Fosfomycin Penetration into Swine Leukocytes

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ABSTRACT

Fosfomycin (FOS) is an antibiotic widely used in Asia and Latin-America for the treatment and prevention of swine infections caused by resistant bacteria. Intracellular and interstitial fluids are biophases for facultative intracellular microorganisms. On the other hand, the choice of an antibiotic depends on its direct antimicrobial activity, which is in vitro determined by the MIC. FOS MIC90 ranges from 0.25 to 0.50 µg/mL for the most important pathogens in pig production. The aim of this research was to study the in vitro and in vivo FOS penetration into swine leukocytes (white blood cells-WBC) and to determine if intracellular concentrations exceed the MIC90 for most swine pathogens. For the in vivo, study six weaning piglets were intramuscularly administered a 15 mg/kg dose of disodium FOS. For the in vitro study, WBC obtained from weaning piglets were incubated with a 150 µg/mL dose of disodium FOS. Intracellular concentrations were analyzed by HPLC MS/MS and they ranged from 0.89 to 3.88 µg/mL for the in vitro assay (Tmax: 4 h) and between 0.16 and 1.33 µg/mL for the in vivo study (Tmax: 0.5 h). The values found in both studies are above the MIC90 for the

most important pathogens in swine production. These values were maintained for more than 24 h for the in vitro study (T> MIC=24 h) and for more than 4 h for the in vivo study (T> MIC=4 h).

INTRODUCTION

Weaning is considered as a critical period for piglets characterized by a decrease in food intake that leads to a status of undernutrition. Other aspects of animal physiology and metabolism are also affected (Dirkzwagera et al., 2005). During this period animals are more susceptible to infectious diseases (Nabuurs et al., 1993), being that the interstitial and intracellular fluids the main sites of infection (biophases) of the pathogens responsible for these disorders. Different antibiotics have been used for decades to reduce pathogen infection in pigs. For this reason, many bacteria have become resistant to the most frequently used antimicrobials (Dirkzwagera et al., 2005; Mathew et al., 1998; Rood et al., 1985).

Fosfomycin (FOS) (cis-1,2-epoxyphosphonic acid) is an antibiotic widely used in intensive production (Serrano, 2002). It is a broad-spectrum drug, structurally unrelated to other classes of antimicrobial agents. It inhibits cell wall synthesis as it interferes with peptidoglycan production at an earlier stage than beta-lactams or glycopeptide antibiotics (Gobernado, 2003; Kahan et al., 1974; Lin, 1976; Popovic et al., 2009). It has a low molecular weight (138.059 Da) and its chemical structure is similar to that of phosphoenol-pyruvate. When compared with other antibiotics, FOS has a broader in vitro spectrum of action than penicillin and semi-synthetic cephalosporins (Mata et al., 1977), and cross-resistance has not been reported (Gobernado, 2003). The use of FOS in animals and humans has been proposed because of its low toxicity and potential efficacy (Gallego et al., 1974), being also widely used in animal production due to its rapid effect, good tolerance, and lack of side effects (Aramayona, 1997; Carramiñana, 2004). Its chemical structure supports different salts: sodium, calcium, and tromethamine (Perez-Rodriguez and Chavez Hernandez Velasco, 1997; Serrano, 2002). The FOS-calcium salt formulation is used orally, whereas the more water-soluble disodium salt can be used intravenously. FOS-tromethamine salt is highly hydrosoluble and offers a good oral bioavailability in humans (Borsa et al., 1988; Patel et al., 1997; Popovic et al., 2009).

Our research group has studied FOS pharmacokinetics (PK) in pigs (Soraci et al, 2011a, Pérez et al., 2012), although other authors have previously studied FOS PK in many species such as humans (Gallego et al., 1974; Damaso et al. 1990; Falagas et al., 2008), rabbits (Fernandez Lastra et al. 1986, 1987), broilers (Aramayona et al., 1997, Soraci et al. 2011b), cattle (Sumano et al., 2007), horses (Zozaya et al., 2008), and dogs (Gutiérrez et al., 2008). Previous studies from our group also established a FOS withdrawal period. It was of 3 days for broiler chicken muscle, liver, and kidney. For pig tissues, the withdrawal period was of 3 days after oral administration and 2 days after intramuscular administration (Pérez et al., 2011, 2012). It has been demonstrated that, besides being a bacterial inhibitor, FOS has other properties such as, inhibition of bacterial adhesion to epithelial cells, penetration of wells in biofilms of exo-polysaccharide, and protection against nephrotoxicity caused by drugs including, cisplatin, cyclosporine, aminoglycosides, vancomycin, teicoplanin, amphotericin B, and polymyxin (Gobernado, 2003). FOS exhibits a time dependent killing, thus it kills bacteria when its concentrations remain constantly above the Minimum Inhibitory Concentration (MIC) (Aliabadi and Lees, 1997; Toutain et al., 2002). In this regard, for an antibiotic to be effective against relevant pathogens, it is essential to reach concentrations higher than the MIC at the site of action (Nix et al., 1991; Schentag and Ballow, 1991; Toutain et al., 2002). FOS is indicated for the treatment of a variety of porcine bacterial pathogens (Haemophilus parasuis, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Staphylococcus hyicus, Escherichia

