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Intracellular Concentrations of Fosfomycin in Alveolar Macrophages from Weaning Piglets

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ABSTRACT

At weaning, piglets are more susceptible to infectious diseases, being respiratory syndromes one of the most frequent conditions. Fosfomycin is indicated for the treatment of a variety of porcine bacterial pathogens, with MIC_{90} ranging between 0.25-0.50 μg mL^{-1}. Fosfomycin exhibits a time-dependent killing. Thus, killing of bacteria occurs when its concentrations remain constantly above the MIC. Fosfomycin concentrations in respiratory cells and epithelial lining fluid of pigs have already been studied. Although, fosfomycin showed clinical efficacy in the treatment of pulmonary diseases, concentrations in alveolar macrophages (biophase for facultative and obligate intracellular microorganisms) have not been established in any species. The present study determined the intracellular concentrations of disodium fosfomycin in alveolar macrophages after a single IM dose of 15 mg kg^{-1} in the gluteal muscle. Concentrations ranged from 0.14-1.52 μg mL^{-1}, being lower than those found in epithelial lining fluid (49.03%) and respiratory cells (54.48%). The C_{max} was 1.52 μg mL^{-1} and T_{max} was 4 h. Concentrations exceeded the MIC_{90} for most pathogens of importance in pig production for up to six hours (T>MIC = 6 h). Therefore, fosfomycin may be useful in the treatment of lung infections caused by facultative intracellular microorganisms.

Key words: Swines, antibiotic, macrophages, cellular drug penetration

INTRODUCTION

In pig production, weaning is considered as a critical period for piglets. It is characterized by a decrease in food intake that leads to a status of under nutrition, affecting other aspects of animal physiology and metabolism (Dirkzwager *et al*., 2005). During this period, animals are more susceptible to infectious diseases (Nabuurs *et al*., 1993). Respiratory syndromes are one of the most frequent conditions that occur at weaning. They are a common cause of morbidity and mortality. Therefore, they are considered one of the most significant problems in modern intensive swine production systems (Gardner and Hird, 1990; Galina *et al*., 1994; Done and Paton, 1995; Christensen *et al*., 1999; Thanawongnuwech *et al*., 2000; Carr, 2001; Cloutier *et al*., 2003; Dosen *et al*., 2007). The intracellular and interstitial fluids are the pulmonary biophase of facultative microorganisms such as *Mycoplasma hyopneumoniae*, *Bordetella bronchisceptica*, *Salmonella cholerasuis*, *Pasteurella multocida* and *Streptococcus* spp. Different antibiotics have

been used for decades to reduce pathogen infections in pigs. For this reason, many bacteria have become resistant to the most frequently used antimicrobials (Dirkzwager *et al*., 2005). Fosfomycin (FOS) (cis-1, 2-epoxyphosphonic acid), a broad-spectrum antibiotic structurally unrelated to other classes of antimicrobial agents, is used in swine production in Central and South America and in various Asian countries. The FOS is indicated for the treatment of a variety of porcine bacterial pathogens (*Haemophilus parasuis*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Staphylococcus hyicus* and *Escherichia coli*), which are usually associated with stress and/or viral diseases. The minimum inhibitory concentration required to inhibit the growth of 90% of the microorganisms (MIC_{90}) for most of the important pathogens in swine production has been established as 0.25-0.5 μg mL^{-1} (Fernandez *et al*., 1995). The FOS exhibits a time-dependent killing. Thus, killing of bacteria occurs when its concentrations remain constantly above the 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).

