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Inflammopharmacology

SHORT COMMUNICATION

Efficacy of some colloidal silver preparations and silver salts against *Proteus* bacteria, one possible cause of rheumatoid arthritis

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Abstract There has been increased interest in the role of anti-*Proteus* antibodies in the aetiology of rheumatoid arthritis (RA) and whether chemotherapeutic agents active against *Proteus* species might reduce the risk and/or exacerbations of RA. We examined the in vitro antibacterial effects of ten different silver preparations which were either ionic silver [Ag(I)] solutions or nanoparticulate silver (NPS) (Ag^0) suspensions against ATCC and two wild (clinical) strains of *Proteus*. The data establish the low minimum inhibitory concentration and minimum bactericidal concentration of all the silver formulations tested against these four *Proteus* strains. In a pilot study, a potent NPS preparation ex vivo showed long-lasting anti-*Proteus* activity in a normal human volunteer.

Keywords *Proteus* · Rheumatoid arthritis · Silver antimicrobials · Nanoparticulate silver

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Introduction

A fundamental way to treat an inflammation is to eradicate its ultimate cause (Whitehouse et al. 2013). *Proteus* infections might be one trigger factor for rheumatoid arthritis (RA) in susceptible individuals (Rashid and Ebringer 2011; Ebringer 2012) evidenced by: (1) isolation of *Proteus* from urine, (2) elevation of antibodies to *Proteus* in the sera of RA patients and (3) cytopathic effects of these serum antibodies upon joint tissues carrying *Proteus* cross-reacting antigens. Treating infections caused by this organism at an early stage might therefore minimise/prevent the joint damage by these *Proteus*-associated cross-reacting antibodies/antigens.

This may provide a new therapeutic approach for RA treatment and/or its prevention using antimicrobials to eradicate *Proteus* sp from the body (Rashid et al. 2001; Ebringer et al. 2003). Effective early anti-*Proteus* therapy might reduce the risk of RA developing, curtailing this disease in the same way that rheumatic fever has been largely eliminated by antibiotics to treat Streptococcal pharyngitis.

This hypothesis has already triggered a search for effective anti-*Proteus* chemotherapeutic agents. They include many conventional and some non-conventional antimicrobials ranging from cranberry juice to carbapenems and some African phytochemicals (Ferrara et al. 2009; Lee et al. 2011; Cock and van Vuuren 2013).

Both silver metal and its ionic salts are effective broad-spectrum antimicrobials active against many Gram-positive and Gram-negative bacteria, as well as some fungi (Marambio-Jones and Hoek 2010; Eckhardt et al. 2013; Laroo 2013). Currently some advantages of using silver nanoparticles as a chemotherapeutic agent are their potency, the minimal development of bacterial resistance and limited toxic side effects (Varner et al. 2010).

Table 1 Range of silver preparations studied

No	Silver preparation (total Ag, ppm)	'Scatter index'[a]	MIC and MBC (mg/L)			
			P.mirabilis 1	*P.mirabilis 2*	*P.vulgaris 1*	*P.vulgaris 2*
1	Silver acetate, Aldrich (8)	04	2	2	2	2
2	Silver sulphate, Brit Drug Hses (10)	01	1.25	1.25	1.25	1.25
3	NPS prepared chemically (100)	54	1.5	1.5	1.5	1.5
4	Mesosilver®, (20)[b]	35	3.13	3.13	3.13	3.13
5	E + PC, LR-203 (6)	62	2.5	2.5	2.5	2.5
6	E + PC, LR-049 (6)	≥36	1.5	1.5	1.5	1.5
7	E + PC, HL-004 (5.3)	17	0.66	0.66	0.66	0.66
8	E + PC, W-7 (3.6)	30	1.8	1.8	1.8	1.8
9	E + PC, W-8 (4.8)	01	1.25	1.25	1.25	1.25
10	E + PC, W-9 (3.5)	06	1.75	1.75	1.75	1.75

NPS nanoparticulate silver, *E + PC* electro- and photochemical preparations of NPS (see text), *ppm* parts per million i.e. mg/L

[a] Intensity of orthogonally scattered green laser light (532 nm) as arbitrary units/ppm total Ag

[b] From purest colloids, Westampton NJ

Methods

Four strains of *Proteus* species were tested against ten silver preparations (salt or nanoparticulate) using microtitre plates to determine the minimum bactericidal concentration (MBC) (the lowest concentration of antibiotic to kill a particular bacterium) and the minimum inhibitory concentration (MIC) (the lowest concentration of an antimicrobial to inhibit visible growth of a microorganism after overnight incubation). The *Proteus* strains were:

1. *Proteus mirabilis* ATCC 7002
2. *Proteus vulgaris* ATCC 6380
3. *Proteus mirabilis,* a clinical isolate
4. *Proteus vulgaris,* a clinical isolate

Silver products

Ten silver preparations were tested against each *Proteus* strain (Table 1). Products 1 and 2 are commercial silver salts. Sample 3 was synthesised by chemical reduction of 'silver carbonate' (Ag NO_3 with $NaHCO_3$), kindly donated by A. White, Brisbane. Sample 4 was a commercial batch of 'colloidal silver' (Meso Silver). Products numbered 5–10 are experimental nanoparticulate silver (NPS) preparations (Laroo Research) synthesised by electrochemistry and controlled radiation or photonic electron transfer.