coli, Salmonella enterica) associated with stress and/or several viral diseases (Martineau, 1997). The MIC90 for the most important pathogens in swine production have been established in 0.25-0.5 µg/mL, reaching to 4 µg/mL for S. enterica (Fernández et al., 1995, Sumano et al., 2007, Ibar et al., 2009). In this regard, Pérez et al. (2012) have demonstrated that FOS is an alternative for the treatment of respiratory and enteric infections in pigs caused by intracellular facultative or obligate pathogens, because it penetrates in HEP-2 and IPEC-J2 reaching concentrations above the MIC90. We have also demonstrated FOS penetration in alveolar macrophages and epithelial lining fluid at concentrations above the MIC90 for important swine pathogens.

On the other hand, authors as Morikawa et al (1993) and Honda et al (1998) have shown that FOS has immunomodulatory effect on lymphocytes. Similarly, Krause et al (2001) studied the effect of FOS on neutrophil function and showed that destruction of microorganisms is increased when incubated with this antibiotic. As shown, studies describing the penetration of FOS into porcine leukocytes (white blood cells - WBC) are not available, and only a few studies analyze FOS effects in human WBC. According to this background, the aim of this work was to determine FOS concentrations in the intracellular fluid of swine WBC.

MATERIALS AND METHODS

Animals

Twelve post-weaning piglets, divided in two groups, were used (A: n=6, in vivo study, not treated with antimicrobials, B: n=6, in vitro study). Clinically healthy, 4-5 weeks old piglets, were weighted, identified, and housed in pens in the weaning room, with free access to water and food.

Antibiotic

Disodium FOS salt was used (sterile powder, purity 99.9%, Bedson S.A., Pilar, Buenos Aires, Argentina).

Dose

- In vivo study (Group A): 15 mg/kg, via IM in the gluteal muscles.
- In vitro study (Group B): 150 µg/mL.

The concentration was calculated considering the average weight of a weaning piglet (15 ± 2 kg), the dose of disodium FOS to be therapeutically administered via IM (15 mg/kg), the F% of this FOS salt (85.50) (Soraci et al., 2011a), and the volume in which the drug is diluted (blood volume = 8% pv).

Sampling

Five mL of blood per animal were collected. Samples were obtained by jugular catheterization technique (Soraci et al., 2010) and placed in glass tubes containing sodium citrate, pH 6.5, as anticoagulant at a 1:4 ratio.

Sampling times: Group A : 0.5; 1; 2; 3; 4; 6; 8; 12; 18 and 24 hours. Group B: time zero.

Cell Separation

Blood samples from both groups were centrifuged 15 min at 2,500 rpm. Using Pasteur pipettes, the buffy coats were obtained and seeded in tubes containing 10 mL of ClNH4. Centrifugation was carried out for 7 min at 3,000 rpm and 4 ºC. The supernatant was discarded and the pellet was lifted with pipette. Pellets were washed with HPLC water and a new centrifugation (10 min. at 1000 rpm) was performed. Then, the pellet was re-suspended in 1 mL of MEM. An aliquot was separated for vital staining and cell counting with trypan blue.

Quantification of FOS Cellular Uptake

For both groups, the buffy coats were seeded into 24-well culture plates, to which 1 mL of MEM supplemented with fetal bovine serum (FBS) was added. For the in vitro study, cell cultures were incubated at 37°C with 1 mL of disodium FOS at a concentration of 150 µg/mL. Supernatants were collected from the wells simulating the sampling times of the in vivo test (0.5, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h). Each well containing WBC was washed twice with HPLC water. The supernatants were centrifuged at 3,500 rpm for 6 min. in order to obtain the extracellular FOS. Pellets (detached cells) were restored

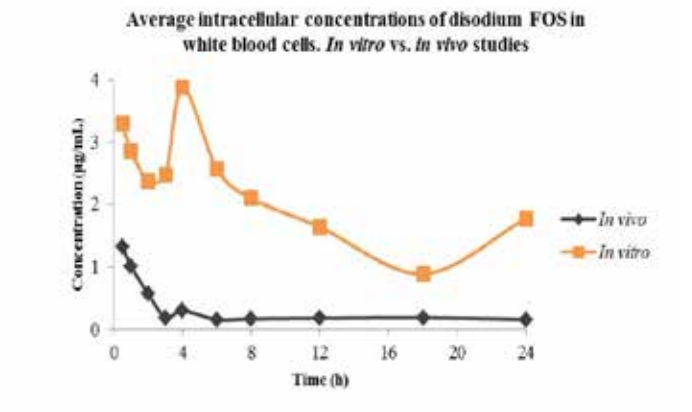
to the corresponding well. After the addition of 2 mL of HPLC water to the wells and with the aim of breaking the cells and releasing FOS to the HPLC water, culture plates were sealed and sonicated for 30 min. Then, the content of each well was centrifuged at 10,000 rpm at 4 ° C. It was filtered through 0.22 µm nylon filters and placed in 1.5ml tubes. The intracellular concentrations of FOS were determined by HPLC-MS/MS. Tests were performed in quadruplicate.

