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Silver nanoparticles: synthesis, effectiveness in treatment of purulent-inflammatory diseases of the maxillofacial area, development of dosage forms

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ABSTRACT

Water dispersion of 30 nm spherical silver nanoparticles (AgNP) has been synthesized and characterized as biosafe according to the parameters of cytotoxicity, genotoxicity, mutagenicity and biochemical markers. AgNP' high bactericidal effectiveness in vitro has been revealed against wide spectra of pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Micrococcus*, *Candida albicans* etc.) isolated from the patients of the Department of Maxillofacial Surgery. High antimicrobial and anti-inflammatory effectiveness in vivo of the AgNP substance has been shown on the model of abscesses of submandibular area in comparison with traditional antiseptic Chlorhexidine. Wistar rats have been used for modeling of abscesses of submandibular area by means of subcutaneous injection in the submandibular area of the suspension containing clinical isolate of *Staphylococcus aureus* and active carbon powder. The composition and technology of different antiseptic dosage forms (gel, ointment and surgical wound irrigation solution) have been developed using water dispersion of AgNP in different concentration.

Key words: silver nanoparticles, biosafety, antimicrobial activity, purulent-inflammatory diseases of the maxillofacial area, dosage forms.

INTRODUCTION

Purulent-inflammatory diseases of the maxillofacial area are complex in treatment and make up more than 30-40% of the total number dental surgical patients. This index is growing by every year due to increasing of widespread of multidrug-resistant pathogen strains, as well as weakening of the immune status and allergization of patients. The same causes are among leading in the case of the emergence and development of complications in dental implantology because of a high risk of surgical wound infection by oral microflora. Purulent-inflammatory processes in the form of abscesses and phlegmons, periostitis, osteomyelitis, suppuration of the bone wounds etc. usually prevail. Among most common pathogens are isolated an aggressive *MDR Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus*, fungi of the genus *Candida albicans* etc. [1-3].

So, search of ways for effective treatment of purulent-inflammatory diseases and complications in the practice of maxillofacial surgery and dental implantology are important today.

Effective solution of this problem is in need of the development and implementation of new alternative antiseptic drugs. Prospective in this way may be silver nanoparticles, because of their well known potent antimicrobial activity against wide spectra of pathogenic microorganisms [4-6].

EXPERIMENTAL SECTION

The aim of present study was synthesis of biosafe silver nanoparticles, estimation of their effectiveness *in vitro* and *in vivo* in treatment of purulent-inflammatory diseases of the maxillofacial area and development of different dosage forms (gel, ointment, surgical wound irrigation solution) based on silver nanoparticles.

Silver nanoparticles have been synthesized by the method of chemical condensation in water medium. Silver nitrate (AgNO_3) (BioXtra, >99% (titration, Sigma-Aldrich), potassium carbonate (K_2CO_3) (99.995% trace metals basis, Sigma-Aldrich), tannin (ACS reagent, Sigma-Aldrich) have been used. The concentration of obtained silver nanoparticles was 8.0 mg/ml by the metal. Size and shape of the nanoparticles have been defined using transmission electron microscopy (TEM) (JEM-1230 «JEOL LTD», Japan) method. The method of energy-dispersive X-ray spectroscopy has been used for chemical composition's X-ray microanalysis of the synthesized nanoparticles (IETEM 250 with detector X-Max 80, Oxford Instruments Analytical, UK for JEM-1230).

The nanoparticles' biosafety level has been estimated *in vitro* using parameters of cytotoxicity, genotoxicity, mutagenicity and biochemical markers according to the Guidelines «Safety assessment of medical nanopreparations» [7].

AgNP antimicrobial effectiveness *in vitro* has been estimated using the clinical isolates of microorganisms isolated from patients who were undergoing treatment in the department of maxillofacial surgery based on Kyiv City Clinical Hospital №12. The microorganisms' specimens were allocated on patients of both sexes, aged from 18 to 70 years old with diagnosis abscesses and phlegmons of various parts of the maxillofacial area.

«Method of serial dilutions in agar» according to Guidelines for Susceptibility Testing of Microorganisms to Antibacterial Agents (4.2.1890-04) has been used for AgNP antimicrobial activity estimation against clinical isolates of the microorganisms [8]. Muller-Hinton agar has been used as determination medium for antimicrobial activity analysis.

Antiseptic solution Chlorhexidine (1 ml containing 0.5 mg of chlorhexidine digluconate) has been used as a comparison drug for *in vitro* and *in vivo* assessing of the AgNP antimicrobial effectiveness.

Chlorhexidine antiseptic solutions belong to the most common traditional bactericidal drugs using in maxillofacial surgery, dentistry, in the treatment of wounds as well as in urology and gynecology [9].

The final concentration of chlorhexidine digluconate in the determination medium under *in vitro* studies was 0.5 mg/ml.