These electro-photochemical (E + PC) preparations were produced from 99.99 % pure silver electrodes by varying (a) DC voltage (28–300 v), (b) duration of electrochemical oxidation and (c) exposure to light at frequencies ≤470 nm. Photo-induced reduction of electrochemical-generated silver cations by hydrated electrons is an important factor determining NPS yield and cluster size (Laroo 2013).

Total silver content was determined by atomic absorption spectrophotometry. Ionic silver was determined either with a silver-specific ion electrode (Ionex) or by fractionation using KSCN or a cation exchanger resin (Amberlite IR-120) (Cock et al. 2012). The NPS preparations containing 5–35 % ionic silver, were polydisperse in their size distribution and ranged from 5 to 230 nm. Some of the larger particle aggregates could be dissociated by gentle agitation or brief sonication. A working mode of characterisation was to measure orthogonal (90°) scattering of green laser incident light (532 nm) in a modified spectrophotometer soon after preparation and again after completing bioassays.

Each of the four *Proteus* strains was tested in triplicate against each silver preparation to determine both the MIC and the MBC.

- A *Proteus* suspension was prepared in nutrient broth from overnight growth on horse blood agar at 35 °C. Turbidity was adjusted to MF 0.5. This was diluted to produce a concentration of 1×10^4 org/ml. 10 µl from the final dilution (~100 organisms) was used to inoculate the dilutions of silver (solution or dispersion).
- Doubling dilutions of each silver preparation in nutrient broth were prepared in microtitre plate wells in triplicate. Each well and the growth control wells were inoculated with 10 µl of *Proteus* suspensions (~100 organisms). Sterility control wells for silver solutions and nutrient broth were included.
- Inoculated microtitre plates were incubated aerobically at 35 °C for 24 h.
- The MIC was determined after examining each well for turbidity.

- Ten micro litre from each well was plated onto McConkey agar using sterile loops and incubated aerobically at 35 °C for 24 h.
- Plates were examined for growth and the number of colonies counted to determine the MBC of each silver product.

In vivo phase I study

Two male volunteers gave informed consent to ingest one NPS preparation, LR-049 containing 6 ppm Ag with proven ex vivo activity again *Proteus*. (Table 1) This pilot study, approved by the WFC Ethics Committee, accorded with recommended practise (Lo 2010). An approximate index of the *Proteus* burden in the lower bowel, ureters, urinary bladder and urethra was determined by non-linear scanning using a MetAtron/Hunter (Institute of Psychophysics, Omsk, Russia). This instrument detects many pathogenic microorganisms in vivo by monitoring their characteristic bio-resonance frequencies (Sylver 2009). The manufacturer claims that it can also provide a semi-quantitative index of infection as a probability index.

LR-049 was taken twice daily (early a.m., late p.m.), each daily dose being 6 μg/kg for 8 days; the maximum total dose being 4 mg silver. *Proteus* levels were recorded on days 0, 7 and 14. Daily records were kept for 2 weeks of possible side effects, e.g. dyspepsia, diarrhoea, malaise, etc. (There were none.) A responsive volunteer was monitored monthly thereafter over the following 12 months.

Results

In vitro studies

All four strains were uniformly susceptible to each of the silver products with identical MICs and MBCs. We could discern no simple correlation between anti-*Proteus* activity, the proportion of silver cations (5–100 %) or with some of the variables investigated in producing different batches of NPS (see Methods).

In vivo study

Table 2 presents the results from a pilot study to ascertain whether an NPS preparation with potent anti-*Proteus* activity in vitro might be effective in vivo. This was essentially an '*N* of 1' probing trial. Two volunteers ingested an NPS preparation for a total dose of ≤4 mg silver taken over 1 week; the levels of *Proteus* infection being monitored with a MetAtron (see Methods).

