorescent microscope (Olympus BX40 equipped with a 515–560-nm excitation filter). Based on the extent of strand breakage, cells were classified according to their tail length in five categories, ranging from 0 (no visible tail) to IV (still a detectable head of the comet, but most of the DNA is in the tail) (Collins 2004; Olive 1999). With the data, an index damage (ID) was obtained according to Collins (2004), using the formula ID = [(I) + 2(II) + 3(III) + 4(IV)], where I–IV represent the nucleoid types. Visual scoring (arbitrary units) is rapid as well as simple, and there is very close agreement between this method and computer image analysis (percentage of DNA in the tail) (Collins 2004).

Cytokinesis-block micronucleus cytome assay

Cytostatic effects and chromosomal damage were assessed by the CBMN cyt assay according to the method of Fenech (2007) with slight modifications. PLs were cultured and treated with EFZ and cytochalasin B (3 μg/mL final concentration) (Sigma, St. Louis, MO, USA) during 24 h. At the end of the culture period, the cell suspension was centrifuged and the pellet was resuspended in 5 mL of fixative (sodium chloride:methanol:acetic acid 6:5:1). The cells were washed two times with fresh fixative 2 (methanol:acetic acid 5:1); they were later resuspended, dropped onto clean slides, and finally stained with 5% Giemsa for 10 min.

Bovine CCs were seeded onto precleaned 22 mm × 22 mm cover slips in a six-well microplate at a density of 1×10^5 cells in a final media volume of 2 mL per well, and cultures were performed 48 h after plating. Then, the cells were treated with EFZ and cytochalasin B (3 μg/mL) for 24 h at 37 °C in 5% CO_2 atmosphere until harvesting. At the end of culture, 1000 μL of methanol:acetic acid (6:1) was added to each dish during a prefixation period of 15 min. Afterward, the supernatant was removed by pipetting and cells were fixed with methanol:acetic acid (6:1) for 15 min in the same well and finally stained with 3% aqueous Giemsa solution after being air-dried. Cover slips were air-dried and then placed down onto precleaned slides using the Depex mounting medium.

The chromosome damage biomarkers scored were MNi, nucleoplasmic bridges (NPBs), and nuclear buds (NBuds). One thousand binucleated (BN) PLs or CCs were analyzed per experimental point. Cytostatic effects were analyzed through the nuclear division index (NDI), estimated by the ratio of mono-, bi-, and multinucleated cells. Five hundred viable cells were scored per experimental point to determine the frequency of cells with one, two, three, or four nuclei and calculate the NDI using the formula $(M_1 + 2M_2 + 3M_3 + 4M_4) / N$, where $M_{1–4}$ represents the number of cells with one to four nuclei and N is the total number of viable cells scored (Fenech 2007). Fenech's scoring criteria for cell determinations of MNi, NPBs, and NBuds were used (Fenech 2007).

All experiments were performed in triplicate to allow an accurate estimate of inter-experimental variation.

Statistical analysis

Data were statistically analyzed using ANOVA and multiple range test for biomarkers with a normal distribution and the Kruskal–Wallis test for biomarkers with non-normal distribution. Statgraphics® 5.1 software (Manugistics, Inc., Rockville, MD, USA) was used for all analyses. Variables were tested for normality with the Kolmogorov–Smirnov test, and the homogeneity of variances between groups was verified by Levene's test. To evaluate the concentration-dependent response to treatments, simple linear regression and correlation analysis were performed. Data were expressed as a mean ± standard error, and a p value < 0.05 was considered statistically significant.

Results

Effect of EFZ on mitochondrial activity

Figure 1 shows the results of MTT analysis in bovine PLs and CCs exposed during 24 h with different EFZ concentrations. The results demonstrated a significant decrease in cell viability evaluated by MTT assay when PLs were exposed to EFZ at any concentration compared with the negative control ($p < 0.05$) (Fig. 1a). On the other hand, an inhibition was achieved only when CCs were treated with 100–150 μg/mL EFZ ($p < 0.05$; Fig. 1b). A regression analysis showed that the viability decreased in a concentration-dependent manner when EFZ was added to CC ($r = -0.47$, $p < 0.01$) and PL ($r = -0.57$, $p < 0.01$) cultures.

