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EFFECTIVE STEPS TOWARDS THE APPLICATION OF SILVER NANOPARTICLES AS SAFE SYSTEMIC INFECTION CONTROLLING AGENTS

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ABSTRACT

Silver nanoparticles (AgNPs) possess potent pan-microbicidal properties with non-specific synergisms for different antimicrobial agents, yet none found suitable for systemic use for potential risk of host cell damage. AgNPs prepared from silver nitrate using dextrose as reducing and human serum as mixture of stabilizing agents, also show all antimicrobial properties. There is least possibility of internalization by receptor-ligand attachment in human cells and liberation of any toxic components after de-capping for bio-mimicking outer coat. For their antimicrobial actions against different multi-drug resistant pathogens at any point of cell cycle and wide range nonspecific synergism with different antibiotics, can be used at safe low dosage with or without synergistic antibiotics. *In-vitro* study on human liver cell-line (WRL-68) and *in-vivo* study in mice model indicated wide margin of safety. Thus, major life-threatening infections caused by super-bugs can be managed in future by such safe systemic useable Nano-antimicrobials.

INTRODUCTION:

An appropriate measure to combat emergence of multidrug-resistant bacteria is a challenge for microbiologist [1]. In-view of dwindling of antibiotic development and increasing evolvement of multi-drug resistant pathogens, the search for developing resistant-proof, non-traditional antibacterial agents is gaining importance. Silver nano-particles (AgNPs) have tremendous potentiality[2,3,4] to be such a drug due to their many fold higher microbial damage[5] after physical transformation of ionic silver (Ag^+) to a different materialistic phase containing plenty of unstable zero valent reduced silver (Ag^0) atoms in robust core of stabilized nanoparticles. This in turn targets different bio-molecules through production of reactive oxygen species (ROS)[6,7]. Resultant colloidal AgNPs have greater surface volume ratio than ionic silver and higher mobilization towards oppositely charged microbial membrane, resulting appreciable damage with higher drug influx and osmotic imbalance. It is profound with smaller size, higher zeta potential and triangular size nanoparticles[8,9]. Microbes may fail to develop resistance[10] by molecular adaptation against such high reactive Ag^0 particles[11], though may develop resistance against metallic silver following overuse[12]. Strong nonspecific synergism of AgNPs with antibiotics of different target action[13] may minimize drug requirement at sub-toxic dose[14]. The use of low dose antibiotics in combinations[2] can lower the chance of resistance development for both. Other advantages of nano-particles (NP) are consistent action on target cell irrespective of static or dividing cell cycle and greater accessibility to bacteria within biofilms[15] or phagosomes [16]. Therefore, AgNPs may not require frequent dosage for longer duration to induce killing effects on microbes in case of systemic use.

Attempt has been made to prepare such heavy metal nano-antimicrobials, using homologous acellular component of blood suitable for intravenous use with acceptable margin of safety to use as drug for man or animal. Plasma components being outer capping structure of such novel AgNPs, may spare host cells for poor receptor-ligand attachment during affinity-based endocytosis. Added advantages of such AgNPs are non-toxicity of bio-mimicking de-capping products and least chance of further adsorption of important functional proteins from circulation. Margin of safety assessment on experimental model may support these propositions, though definitive studies can explore real mechanisms in future.

MATERIALS AND METHODS:

Preparation of systemic usable AgNPs:

To prepare systemic usable AgNPs by chemical reduction method, we used aqueous solution of silver nitrate ($AgNO_3$) (Sigma Aldrich, USA) with pooled sterile human serum (from discarded samples after serological testing in our laboratory) as primary capping or anionic surfactant stabilizing agent and dextrose (Sigma Aldrich, USA) as reducing agent. To avoid precipitation of $AgNO_3$ in presence of sodium chloride in serum the following method was followed by which prompt reduction of silver blocked precipitation reaction in presence of reducing content of serum. In a beaker with magnetic stirrer, 50 ml of sterile serum was taken. To this, 50 ml 4 mM $AgNO_3$ solution (1 mM effective conc. in 200 ml AgNP, 106.8 mg/ L silver or 168 mg $AgNO_3$ /L) in de-ionized water was slowly added with continuous stirring under refluxing condition at 56°C. Then 100ml 4mM dextrose (2260 mg/ L) was added forcefully into the mixture and stirring was continued for 30 minutes. The nano state transformation was indicated by change of color to light brown. Resultant mixture was stored in amber colored bottle, one at room temperature another at 4°C refrigerator and another at oxygen depleted environment inside two-step combustion modified candle jar [17] with loosely tight screw cap. As bio-mimicking agents were used, dialysis to remove excess capping agents after preparation was skipped out for novel nano. Human serum without silver salt and one equivalent aquatic solution of silver salt were included as test controls.