The FOS penetration has been demonstrated in phagocytic cells, where it reaches high concentrations, showing an intracellular activity close to that of rifampicin (Trautmann *et al*., 1992). Antibiotic penetration does not alter the ability of ingestion and destruction by these cells, as it has been demonstrated by *in vitro* studies (Hoger, 1985). Morikawa *et al*. (1993) and Honda *et al*. (1998) have shown that FOS has immune-modulatory effects on lymphocytes. Similarly, Krause *et al*. (2001) studied the effect of FOS on neutrophil function and showed that the destruction of microorganisms is increased when incubated with the antibiotic. Perez *et al*. (2013) studied FOS penetration in swine intestinal cells (IPEC-J2) and they found that FOS concentrations exceed the MIC_{90} for the most important pathogens in swine intestinal infections. Therefore, it is apparent that this antibiotic is an alternative for the treatment of intestinal infections in pigs. Martinez *et al*. (2013) studied the penetration of FOS into IPEC-J2 cells in the presence or absence of the mycotoxin deoxynivalenol (DON) and they concluded that the non-toxic concentration of DON on IPEC-J2 cells after short-term exposure interferes with the pharmacokinetics of the antibiotic. Perez *et al*. (2012) showed that FOS concentrations in HEP-2 cells, a respiratory cell line, exceeded the MIC_{90} for the pathogens which frequently cause swine respiratory infections. Therefore, it is apparent that FOS is an alternative for the treatment of intracellular respiratory infections in pigs. Furthermore, determined FOS penetration in HEP-2 cell cultures and their interactive effect with DON and they concluded that the presence of the mycotoxin does not alter the intracellular distribution of the antibiotic. On the other hand, Soraci *et al*. (2012) demonstrated that FOS concentrations in Epithelial Lining Fluid (ELF) were above the MIC_{90} value for *Streptococcus* up to 8 h post IM administration of 15 mg kg^{-1} of FOS in weaning piglets.

The FOS showed clinical efficacy in the treatment of pulmonary diseases. Nevertheless, concentrations in Alveolar Macrophages (AM) (biophase for facultative and obligate intracellular microorganisms) have not been established in any species yet. Assuming that the determination of FOS concentration in the biophases represents the key parameter for establishing efficacy of antibiotics, the objective of this study was to characterize the penetrating capacity of FOS in alveolar macrophages and its relationship with serum concentrations in weaning piglets.

MATERIALS AND METHODS

Six weaning piglets (three males and three females), clinically healthy, 25-28 days of age, were used in this assay. Disodium FOS was supplied by Bedson Laboratories S.A., Pilar, Buenos Aires,

Argentina. It was dissolved in a 10% sodium citrate solution (pH: 6.8). To minimize stress and facilitate blood sampling, a permanent long catheter was placed in the left external jugular vein of each piglet. The study was carried out according to the rules of ethics approval of the Experimental Ethics Committee of the Faculty of Veterinary Science, UNCPBA, Argentina.

Alveolar macrophages intracellular concentrations of disodium FOS after a single IM dose of 15 mg kg^{-1} in the gluteal muscle were evaluated. Macrophages were obtained from bronchoalveolar lavage fluid (BALF) samples. To procure BALF samples, a flexible fiber optic bronchoscope (Olympus BF- P20D) was used. The bronchoscope was pushed into the bronchus trachealis which leads into the right cranial lung lobe (Shields and Riedler, 2000; Baltes *et al*., 2001; Scollo *et al*., 2011; Soraci *et al*., 2011). Seven milliliters of 0.9% sterile saline solution (prewarmed to 30°C) were introduced and recovered by using a vacuum pump aspiration with a maximum of 15 kPa (Shields and Riedler, 2000; Baltes *et al*., 2001). A range between 15 and 18 mL of BALF were obtained after repeating the washings three times (Shields and Riedler, 2000). The procedure of instillation and collection was completed in <1 min (Baughman *et al*., 1983; Dohn and Baughman, 1985; Rennard *et al*., 1986; Grigg *et al*., 1991; Lamer *et al*., 1993; Baughman, 1997; Mombarg *et al*., 2002). The BALF samples were collected after 1, 2, 3 and 6 h of IM administration of FOS. Lavage sample were immediately centrifuged at 2000 rpm for 10 min and the supernatant was separated from the pellets. The BALF samples, stored in 1.5 mL tubes, were centrifuged at 4°C and 3500 rpm for 15 min. Supernatants were removed and the pellets were resuspended in 500 μL of HPLC water. Ten microliter of the resuspended pellets were taken and mixed with 10 μL of trypan blue to determine cell viability. Cell count was performed in a Neubauer chamber. Samples were ultrasonicated for 30 min to release FOS from AM. Vials were filtered with 0.22 μm filters and finally analyzed by HPLC-MS/MS. Since, the number of cells in each sample was different, results were extrapolated to 400,000 cells, corresponding to the number of macrophages present in a well monolayer of a 6-well plate. To calculate the final intracellular concentrations of FOS in AM, it was considered that the pellet was resuspended in 500 μL of HPLC water and that there is an intracellular water volume of 1.50 μL in 400,000 macrophages (dilution factor: 0.5015).