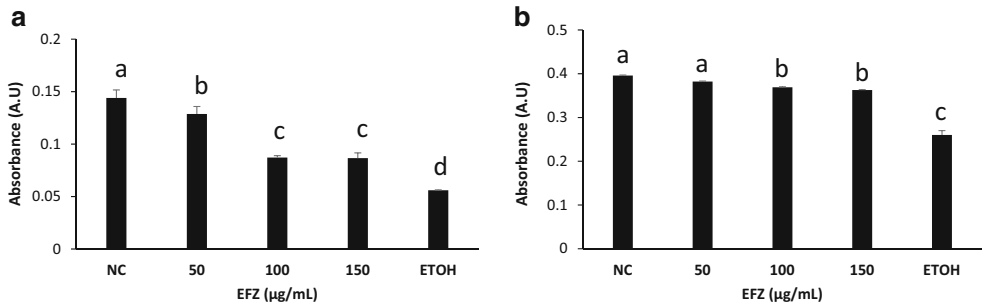

Fig. 1 Viability evaluated by MTT assay in bovine peripheral lymphocytes (**a**) and cumulus cells (**b**) treated with 50 μg/mL, 100 μg/mL, and 150 μg/mL of enrofloxacin (EZF)-based antibiotic formulation during 24 h. Cultures were incubated for 3 h with MTT dye after EFZ treatment. Results are expressed as mean of absorbance values at 550 nm from three independent experiments ± SEM. A.U (arbitrary units). Culture media without any treatment were used as a negative control (NC). Ten percent of ethanol (ETOH) was used as a positive control. Bars with different lowercase letters (a–d) differ statistically ($p < 0.05$)

Effect of EFZ on DNA damage

Data of the SCGE assay obtained in bovine PLs and CCs treated with EFZ are presented in Table 1. PLs treated with 50–150 μg/mL EFZ showed an enhancement of genetic ID values ($p < 0.05$). Statistical analyses show that the ID increase induced by 50 μg/mL EFZ was due to an enhancement in the frequency of grade I ($p < 0.05$) and a decrease of grade 0 ($p < 0.05$) generating slight damage to the cells (Table 1). Instead, the increase in ID ratio induced by 100 μg/mL and 150 μg/mL EFZ was due to an increase in the frequency of grade II, III, and IV ($p < 0.05$) and a decrease of grade 0 and I comets ($p < 0.05$) (Table 1). Overall, regression analysis demonstrated that the ID varied as positive function of the EFZ concentration ($r = 0.81$, $p < 0.01$). In contrast, no statistical differences in genetic ID were observed when CCs were exposed to all assayed concentrations of EFZ ($p > 0.05$) (Table 1).

Effect of EFZ on micronuclei and other nuclear abnormalities

Table 2 shows the results of antibiotic-induced MNi, NPBs, NBuds, and NDI in BN cytokinesis-blocked cells. There is an increase in frequencies of MNi when PLs were treated with 50 μg/mL and 100 μg/mL EFZ compared with control values ($p < 0.05$). However, 150 μg/mL EFZ did not modify the frequency of MNi ($p > 0.05$). A regression test showed that the increase observed with EFZ treatment was independent of the concentration used ($r = 0.14$, $p > 0.05$). Increased frequencies of NBuds were observed

Table 1 Analysis of DNA damage measured by comet assay in bovine peripheral lymphocytes (PLs) and bovine cumulus cells (CCs) treated with enrofloxacin (EFZ)-based antibiotic formulation during 24 h