To estimate the influence of the intracellular water of the WBC in the intracellular concentrations of FOS the cell count per well (400,000 WBC) and the average cell volume (2.75 x 10-6) were considered, as described by Kiem and Shentag (2008). Therefore, the data obtained based on the Xcalibur software were re-calculated considering the intracellular volume of water. The degree of penetration of FOS into WBC was determined by comparing its AUC0-t with plasma AUC0-t.

RESULTS

For the in vitro assay, intracellular concentrations of the incubated antibiotic were maintained between 0.89 and 3.88 µg/mL at different times. Only 2.6% of the antibiotic incubated with the WBC was able to enter intracellularly. The highest concentration of the antibiotic remained in the extracellular space. The Cmax was 3.88 µg/mL and the Tmax was 4 h. For the in vivo assay, intracellular concentration of the antibiotic ranged between 0.16 and 1.33 µg/mL at different times. The Cmax was 1.33 µg/mL and the Tmax was 0.5 h. Figure 1 shows the average intracellular penetration of disodium FOS in WBC in vitro vs. in vivo after incubation with a concentration of 150 µg/mL and an IM administration of 15 mg/kg to weaning piglets, respectively.

Figure 1. *In vitro vs. in vivo average intracellular concentrations of disodium FOS in WBC after incubation with a dose of 150 µg/mL and an IM administration of 15 mg/kg, respectively.*



FOS AUC0-t were also calculated. The AUC0-t after incubation with 150 µg/mL of disodium FOS was 31.6 µg-h/mL and the AUC0-t after the administration of disodium FOS at a rate of 15 mg/kg was 5.94 µg-h/mL. Table 1 summarizes the pharmacokinetic (PK) parameters found in WBC by in vivo and in vitro studies.

DISCUSSION

Penetration of drugs in various tissues is best described by the use of AUCs, which respond to variations in concentration over time (Schentag and Ballow, 1991, Kiem and Schentag, 2008). In previous studies from our research group (Soraci, 2011a), plasma pharmacokinetics parameters as

Table 1. *PK parameters of disodium FOS found on in vivo and in vitro studies.*

	WBC	
	In vitro: 150 µg/mL	*In vivo*: IM, 15 mg/kg
C_{max} µg/mL	3.88	1.33
T_{max} (hs)	4.00	0.50
AUC_{0-t} µg.h/mL	31.60	5.94

Cmax, Tmax and AUC were determined for disodium FOS. When comparing with the disodium FOS Cmax in plasma (43.00 µg/mL), the values found on the in vitro and in vivo assays were significantly lower. The similarity between the Tmax values was expected when dealing with the penetration of FOS into cellular elements present in the blood. The degree of FOS penetration into WBC was determined by comparing their AUC0-t with the plasma AUC0-t (99.00 µg-h/mL). The ratio of AUC to WBC in vivo compared to plasma AUC (AUCWBC/AUCplasma) was 0.06. For the 150 µg mL of disodium FOS concentration, the value of AUCWBC vs AUCplasma was 0.32. As seen, for the in vitro study, it is observed that the value of AUCWBC vs AUCplasma is 18.75% of that found in the in vitro study (0.06 vs. 0.32). Nevertheless, the comparison AUCWBC vs. AUCplasma was considered significant ($p < 0.05$) for both studies.

When comparing the AUCWBC obtained in the in vitro test with the results obtained for other cell cultures analyzed in previous studies of the research group, it is observed that the value of AUCWBC vs AUCplasma with a dose of 150 µg/mL of disodium FOS is similar to that found for a dose of 130 µg/mL of the same salt (AUCHEP-2 vs. AUCplasma=0.27), and lower than that found for a dose of 280 µg/mL of disodium FOS (AUCHEP-2 vs AUCplasma=0.48), 130 µg/mL of calcium FOS (AUCHEP-2 vs AUCplasma=0.49) and 580 µg/mL of calcium FOS in IPEC-J2 cells (AUCIPEC-J2 vs AUCplasma=16.04) (Pérez et al., 2012; Pérez et al., 2013). As shown, the proportion value of AUC with respect to the AUC in plasma obtained for the WBC in vivo, is the lowest of all the estimations performed.

As previously reported, there are no studies describing the penetration of FOS into porcine WBC, and there are few studies regarding FOS effect in human WBC. As previously described, FOS exhibits a time dependent killing. Thus, bacteria killing occurs when its concentrations remain constantly above MIC (Aliabadi and Lees, 1997; Toutain et al., 2002). In this regard, for an antibiotic to be effective against relevant pathogens, it is essential to reach concentrations higher than the MIC at the site of action (Nix et al., 1991; Schentag and Ballow, 1991; Toutain et al., 2002). Values found in both studies were above the MIC90 for the most important pathogens in swine production and they remained above MIC90 for more than 24 h for the in vitro study (T> MIC=24 h) and for more than 4 h for the in vivo study (T> CIM=4 h).

CONFLICT OF INTEREST STATEMENT

Authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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