The FOS concentrations were measured using a High Performance Liquid Chromatography-Mass-Mass Spectrometry (HPLC-MS/MS) according to the method determined by Soraci *et al*. (2012). The HPLC-MS/MS (Thermo Electron Corporation) system consisted of a Finnigan Surveyor autosampler and a quaternary pump Finnigan MS. The detector was a mass spectrometer quantum triple quadrupole discovery thermo max, equipped with an ESI source. The nitrogen used as nebulizer gas and envelope was obtained by a nitrogen generator peak scientific (Inchinnan). Data processing was performed using the Xcalibur software, also purchased from Thermo. A workstation Turbo Vap (caliper) with bath temperature control and airflow was used for solvent evaporation. The mass spectrometer was operated in the negative ionization mode. The adjustment parameters were optimized with individual aqueous solutions of 10 mg mL^{-1} FOS. An injection pump directly infused solutions to ion source, while the mobile phase was delivered from the LC pump via a T-connection to give the corresponding chromatographic flow rate. The spray voltage was set to 3800 eV, the capillary temperature was 350°C. Argon (purity 99.99%) at 1.6 m torr was used for Collision-Induced Dissociation (CID) in the collision cell. The CID energy source was set at -8 eV. Detection and quantification of FOS was achieved by single reaction monitoring of the transitions m/z 137→79 with optimized collision energy of 25. Separation was performed on a Phenomenex CN (cyano) (411 Madrid Avenue Torrance, CA90501-1430, USA), stationary phase, 4.6×75 mm id, 5 μm column. The mobile phase consisted of acetonitrile: water

20:80 isocratic mode at a flow rate of 100 μL min^{-1}. The column was maintained at 30°C. The samples were kept at 10°C in the autosampler. The injection volume of the sample was 20 μL and the chromatographic run was 6 min.

RESULTS

Concentrations ranged from 0.14-1.52 μg mL^{-1}. The C_{max} was 1.52 μg mL^{-1} and T_{max} was 4 h. Table 1 shows FOS average intracellular concentrations found for each sampling time and some pharmacokinetics parameters (Tmax; Cmax). Figure 1 shows FOS average intracellular concentrations in AM after an IM administration of disodium FOS at 15 mg kg^{-1} in weaning piglets.

DISCUSSION

Intracellular penetration, accumulation and disposition of antibacterial agents are crucial for effective treatment of infections caused by intracellular bacteria. Intracellular concentrations and locations of both antibacterials and bacteria remain poorly understood and further research is needed to establish the importance of these concepts (Butts, 1994). Measurement of antibiotic concentrations achieved in lung parenchyma, ELF, bronchial mucosa of bronchial secretions has shown variable levels for the same drug. Many respiratory infections are caused by obligate or facultative intracellular pathogens, which may be eradicated as a result of drug intracellular penetration and accumulation, as shown in several models of phagocytic cells and as a result of intracellular antibacterial activities (Bergogne-Berezin, 1995). In previous works from our research group, disodium FOS concentrations in serum (Soraci *et al*., 2011), respiratory cells (HEP-2) (Perez *et al*., 2012) and ELF (Soraci *et al*., 2011) have been determined. Considering our previous studies, it was found that FOS concentrations were significantly lower ($p<0.05$) in AM than in ELF

Table 1: FOS average intracellular concentrations and standard deviations found for each sampling time and some pharmacokinetics parameters (Tmax; Cmax) in AM after IM administration of disodium FOS at 15 mg kg^{-1} in weaning piglets

Time (h)	FOS average concentration (μg mL^{-1})	SD
1	0.15	0.01
2	0.45	0.02
3	0.60	0.04
4*	1.52^	0.02
6	1.04	0.01