Cell types	EFZ treatment (μg/mL)	Proportion of damaged nucleoids (%)					DNA damage (%)	ID ± SEM
		Grade 0	Grade I	Grade II	Grade III	Grade IV	(II + III + IV)	
PLs	NC	93.00^{a}	6.75^{a}	0.25^{a}	0.00^{a}	0.00^{a}	0.25	7.25 ± 2.56^{a}
	50	76.50^{b}	23.25^{b}	0.25^{a}	0.00^{a}	0.00^{a}	0.25	23.75 ± 1.59^{b}
	100	76.00^{b}	16.00^{b}	7.75^{b}	2.25^{b}	0.00^{a}	10.00	38.25 ± 5.73^{c}
	150	76.50^{b}	16.75^{b}	3.25^{b}	1.25^{b}	2.25^{b}	6.75	36.00 ± 6.30^{c}
	PC	40.50^{c}	48.75^{c}	8.00^{c}	1.50^{b}	0.75^{a}	10.25	72.25 ± 4.36^{d}
CCs	NC	76.48^{a}	12.16^{a}	3.09^{a}	1.89^{a}	6.38^{a}	11.36	49.53 ± 10.55^{a}
	50	59.86^{a}	28.00^{a}	3.00^{a}	2.14^{a}	7.00^{a}	12.14	68.42 ± 12.61^{a}
	100	67.32^{a}	20.09^{a}	3.49^{a}	3.49^{a}	5.61^{a}	12.59	59.98 ± 11.79^{a}
	150	55.89^{a}	26.29^{a}	7.37^{a}	5.17^{a}	5.28^{a}	17.82	77.66 ± 11.12^{a}
	PC	48.54^{b}	23.78^{b}	18.17^{b}	5.73^{b}	3.78^{b}	27.68	92.43 ± 20.45^{b}

Bovine PLs and CCs were incubated with 50 μg/mL, 100 μg/mL, and 150 μg/mL of EFZ during 24 h. DNA damage was evaluated by SCGE assay in three independent experiments. The length of DNA migration quantified the extent of DNA damage, which was visually determined in 200 randomly selected and non-overlapping cells per replicate. DNA damage was classified into four grades: 0–I (undamaged), II (minimum damage), III (medium damage), and IV (maximum damage). Results are expressed as the mean comet score for each treatment group and the mean DNA damage (sum of classes II, III, and IV). Culture media alone were used as a negative control (NC). Bleomycin (1 μg/mL) was used as a positive control (PC). Index damage (ID) of each treatment was determined with the formula ID = [(I) + 2(II) + 3(III) + 4(IV)] where 0–IV represent the nucleoid type. Values with different superscript lowercase letters (a–d) in the same column and group (PL or CC) differ statistically ($p < 0.05$)

Table 2 Effect of enrofloxacin (EFZ)-based antibiotic formulation on induction of micronuclei (MNi), nuclear abnormalities, and nuclear division index (NDI) on binucleated cytokinesis-blocked peripheral lymphocytes (PLs) and bovine cumulus cells (CCs)

Cell types	EFZ treatment (μg/mL)	Frequencies of MNi	Nuclear abnormalities		NDI
			NPBs	NBuds	
PLs	NC	39 ± 2.5[a]	4.0 ± 3.4[a]	0.00 ± 0.0[a]	1.57 ± 0.06[a]
	50	60 ± 6.9[bc]	7.5 ± 3.8[b]	1.0 ± 0.8[b]	1.35 ± 0.04[a]
	100	64 ± 5.1[c]	12.0 ± 0.0[b]	3.0 ± 0.8[c]	1.34 ± 0.09[a]
	150	44 ± 12.1[ab]	6.0 ± 1.7[ab]	1.5 ± 0.4[b]	1.33 ± 0.08[a]
	PC	86 ± 3.4[d]	13.0 ± 8.6[b]	3.0 ± 1.7[c]	1.46 ± 0.06[a]
CCs	NC	30.0 ± 2.0[a]	1.0 ± 0.5[a]	2.0 ± 0.0[a]	1.76 ± 0.04[a]
	50	30.0 ± 1.0[a]	0.0 ± 0.0[a]	2.0 ± 05[a]	1.65 ± 0.01[a]
	100	23.5 ± 1.5[a]	0.0 ± 0.0[a]	4.0 ± 1.0[a]	1.65 ± 0.01[a]
	150	26.0 ± 6.0[a]	1.0 ± 0.5[a]	2.0 ± 1.0[a]	1.70 ± 0.02[a]
	PC	46.5 ± 7.5[b]	1.0 ± 0.5[a]	3.0 ± 0.5[a]	1.62 ± 0.00[a]

