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Enhanced antibacterial effect of antibiotics in combination with silver nanoparticles against animal pathogens

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ABSTRACT

Antibiotic resistant bacteria are a serious health risk in both human and veterinary medicine. Several studies have shown that silver nanoparticles (AgNPs) exert a high level of antibacterial activity against antibiotic resistant strains in humans. The aim of this study was to evaluate the antibacterial effects of a combined therapy of AgNPs and antibiotics against veterinary bacteria that show resistance to antibiotics. A microdilution checkerboard method was used to determine the minimal inhibitory concentrations of both types of antimicrobials, alone and in combination. The fractional inhibitory concentration index was calculated and used to classify observed collective antibacterial activity as synergistic, additive (only the sum of separate effects of drugs), indifferent (no effect) or antagonistic.

From the 40 performed tests, seven were synergistic, 17 additive and 16 indifferent. None of the tested combinations showed an antagonistic effect. The majority of synergistic effects were observed for combinations of AgNPs given together with gentamicin, but the highest enhancement of antibacterial activity was found with combined therapy together with penicillin G against *Actinobacillus pleuropneumoniae*. *A. pleuropneumoniae* and *Pasteurella multocida* originally resistant to amoxycillin, gentamicin and colistin were sensitive to these antibiotics when combined with AgNPs. The study shows that AgNPs have potential as adjuvants for the treatment of animal bacterial diseases.

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Introduction

Extensive use of antimicrobial agents contributes to the development and rapid spread of bacterial resistance, which implies a decrease in antibiotic efficacy in both human and veterinary medicine (Schwarz et al., 2001). Resistance to antimicrobial agents in commensal bacteria (e.g. *Escherichia coli*), zoonotic enteropathogens (e.g. *Salmonella* spp.) and animal pathogens (e.g. *Pasteurella multocida* or *Actinobacillus* spp.) has been reported (Abd-Elghany et al., 2014; Chantziaras et al., 2014; Dayao et al., 2014). One approach to control bacterial infections is combination therapy in which antibiotics are given together with other antimicrobial or non-antimicrobial agents. These adjuvants include other antibiotics, non-antibiotic substances (e.g. cardiovascular drugs), resistance inhibitors, such as β-lactamase inhibitors, and inhibitors of biofilm formation (Kalan and Wright, 2011).

Silver nanoparticles (AgNPs) have bactericidal effects against many species of human bacteria (Kim et al., 2007; Taglietti et al., 2012) and their veterinary counterparts (Soltani et al., 2009), including highly resistant strains, such as methicillin resistant *Staphylococcus aureus* (MRSA) (Panáček et al., 2006; Lara et al., 2009). AgNPs are efficient as antimicrobial agents at low concentrations (mg/L) and are not cytotoxic to eukaryotic cells, including human erythrocytes (Krajewski et al., 2013). Moreover, because of the non-specific mechanism of AgNP-mediated antibacterial activity, the risk for development of resistance is not as high as for antibiotics.

The mechanism whereby AgNPs interact with bacteria cannot be described in terms of a single and specific mode, as with antibiotics. AgNPs damage the bacterial cell wall, change membrane permeability, and cause a collapse of plasma membrane potential (Sondi and Salopek-Sondi, 2004; Lok et al., 2006). Furthermore, AgNPs interact with DNA, inactivate enzymes, influence metabolic processes, change protein expression and damage the respiratory chain (Lara et al., 2009; Li et al., 2011; Cui et al., 2013). Silver ions are released from the nanoparticle surface and enter the bacterial cell to generate reactive oxygen species (ROS), which destroy biomacromolecules (Choi and Hu, 2008).

AgNPs facilitate the interaction of antibiotics with cells in numerous ways. For example, they may help the penetration of antibiotics into the bacterial cell by changing membrane permeability;

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alternatively, both AgNP and antibiotic may cooperate in the destruction of the cell wall. In the case of β-lactam antibiotics, AgNPs may inhibit hydrolytic β-lactamases produced by bacteria. Damage and weakness by simultaneous action of antibiotic and AgNP will lead to cell death. Hwang et al. (2012) suggested that this synergism is associated with generation of hydroxyl radicals, the alteration of protective cellular functions and an anti-biofilm potential. Therefore, the combination of antibiotics with AgNPs seems to be a more effective method for enhancing antibiotic efficacy in comparison with other adjuvants currently used in clinical practice. The combination implies reduced antibiotic dose requirements, reduced development of bacterial resistance and increased efficiency of co-administered antibiotics.

Many antibiotics, with differing mechanisms of action, are more effective against human bacteria when combined with AgNPs (Li et al., 2005; Jain et al., 2009; Brown et al., 2012; Ghosh et al., 2012; Hwang et al., 2012). Unfortunately, several of these studies evaluated combined antimicrobial effects using the disc diffusion method employing standard discs with predetermined concentration of antibiotics. In most cases, a zone of inhibition was apparent indicating that the antibiotic was itself effective (Shahverdi et al., 2007; Birla et al., 2009; Fayaz et al., 2010). There would appear to be no reason to combine drugs if a single drug at a specific concentration is effective.

In the present study, we evaluated the synergistic effects of antibiotics administered at doses lower than their minimum inhibitory concentrations (MICs) with AgNPs of two different sizes to test whether it was possible to enhance the antibacterial activity of antibiotics that targeted veterinary-relevant bacteria, including resistant species. Potential synergistic antibacterial effects were quantified by the fractional inhibitory concentration (FIC) index, which was determined using MICs obtained by the microdilution checkerboard method (Lorian, 2005).