FOS: Fosfomycin, SD: Standard deviation, AM: Alveolar macrophages

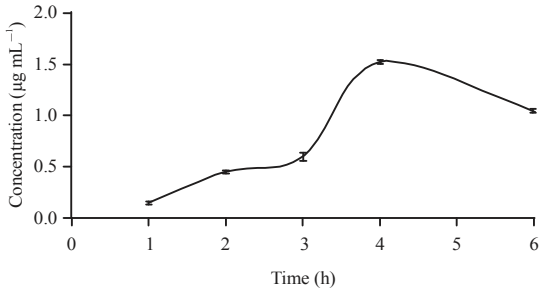

Fig. 1: Fosfomycin average intracellular concentrations (+1 SD) in AM after IM administration of disodium Fosfomycin at 15 mg kg^{-1} in weaning piglets

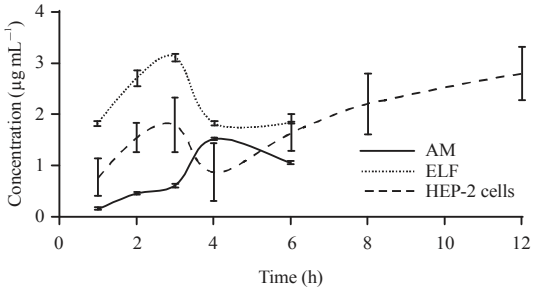

Fig. 2: Differences between fosfomycin concentrations (mean +1 SD) in AM, ELF and HEP-2 cells. ELF and HEP-2 cells FOS intracellular concentrations were determined on previous works of our research group (Soraci *et al*., 2012; Perez *et al*., 2012)

after the IM administration of disodium FOS at a dose of 15 mg kg^{-1} at every sampling time points. However, the intracellular concentrations in AM correspond to 49.03% of the concentration achieved in bronchial fluid (AM: 1.52 μg mL^{-1} vs ELF: 3.10 μg mL^{-1}). Concentrations found in AM were also lower (54.48%) than those found in HEP-2 cells (2.79 μg mL^{-1}) after incubation of the cells with a dose of 280 μg mL^{-1} (which corresponds to an *in vivo* dose of 15 mg kg^{-1} of disodium FOS). Tmax (4 h) was higher than that found in ELF (2.50 h), although it is lower than the Tmax found in HEP-2 cells (12 h). Figure 2 shows the differences between FOS concentrations in AM, ELF and HEP-2 cells. It is concluded that concentrations found in AM exceeded the MIC_{90} for most pathogens of importance in pig production (0.25-0.50 μg mL^{-1}) (Fernandez *et al*., 1995) (T>MIC = 6 h). Therefore, FOS may be useful in the treatment of lung infections caused by facultative intracellular microorganisms. However, it is well-known that the pathophysiological changes induced by inflammation that occur in the lungs of infected pigs (dilation of the capillaries and increased vascular supply to the infected region) may influence the antibiotic pharmacokinetics in the respiratory tract of swines (Baggot, 1977; Agerso and Friis, 1998). Thus, additional studies on FOS pharmacokinetics should be carried out in diseased pigs.

REFERENCES

Agerso, H. and C. Friis, 1998. Penetration of amoxycillin into the respiratory tract tissues and secretions in pigs. Res. Vet. Sci., 64: 245-250.

Aliabadi, F.S. and P. Lees, 1997. Pharmacodynamic and pharmacokinetic inter-relationships of antibacterial drugs. J. Vet. Pharmacol. Therapeut., 20: 14-17.

Baggot, D., 1977. Principles of Drug Disposition in Domestic Animals. In: The Physiological Basis of Veterinary Clinical Pharmacology, Baggot, J.D. (Ed.). WB Saunders, Philadelphia, PA., pp: 1-22.

Baltes, N., W. Tonpitak, G.F. Gerlach, I. Hennig-Pauka, A. Hoffmann-Moujahid, M. Ganter and H.J. Rothkotter, 2001. *Actinobacillus pleuropneumoniae* iron transport and urease activity: Effects on bacterial virulence and host immune response. Infect. Immun., 69: 472-478.