Bovine PLs and CCs were treated during 24 h after seeding with EFZ antibiotic and cytochalasin B. Values for micronuclei (MNi) are expressed as the mean of MNi/1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiment ± SEM. Nuclear abnormalities were observed in 1000 binucleated cells at × 1000 magnification per experimental point from each experiment and classified as nucleoplasmic bridges (NPBs) or nuclear buds (NBuds). Nuclear division index (NDI) was calculated with the formula $NDI = (M_1 + 2M_2 + 3M_3 + 4M_4) / N$, where $M_{1–4}$ represents the number of cells with one to four nuclei and N is the total number of viable cells scored. Culture media alone were used as a negative control (NC). Bleomycin (1 μg/mL) was used as a positive control (PC). Values with different superscript lowercase letters (a–d) in the same column and group (PL or CC) differ statistically ($p < 0.05$)

in antibiotic-treated cultures at any concentration assayed ($p < 0.05$). Concentrations of 50 μg/mL and 100 μg/mL EFZ induce higher NPB frequencies compared with control values ($p < 0.05$). Regardless of assayed concentrations, EFZ treatment did not modify the NDI values compared to control (Table 2) ($p > 0.05$). In CCs, EFZ did not modify frequencies of MNi, NPBs, and NBuds nor NDI (Table 2) ($p > 0.05$).

Discussion

EFZ is a commonly used drug in veterinary medicine. In the present study, three different assays were used to investigate the cytotoxic and genotoxic properties of EFZ-based antibiotic formulation on bovine PL and CC in vitro culture. The collection of PLs is very easy, and it is encouraged to use them as an alternative for target tissues with the intention of investigating the effect of environmental genotoxic agent on animal/human or to explore the in vitro metabolism of that agent (Pavanello and Levis 1994). Previously, we demonstrated that CC culture was useful for evaluating cytotoxicity and genotoxicity (Anchordoquy et al. 2017). In the present study, we established that EFZ induced genotoxic effects in PL and cytotoxic effects in both PL and CC cultures.

EZF is a synthetic antibacterial agent which inhibits bacterial DNA gyrase, interfering with the supercoiling of chromosomal material (Brown 1996). It has been revealed that EZF is not mutagenic when it was evaluated by the Ames test, Chinese hamster ovary–HGPRT forward mutation assay, and unscheduled DNA synthesis test (Altreuther 1987). It has been suggested that FQs produce genotoxicity by increasing mammalian topoisomerase II enzyme activity (Hooper and Wolfson 1991) and cytotoxicity (Radko et al. 2013). In our study, we evaluated EFZ cytotoxicity by MTT assay, which allows us to determine alterations in mitochondrial function. EFZ resulted in PL cytotoxicity which was clearly demonstrated by cellular death in a dose-dependent manner. In CCs, although the viability reduction occurred in a dose-dependent manner, this decrease was significant only for higher concentrations of EFZ (100 μg/mL and 150 μg/mL). Certainly, a PL cell model appeared to be more sensitive than a CC model for detecting EFZ cytotoxicity. The differences in cell line sensitivity to FQs have been reported in the literature (Radko et al. 2013). Numerous studies have evaluated the cytotoxicity and genotoxicity of FQs. However, EFZ information of cytotoxic and genotoxic properties is scarce. Our results are in agreement with other reports that point out the deleterious effect of FQs on cell viability (Ayaki et al. 2010; Tsai et al. 2010). Tsai et al. (2010) demonstrated that ciprofloxacin and gatifloxacin decrease dramatically the viability of human corneal epithelial cells after 30 min by using MTT assay. Furthermore, levofloxacin, moxifloxacin, gatifloxacin, norfloxacin, and tosufloxacin decrease cell viability evaluated by MTT assay in corneoconjunctival cell lines (Ayaki et al. 2010). Radko et al. (2013) showed that cytotoxic effects of FQs on cell

culture are not only concentration dependent but also time dependent. In agreement with our findings, they observed cytotoxicity on cell culture after 24 h of exposure to 100 μg/mL EFZ.