Characterization of AgNPs:

Physical characterization of our serum-based silver nanoparticles for hydrodynamic size was determined by UV-Vis absorption spectrophotometer (Thermo Scientific Genesys 10S Vis). The size ranges of nano mixture with narrow size distributions were indicated by lognormal size distribution curves obtained from DLS (Malvern Zen 3600 Zetasizer, USA) measurements. Negative zeta potential (Malvern Zen 3600 Zetasizer, USA) values -8 mV ensure high aggregation stability of such mixture of AgNPs in aqueous dispersion, while actual size and shape were determined by transmission electron microscope (JEOL JEM 2100 HR with EELS, USA) images.

Study on anti-microbial properties:

Multidrug resistant clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida*

albicans from stock culture of our laboratory were included as test organisms along with a sensitive reference strain *Escherichia coli* ATCC-25922 (Microbiologics-Inc, USA) as control. Clinical isolates from blood samples of sepsis patients were selected after isolation, identification and antimicrobial susceptibility testing in automated system. Minimum inhibitory concentrations (MIC) of each organism for native silver nitrate solution and equivalent silver-containing nano-particles were determined by two-fold serial broth micro-dilution method. In one row for each microorganism of 96 wells plates, aliquots of 100 µl of two-fold serially diluted AgNPs in de-ionized water were added to 100 µl of 0.5 Mf bacterial / 1 Mf yeast suspension in double strength brain heart infusion broth. After incubation at 37°C for 24 h / (48 h for yeast), plates were observed for turbidity to note MIC as end point concentration in well without turbidity. Five repetitions of the study were done.

The higher antimicrobial action of AgNPs over equivalent ionic silver i.e. "Bonus Effects" for different bacteria /yeast were calculated as increase of dilution factors of respective MIC values in two sets broth dilution study. Higher "bonus effect" indicates acquisition of better nano character which may be proportionate to some physical parameters of NPs.[18]

Margin of safety study in animal model and cell-line:

For animal experiment 10 fold higher AgNP were prepared and checked for comparable antimicrobial action at $1/10^{th}$ dilution. Four experimental groups of 25-30g male Swiss albino mice of 5-6 months of age, five for each group were treated with 100 µl of strong AgNP as single intra-venous injections., with one untreated as control. Thus about 107 µg silver equivalent nano particles were given per 30 g mouse by I/V route or 3570 µg/kg body weight. Any behavioural changes were noted, and animals were sacrificed on 7, 14, 21& 28^{th} day one from each group. Each was aseptically dissected for Histo-pathological examinations of liver tissue. Eosin-Haematoxylin stained slides from tissues were examined to note any difference than that of control.

In a preliminary study for antibacterial efficacy on infected mice model, 10 Swiss albino mice were infected by intra-peritoneal injection of 200 µl fresh 0.5 Mf. turbid growth suspension of septicaemia causing strain *Salmonella typhimurium* (from laboratory stock). First five test animals also received simultaneous 100 µl of prepared undiluted strong AgNP as single intra-venous injection, rest 5 were kept separately as control. Fate of animals was observed for 10 days.

Cytotoxicity study was done on human liver cell line (WRL-68) procured from NCCS Pune. MEM medium, 2 ml was added into each well tissue culture plate (flat bottom 6 wells), followed by addition of 10µl of confluent cells. Plates after 24 h incubation at 37°C in CO_2 incubator were checked for cell adherence and continued for another 3 days incubation for colony formation. Wells were washed with incomplete MEM medium (without FBS), then 2ml of fresh complete MEM medium were added with strong AgNPs (containing 10 M silver and dextrose), at effective concentrations of silver 0 (2 ml MEM added in control), 535 µg/ ml (1 ml test AgNP + 1 ml MEM), 800 (1.5 ml AgNP + 0.5 ml MEM) and 1075 µg of silver / ml (added 2 ml AgNP) into wells in chronological order. After 24 h incubation at 37°C in CO_2 incubator, wells were washed with PBS, then fixed with 500 µl of methanol for 15 m. Wells after washing twice with PBS, stained with Hematoxyline for 2 m, washed in tap water and imaging done at 4X inverted microscope. Tests were repeated thrice and CFU % inhibition in respect of control were calculated.