Baughman, R.P., 1997. The uncertainties of bronchoalveolar lavage. Eur. Respir. J., 10: 1940-1942.

Baughman, R.P., C.H. Bosken, R.G. Loudon, P. Hurtubise and T. Wesseler, 1983. Quantitation of bronchoalveolar lavage with methylene blue. Am. Rev. Respir. Dis., 128: 266-270.

Bergogne-Berezin, E., 1995. New concepts in the pulmonary disposition of antibiotics. Pulm. Pharmacol., 8: 65-81.

Butts, J.D., 1994. Intracellular concentrations of antibacterial agents and related clinical implications. Clin. Pharmacokinet., 27: 63-84.

Carr, J., 2001. Porcine respiratory disease syndrome. Int. Pig Top., 16: 11-13.

Christensen, C., V. Soerensen and J. Mousing, 1999. Diseases of the Respiratory System. In: Diseases of Swine, Straw, B.E., S. D'Allaire, W.L. Mengeling and D.J. Taylor (Eds.). Wiley, New York, USA., ISBN-13: 9780813803388, pp: 913-940.

Cloutier, G., S. D'Allaire, G. Martinez, C. Surprenant, S. Lacouture and M. Gottschalk, 2003. Epidemiology of *Streptococcus suis serotype* 5 infection in a pig herd with and without clinical disease. Vet. Microbiol., 97: 135-151.

Dirkzwager, A., B. Veldmana and P.A. Bikkera, 2005. A nutritional approach for the prevention of post weaning syndrome in piglets. Anim. Res., 54: 231-236.

Dohn, M.N. and R.P. Baughman, 1985. Effect of changing instilled volume for bronchoalveolar lavage in patients with interstitial lung disease. Am. Rev. Respir. Dis., 132: 390-392.

Done, S.H. and D.J. Paton, 1995. Porcine reproductive and respiratory syndrome: Clinical disease, pathology and immunosuppression. Vet. Rec., 136: 32-35.

Dosen, R., J. Prodanov, D. Milanov, I. Stojanov and I. Pusic, 2007. The bacterial infections of respiratory tract of swine. Biotech. Anim. Husb., 23: 237-243.

Fernandez, P., I. Herrera, P. Martinez, L. Gomez-Lus and J. Prieto, 1995. Enhancement of the susceptibility of *Staphylococcus aureus* to phagocytosis after treatment with fosfomycin compared with other antimicrobial agents. Chemotherapy, 41: 45-49.

Galina, L., C. Pijoan, M. Sitjar, W.T. Christianson, K. Rossow and J.E. Collins, 1994. Interaction between *Streptococcus suis* serotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets. Vet. Rec., 134: 60-64.

Gardner, I.A. and D.W. Hird, 1990. Host determinants of pneumonia in slaughter weight swine. Am. J. Vet. Res., 51: 1306-1311.

Grigg, J., J. Beacham and M. Silverman, 1991. Estimation of lung epithelial lining fluid volume by urea dilution technique in ventilated neonates. Proceedings of the 3rd International Conference on Bronchoalveolar Lavage, June 20-22, 1991, Vienna.

Hoger, P.H., 1985. Influence of intracellularly active antibiotics (fosfomycin, rifampin, sulfamethoxazole, trimethoprim) on normal neutrophil function *in vitro*. Proceedings of the 5th International Mesostructured Materials Symposium, July 4-6, 1985, Mexico, Libro Resumen, pp: 46-57.

Honda, J., Y. Okubo, M. Kusaba, M. Kumagai, N. Saruwatari and K. Oizumi, 1998. Fosfomycin (FOM: 1*R*-2*S*-epoxypropylphosphonic acid) suppress the production of IL-8 from monocytes via the suppression of neutrophil function. Immunopharmacology, 39: 149-155.

Krause, R., S. Patruta, F. Daxbock, P. Fladerer and C. Wenisch, 2001. The effect of fosfomycin on neutrophil function. J. Antimicrob. Chemother., 47: 141-146.