Genotoxicity tests as SCGE and CBMN cyt assays have become extensively valuable as biomarkers for different cells in vitro (Vasquez 2010; Araldi et al. 2015). SCGE detects single- and double-strand breaks as well as apurinic sites (Tice et al. 2000). In the present study, EFZ induced DNA damage in bovine PLs exposed to all concentrations evaluated, whereas no damage was found in CCs. In order to find out whether EFZ causes chromosomal damage, we conducted the CBMN cyt assay. This test detects small chromosomal fragments such as acentric chromosome/chromatid fragments in interphase cells induced by clastogens, or chromosome laggings at anaphase produced by aneugens as well as other nuclear anomalies (Fenech et al. 2011). In the present study, EFZ showed no induction of MNi on bovine CCs after 24 h of exposure. However, 50 μg/mL and 100 μg/mL EFZ increased frequencies of MNi in binucleated bovine PLs, while the frequency of MNi with 150 μg/mL EFZ was similar to control values. The possibility of induction of an involuntary loss of dead or severely damaged cells produced by the higher EFZ concentration during the test, and causing an alteration in the proportion of micronucleated cells with the consequent underestimation of the actual damage, could not be ruled out. However, future works are needed to confirm if the latter hypothesis is true or not. In addition, the rates of nuclear anomalies (NBuds and NPBs) were elevated in PLs after EFZ treatment. It is known that these anomalies reflect genetic instability; i.e., NPBs are formed because of dicentric chromosomes while NBuds reflect gene amplification events (Fenech et al. 2011). The biological consequences of the formation of these anomalies are not known until present (Fenech 2007; Dutra et al. 2010). However, Dutra et al. (2010) showed that nuclear buds could be detached from the original nucleus before reaching apoptosis.

Bovine ovary is a popular experimental model for studying mechanisms and properties related to ovarian function even in human (Babaei et al. 2012; Santos et al. 2014; Ceko et al. 2015). To our knowledge, we have conducted the first genotoxic, cytostatic, and cytotoxic evaluation of EFZ in bovine CCs in vitro. In mammals, CC damage impairs early embryo development (Høst et al. 2002; Seino et al. 2002; Corn et al. 2005). The close contact between CCs and oocytes through gap junctions allows the bidirectional interchange of molecules regulating nucleus and cytoplasm oocyte maturation, oocyte quality, and subsequent embryo development (Tanghe et al. 2002; Krisher 2004; Yuan et al. 2005). The toxic effect of EFZ on CCs might affect normal early embryonic development.

On the other hand, we must not ignore the fact that EFZ is widely used to treat bacterial infections in livestock with deleterious consequences by increasing environmental residues. Generally, parent compounds and/or metabolites are excreted via urine and feces and released directly in the environment. These compounds may also contaminate the environment by emissions during their fabrication process, water contamination through aquaculture, and the packaging disposal in inappropriate places. Moreover, animal manure is frequently used as a fertilizer because of its nutrients and organic matter content. Nevertheless, antibiotic-treated animal manure has important amounts of antibiotics that can affect other organisms including human (Halling-Sørensen et al. 1998; Hamscher et al. 2002; Boxall 2010; Botelho et al. 2015). Taken into account the recent recognition of environmental contamination problems by veterinary drugs, the results of this study may contribute to define the genotoxic and cytotoxic properties of EFZ in mammalian cells. Furthermore, the genotoxicity test battery used in this research is a suitable tool for compiling a comprehensive hazard profile for environmentally relevant contaminants.

It is important to mention that EFZ-based antibiotic containing 5% of the active compound within the formulation was assayed in our study. It is well known that antibiotics used in veterinarian medicine are a combination of the active ingredient and excipients. The excipients might affect drug performance and even have genotoxic, mutagenic, and carcinogenic potentials (Madhuresh et al. 2016). Hence, in a commercial formulation, risk assessment must also consider additional toxic effects caused by the excipient(s). Unfortunately, the manufacturers did not make the identities of the additive compounds present in the commercial formulation of Floxagen® available to us. For that reason, in a future work, we consider it necessary to determine if the effect observed in this study is totally due to EFZ or due in part to the excipients present in the formulation.

In conclusion, the results from the present study demonstrated that EFZ decreased cell viability, induced DNA damage, and increased frequencies of MNi in PLs. Moreover, EFZ prompted cellular cytotoxicity in CCs as evidenced by MTT assay. Results of this investigation could provide useful data for hazard assessments related to this compound commonly used in veterinary medicine.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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