Materials and methods

Preparation of silver nanoparticles

AgNPs with diameters of 28 nm (AgNP-28 nm) and 8 nm (AgNP-8 nm) were synthesised through the reduction of complex cation $[Ag(NH_3)_2]^+$ by D-maltose (Sivera et al., 2014) and sodium borohydride, respectively. Detailed description of synthetic procedures and characterisation techniques is included in the Appendix: Supplementary material.

Evaluation of synergistic effect of antibiotics combined with AgNPs

Bacterial strains *Salmonella enterica* LT2, *Staphylococcus aureus* GP0004, *Escherichia coli eae+* GN2514, *Actinobacillus pleuropneumoniae* 17/06L, *Pasteurella multocida* P-813 and *Streptococcus uberis* GP1037 were obtained from the Centre de Recerca en Sanitat Animal, Barcelona, Spain.

The antibiotic sensitivity of bacterial strains was assessed according to Clinical Laboratory Standards Institute (CLSI) document VET01-A3 (CLSI, 2008). MICs of antibiotics, AgNPs and their combinations were determined using the microdilution checkerboard method. *S. enterica*, *S. aureus*, *S. uberis* and *E. coli* were tested in MH (pH 7.3), while *A. pleuropneumoniae* and *P. multocida* were tested in BHI (pH 7.4). BHI was supplemented with 1% IsoVitaleX (BBL) for cultivation of *A. pleuropneumoniae*. The microtitre plates were incubated at 37 °C for 24 h (see Appendix: Supplementary material).

The FIC index was calculated to evaluate the combined antimicrobial effect of antibiotics and AgNPs (Lorian, 2005):

$$\text{FIC} = \frac{\text{MIC of drug A in the combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in the combination}}{\text{MIC of drug B alone}}$$

There are differences in the interpretation of the FIC index (Botelho, 2000; Orhan et al., 2005; Kurek et al., 2012). In the present study, the combined antibacterial effects of antibiotics and AgNPs were considered to be synergistic, additive, indifferent or antagonistic when the FIC indices were ≤0.5, >0.5 to ≤1, >1 to ≤2 and >2, respectively (Kurek et al., 2012).

Results

Silver nanoparticles

The results of antibacterial activity of AgNPs are summarised in Table 1. AgNPs exhibited antimicrobial activity against Gram negative organisms. The MICs of AgNPs were 6.3–100 μg/mL depending on the tested strains and size of AgNPs. The growth of Gram positive bacteria was inhibited less by AgNPs than Gram negative bacteria. However, the final MICs of AgNP-28 nm could not be determined because the MIC was greater than the highest silver concentration used in the dilution method (>50 μg/mL).

The antibacterial activity of smaller nanoparticles was stronger than the antibacterial activity of larger AgNPs. Both AgNP-28 nm and AgNP-8 nm showed the most antibacterial effects against the Gram negative bacteria *P. multocida* and *E. coli* (AgNP-8 nm), whereas the Gram positive bacterium *S. uberis* was the least sensitive of the tested strains.

Amoxycillin

The MIC of amoxycillin against the tested bacteria was 0.25 to >32 μg/mL (Table 2). *E. coli* and *A. pleuropneumoniae* had MICs >32 μg/mL (breakpoint MIC ≥32 μg/mL) and were resistant to amoxycillin. When 8 μg/mL amoxycillin was combined with 12.5 μg/mL AgNP-28 nm or AgNP-8 nm, antibiotic resistant *A. pleuropneumoniae* becomes sensitive. The antibacterial activity of amoxycillin combined with AgNP-28 nm was synergistic (FIC index 0.4). An FIC index of 0.6 was obtained when AgNP-8 nm was combined with

Table 1
Minimum inhibitory concentrations (μg/mL) of silver nanoparticles with diameters of 28 nm (AgNP-28 nm) or 8 nm (AgNP-8 nm).

Bacteria	AgNP-28 nm	AgNP-8 nm
Salmonella enterica	25	12.5
Staphylococcus aureus	>50	25
Escherichia coli	25	6.3
Actinobacillus pleuropneumoniae	50	25
Streptococcus uberis	>50	100
Pasteurella multocida	6.3	6.3

Table 2
Minimum inhibitory concentrations (MICs; μg/mL) of amoxycillin alone or with silver nanoparticles with diameters of 28 nm (AgNP-28 nm) or 8 nm (AgNP-8 nm).

Bacteria	Amoxycillin	Drug combination MIC		FIC	Effect	Drug combination MIC		FIC	Effect
		AgNP-28 nm	Amoxycillin			AgNP-8 nm	Amoxycillin		
Salmonella enterica	1	25	1	2.0	I	12.5	1	2.0	I
Staphylococcus aureus	2	25	1	0.8	A	12.5	0.5	0.8	A
Escherichia coli (R)	>32	25	>32	2.0	I	6.3	>32	2.0	I
Actinobacillus pleuropneumoniae (R)	>32	12.5	8	0.4	S	12.5	8	0.6	A
Pasteurella multocida	0.25	6.3	0.25	2.0	I	6.3	0.25	2.0	I
Streptococcus uberis	0.25	>50	0.25	2.0	I	50	0.125	1.0	A

FIC, fractional inhibitory concentration index; S, synergy; A, additivity; I, indifference; R, resistant strain.

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