RESULTS AND DISCUSSION:

Characterization of AgNPs:

UV-Vis absorption spectra of serum stabilized silver nano-antimicrobial displayed maximum absorption peak around 410 nm and peak width 20nm. Cluster size distributions obtained from DLS, negative zeta potential values -8 mV ensure high aggregation stability of such mixture of AgNPs in aqueous dispersion. Transmission electron microscopy (TEM) imaging showed that AgNPs were closed to round in shape and average size was 18 nm.

Antimicrobial assay:

The prepared serum capped AgNPs showed 256, 256, 128 and 128 folds higher antimicrobial action respectively (Table 1) than those of equivalent ionic silver solutions for tested *E. coli, S. aureus, P. aeruginosa* and *C. albicans*. We called it "bonus effect" which was taken as the "biological marker" of the nano-antimicrobial mainly due to added antimicrobial action of Ag0 by creating oxidative stress.

Table 1. Anti-microbial enhancement of silver by shifting MIC following nano conversion

Organism	MIC of colloidal AgNPs (effective concentration 106.8 mg/L silver)	MIC of $AgNO_3$ solution (106.8 mg/L silver)	"Bonus effect"
MDR *S. aureus*	1/512 dil (~0.208 mg/L)	1/2 dil (53.4 mg/L)	256-fold
MDR *E. coli*	1/512 dil (~0.208mg/L)	1/2 dil (53.4 mg/L)	256-fold
MDR *P. aeruginosa*	1/512 dil (~0.208 mg/L)	1/4 dil (26.7 mg/L)	128-fold
MDR *C. albicans*	1/256 dil (0.417 mg/L)	1/2 dil (53.4 mg/L)	128-fold

Cytotoxicity study in mice model:

Preliminary study for cytotoxicity on mice model revealed no behavioral change of animal nor histo-pathological changes in liver tissue even in 3570 µg silver equivalent AgNPs /kg body weight I.V injected mice. With this dose of systemic application, the minimum achievable plasma concentration of novel nano-antimicrobial will be much higher than highest MIC value of all test bacteria (≤ 0.208mg/L). It also reiterates that human serum at low dose is well tolerated in murine model. The homologous serum or self-serum is likely to be well tolerated without any antibody related complications.

Cytotoxicity study on human liver cell line:

The mean CFU on control wells were 155 / field (100%) , 150 (97%), 134 (86%) and 50 (32%) CFU/ field on wells with increasing concentrations of AgNPs. Extent of inhibition in terms of CFU reduction after treatment with different concentrations of AgNPs compared with control clearly demonstrated almost no inhibition at concentration of AgNPs as high as 535 µg/ ml silver equivalent, and 50^{th} percentile inhibition at >800 µg/ ml silver equivalent concentrations.

Study for pathogenicity of *S. typhimurium* resulted death of all 5-control mice, 4 on 1^{st} day, one on 2^{nd} day, while all five test animals survived even after 10 days indicating capability of controlling septicaemia and peritonitis in mice model.

DISCUSSION:

Although silver nano-particles are used commercially for coating various devices to impart anti-microbial properties on them to prevent biofilm colonization but are used limitedly on body as infection controlling agents like burn or surface wound dressing[19]. Though by in-vitro testing various preparations of AgNPs are found to be susceptible for wide range bacteria, fungi and viruses, yet have never been used for controlling systemic infections. This is partly due to adverse effects of ingredients used for nano preparation, either from unbound excess or liberated after de-capping[20,21] before endocytosis. Other factors are instability or short half-life, unmatched viscous colloidal state and adsorption of various essential protein components from circulation[22-25]. It has been observed that bovine albumin capped AgNPs show

corona formation with protein molecules at surface[26] and such AgNPs cause least hemolysis than others[27]. Present novel AgNPs are expected to be free of such adverse effects, being coated with plasma proteins[28,29] and may spare attachment on host cells for internalization, after contact with self-protein capped nano particles.