Table 2 (Proof of concept): Pilot study of an NPS preparation in vivo

Proteus in	Probabilities[a]		
	NPS	V-1	V-2
Large bowel	–	2.8	0.47
	+	0.28 (0.38)	0.44
Urinary bladder	–	0.67	0.18
	+	0.09 (0.01)	0.18

NPS = LR-049, 6 mg/L Ag, mean size of particles = 5.5 nm (9 % w/w), and 42 nm (91 % w/w). Daily dose ≤0.5 mg p.o. for 8 days

Volunteer 1, elderly male without arthritis; Volunteer 2, elderly male with RA, prescribed methotrexate and hydroxychloroquine for ≥12 years

[a] 'Probabilities' of *B. Proteus* infection, computed by the MetAtron sensor before (−) and 8 days after (+) ingesting NPS. The data in brackets for V-1 were obtained 12 months later

In volunteer 1, ingesting LR-049 drastically reduced the *Proteus* burden in the lower bowel (by 90 %) and in the bladder (by 87 %) after 1 week. One year later, with no intervening antibiotic treatment or contrived urinary disinfection, the *Proteus* levels were only 14 and 2 % of the original pre-treatment values.

In volunteer 2, a patient with controlled RA who continued to take two anti-arthritic medications (DMARDs), the much lower (initial) *Proteus* levels were not affected by the NPS treatment. [These DMARDs were methotrexate and hydroxychloroquine, both originally developed as antibiotics.]

No side effects were experienced by either volunteer during and following oral dosing with this NPS preparation.

Discussion

Concerning proteus

Proteus species cause 6–10 % of urinary tract infections (Fairley et al. 1971), confirmed by our own observations in this hospital. They are found as asymptomatic as well as symptomatic isolates in the urinary tract of RA patients (Rashid and Ebringer 2011). The genus *Proteus* currently consists of five named species (O'Hara et al. 2000).

Evidence for a link between *Proteus* microbes and RA, based upon raised *Proteus* antibodies, was first reported by Chandler et al. (1971). The specificity of *Proteus* antibodies was confirmed in several subsequent studies of patients with RA (Deighton et al. 1992a, b; Tiwana et al. 1996; Rashid and Ebringer 2007).

The urinary tract is a likely source of the antigenic *Proteus* (Fairley et al. 1971). RA patients often have

asymptomatic 'non-significant' *P. mirabilis* bacteriuria more frequently than healthy controls (Wilson et al. 1997; Senior et al. 1999). So eliminating *Proteus* might be beneficial for the management of RA patients, used alongside conventional anti-rheumatic drugs.

Concerning silver pharmaceuticals

This term embraces both un-ionised silver in its zerovalent state (Ag^0) and oxidised silver [Ag(I)] either as soluble salts or insoluble oxides, phosphates, etc. Pharmaco-active Ag^0 includes (a) bulk silver (containers, cutlery, coins, rods) used as sterilants or (b) stable aqueous suspensions prepared either chemically or by physical procedures Topical silver therapy for treating infections is well recognised, silver-impregnated dressings being used extensively for wound management particularly in patients with burns, chronic leg ulcers and diabetic wounds. Historically, 'colloidal silver' preparations (usually silver-impregnated proteins or peptides) were ingested to treat various chronic disorders but with the introduction of modern antibiotics in the 1940s, this practise was largely abandoned.

There is now considerable interest in silver nanomaterials and the broad-spectrum microbicidal activities of the nanoparticles produced by various methods (Kim et al. 2007; Marambio-Jones and Hoek 2010; Dayanand et al. 2010; Eckhardt et al. 2013). *Proteus* species have been included among other bacteria in such studies and shown to be inhibited by several silver formulations (Cock et al. 2012). In this study with four different strains of *Proteus* we found that both the ionic and the NPS preparations have appreciable anti-*Proteus* in vitro. This suggests that both NPS and silver salts could be an effective chemotherapeutic agents against *Proteus* spp. NPS particles that are small clusters of atomic silver can release low concentrations of reactive silver cations after controlled oxidation with (dissolved) oxidants or direct interactions with microbial membranes (Liu et al. 2010; Xiu et al. 2012; Eckhardt et al. 2013). In this latter context, the amount of silver solubilised by local oxidation may only be small—but within the contact zone of an NPS particle and a bacterial substrate, the local concentration of newly generated silver cations might be (membrane) toxic. Therefore, an advantage of using NPS as an antibiotic is that it can act as a slow-release pro-drug, delivering microbicidal silver cations when in contact with targeted *Proteus* (and/or its protective biofilms) colonising the lower bowel. Unlike other antibiotics, it will not be so readily lost by intestinal absorption. In rats, orally administered NPS preparations showed anti-arthritic activity, in contrast to silver salts which did not (Whitehouse et al. 2013). Compared to reactive/corrosive silver cations, a bio-effective NPS formulation may only need to be given orally as an intermittent 'purge' i.e. short-term therapy (perhaps for only 3–5 days) as suggested by preliminary data (Table 2).

Whilst silver could be a powerful adjunctive therapy for helping control/eliminate RA, studies will be needed to determine the safety and efficacy of silver nanoparticles in patients, rather than 'normal' subjects.

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