Lamer, C., V. de Beco, P. Soler, S. Calvat and J.Y. Fagon *et al*., 1993. Analysis of vancomycin entry into pulmonary lining fluid by bronchoalveolar lavage in critically ill patients. Antimicrob. Agents Chemother., 37: 281-286.

Martinez, G., D.S. Perez, A.L. Soraci and M.O. Tapia, 2013. Penetration of fosfomycin into IPEC-J2 cells in the presence or absence of deoxynivalenol. Plos One, Vol. 8. 10.1371/journal.pone.0075068

Mombarg, M.J., T.A. Niewold, N. Stockhofe-Zurwieden, L.A.M.G. van Leengoed and J.H.M. Verheijden, 2002. Assessment of respiratory herd health in weaner pigs by measuring cellular composition of bronchoalveolar lavage fluid. J. Vet. Med. B Infect. Dis. Vet. Public Health, 49: 424-428.

Morikawa, K., F. Oseko, S. Morikawa and M. Sawada, 1993. Immunosuppressive activity of fosfomycin on human T-lymphocyte function *in vitro*. Antimicrob. Agents Chemother., 37: 2684-2687.

Nabuurs, M.J.A., A. Hoogendoorn, E.J. van der Molen and A.L.M. van Osta, 1993. Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. Res. Vet. Sci., 55: 78-84.

Nix, D.E., S.D. Goodwin, C.A. Peloquin, D.L. Rotella and J.J. Schentag, 1991. Antibiotic tissue penetration and its relevance: Impact of tissue penetration on infection response. Antimicrob. Agents Chemother., 35: 1953-1959.

Perez, D.S., A.L. Soraci and M.O. Tapia, 2012. *In vitro* penetration of fosfomycin in respiratory cells. Pig J., 67: 43-53.

Perez, D.S., G. Martinez, A.L. Soraci and M.O. Tapia, 2013. *In vitro* penetration of the antibiotic fosfomycinin swine intestinal cells (IPEC J2). Int. J. Med. Pharma. Sci., 4: 6-16.

Rennard, S.I., G. Basset, D.O. Lecossier, K.M. Donnell, P. Pinkston, P.G. Martin and R.G. Crystal, 1986. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J. Applied Physiol., 60: 532-538.

Schentag, J.J. and C.H. Ballow, 1991. Tissue-directed pharmacokinetics. Am. J. Med., 91: 5-11.

Scollo, A., C. Mazzoni, F. Tonon, E. Borri, V. Raffi, R. Donn and M. Gherpelli, 2011. Bronchoalveolar Lavage (BAL). Swine: Two Techniques in Comparison and New Proposals for Concrete Field Employment, pp: 94-102.

Shields, M.D. and J. Riedler, 2000. Bronchoalveolar lavage and tracheal aspirate for assessing airway inflammation in children. Am. J. Respir. Crit. Care Med., 162: 15-17.

Soraci, A.L., D.S. Perez, G. Martinez, S.N. Dieguez and M.O. Tapia *et al*., 2011. Disodium-fosfomycin pharmacokinetics and bioavailability in post weaning piglets. Res. Vet. Sci., 90: 498-502.

Soraci, A.L., D.S. Perez, G. Martinez, F. Amanto and M.O. Tapia *et al*., 2012. Fosfomycin concentrations in epithelial lining fluid in weaning piglets. J. Vet. Pharmacol. Therapeut., 35: 406-409.

Thanawongnuwech, R., G.B. Brown, P.G. Halbur, J.A. Roth, R.L. Royer and B.J. Thacker, 2000. Pathogenesis of porcine reproductive and respiratory syndrome virus-induced increase in susceptibility to *Streptococcus suis* infection. Vet. Pathol., 37: 143-152.

Toutain, P.L., J.R.E. del Castillo and A. Bousquet-Melou, 2002. The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. Res. Vet. Sci., 73: 105-114.

Trautmann, M., C. Meincke, K. Vogt, A.M. Ruhnke and A.M. Lajous-Petter, 1992. Intracellular bactericidal activity of fosfomycin against Staphylococci: A comparison with other antibiotics. Infection, 20: 350-354.