Most researchers have tried to apply nano-technology as infection controlling measure using carbon based nano sheets or tubes tagged with a target antibiotic. The basic difference between nano-particles carrying antibiotic and antimicrobial nano-particles is, enhanced target drug delivery for selective microbe in former[10] while many folds higher antimicrobial action on wide range microbe in resistant-proof manner for later.

Nano-antimicrobials cannot be used for treatment purpose through intra-muscular or oral route[30-32] for chance of rapid de-capping by acid or enzyme exposure. This may lead to systemic effects of absorbed ionic silver; while after intravenous application maximal nano like effect can exert on host cells and invading microbes. However bacterial cells are more susceptible for greater physical affinity[33,34] and unusual chemical reactions targeting energy transport mechanism at cell membrane level. These occur rapidly followed by slow chemical reactions leading to apoptosis[35].

Thus three-pronged approach by developing least toxic nano-antimicrobials, synergistic use with other antimicrobials and less frequent dosage schedule, may open up scope for systemic use of such AgNPs. Before their therapeutic use further studies are required on infected animal models[36].

Adjusted 100 ml colloidal solution of novel AgNPs containing effective concentration of 1 mM silver (108 mg silver / L) if infused through intra-venous route in an adult multi-drug resistant septicemia patient, the achievable plasma concentration (diluted 50 folds in about 5000 ml blood) can reach around 2.16 mg/l silver, which is 5 to 10 times higher MIC values (~0.2- 0.4mg/L) of all tested multi-drug resistant micro-organisms. So, such single dose may be sufficient either alone or in combination with a course of so called "resistant" drug for controlling such dreadful infections. The same dose after infusion into average 100 kg adult individual will deliver 10.8 µg silver / kg body weight, which is much less than minimum lethal dose (> 3570µg/kg) in experimental model. In our mice model study minimum lethal dose could not be determined, which is much less than that of a similar in-vivo study with another nanoparticle at 100 mM/L concentration [37]. The highest tolerable dose in study on human liver cell line (535 mg/l silver equivalent) is much higher than achievable plasma concentration (2.16mg/L) after infusion of 100 ml AgNPs. So, no cytotoxic effect is expected with treatment of AgNPs alone as infection controlling agent against most of the superbugs, if not all.

By using serum as capping agent, excess amount will be available while in use through blood circulation; thereby important role of capping agent[38] for imparting stability to nanoparticles by preventing aggregation for a sustainable time will be assured.

CONCLUSION:

The possible advantages of serum-capped AgNPs for systemic use are: i) before establishing etiological diagnosis on the basis of investigation reports it is empirically usable irrespective of bacterial, fungal or viral infections; ii) it can be used at low sub-toxic dose with a rational synergistic combination of conventional antimicrobial agent; iii) there is low chance of future drug resistance problem; iv) less frequent dosage will require for eradication of infections by novel AgNPs unlike conventional antimicrobial agents; v) it is also effective on dormant persisting cells within biofilms; vi) better physically compatible with another colloidal solution like blood; vii) least chance of killing anaerobic commensal of gut for their reduced state and acidic environment; viii) As end-products are trace amount of reverted Ag+, no adverse environmental impact is expected, if excreted as silver chloride with urine. The possible disadvantages are: i) potential carcinogenicity which is not critically evaluated by long term study; ii) interventions on other drug actions which are not tested; iii) poor information on pharmaco-dynamics and pharmaco-kinetics of novel AgNPs, iv) chance of slight lot to lot variation of action if the drug is not prepared from self-plasma or serum.

Ethics Statement

All animal experiments and sample collection were performed at Chittaranjan National Cancer Institute (CNCI). The protocols were approved by the Institutional Animal Ethics Committee (IAEC) at CNCI and all methods were performed in accordance with the relevant guidelines and regulations.

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Author Contributions:

P.K.M. conceived the project and designed the experiments dealing with synthesis, storage of novel AgNPs and new methods for determining anti-microbial properties. RP carried out experiments dealing with synthesis and microbiological experiments with AgNPs. R.P. and P.G. carried out the animal experiments related to cytotoxicity and antimicrobial activity. R. P. carried out the experiments related to physical characterization of AgNPs. All authors were involved with interpretation of obtained results, preparation and editing the manuscript.

Conflict of interest: Applied for Patent in India by Prasanta Kumar Maiti; Application no. 201831002809, Date of publication 16/02/2018.

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