In vivo studies were carried out using 40 certified laboratory *Wistar* rats weighing 250-300 g from vivarium of National Scientific Centre "N.D. Strazhesko Institute of Cardiology" of NAMS of Ukraine (Kiev, Ukraine).

All experiments on animals were carried out according to the accepted ethical norms and standards for the care and use of laboratory animals (Expert Opinion of the Commission of Ethical questions of the O.O. Bohomolets National Medical University, Protocol No. 80 from 03.06.2014).

Modeling of jaw abscess area was carried out according to the standard method by means of subcutaneous injection in the submandibular area of the laboratory animals 0.5 ml of the suspension containing clinical isolate of *Staphylococcus aureus* (5×10^7 CFU/ml) and active carbon powder (9%).

Expansion of the abscess was performed by outside access on the 4th day after infection. The skin incision was performed over the infiltration center. The length of the section was not less than the length of infiltration.

Rinsing of uncovered abscesses by experimental substance of AgNP and comparison antiseptic solution Chlorhexidine was performed 1 time per day during 3 days. The disclosed abscesses were irrigated with spray using 4 ml of each substance per 1 animal.

Experimental animals were divided into 4 groups:

Group 1 - control group - healthy animals;

Group 2 - control group - animals with untreated disclosed abscess;

Group 3 - experimental group – animals in which revealed abscess was irrigated by substance of silver nanoparticles (AgNP) at concentration of 0.8 mg/ml by the metal;

Group 4 - experimental group - animals in which revealed abscess was irrigated by a commercial antiseptic solution Chlorhexidine (1 ml containing 0.5 mg of chlorhexidine digluconate).

Slaughter of animals of the control and experimental groups was performed on the 5 day after last rinsing uncovered abscesses by decapitation using general anesthesia.

For histological analysis of the tissues the parts of mandible areas with lesion have been isolated, fixed in 4% formaldehyde solution, dehydrated and inserted in paraffin according to the conventional protocol. The specimens of histological sections have been stained by hematoxylin-eosin dye.

For development of AgNP different dosage forms methods of information research, literature data analysis and technological methods have been used.

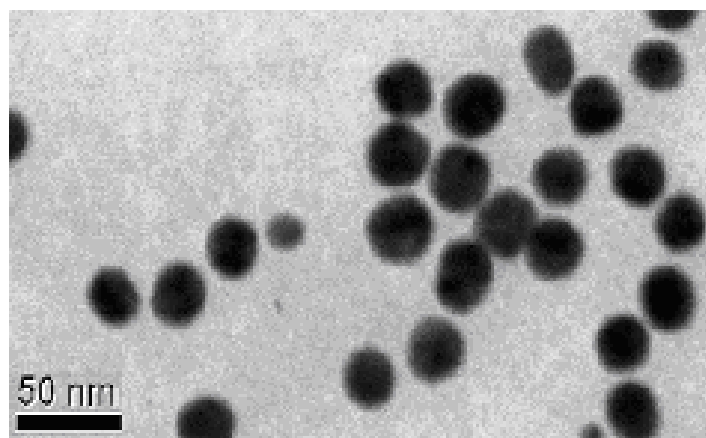
RESULTS AND DISCUSSION

Silver nanoparticles (AgNP) today are most widespread among different nanomaterials which can be used for antiseptic drugs development. Well known antimicrobial properties of AgNP make them perspective components not only for manufacture of new high effective antiseptics, but also for production of cosmetics, antimicrobial sprays, detergents etc. [10, 11].

Application of AgNP in new antimicrobial drugs' manufacture indicates necessity to obtain monodisperse, stable in size, highly concentrated, sterile, biosafe and biocompatible nanoparticles.

Sterile water dispersion of AgNP has been synthesized for estimation of its effectiveness in treatment of purulent-inflammatory diseases of the maxillofacial area. By the method of transmission electron microscopy it has been determined that synthesized nanoparticles have spherical form and average particles' size 30 ± 1 nm (Fig. 1).

Figure 1. Electron-microscopic images of the synthesized silver nanoparticles with average size 30 nm



Chemical composition's X-ray microanalysis of the synthesized silver nanoparticles specified by the method of energy-dispersive X-ray spectroscopy has shown 100% of Ag content in the nanoparticles. The presence of oxygen in the particles' structure has not been fixed.

On the next step AgNP' biosafety level has been estimated *in vitro* using wide spectrum of biosafety parameters according to the protocols and conditions of the Guidelines «Safety assessment of medical nanopreparations» [7]. The data of AgNP biosafety analysis are presented in the Table 1.

Table 1. Biosafety characteristics of the synthesized 30 nm spherical silver nanoparticles

PARAMETERS OF BIOSAFETY	TEST-METHOD	BIOSAFETY CHARACTERISTIC
Cytotoxicity	Crystal violet staining assay	not cytotoxic
Cytotoxicity	MTT assay	not cytotoxic
Genotoxicity	Comet-assay	not genotoxic
Mutagenicity	Allium-test	not mutagenic
Biochemical marker: ATPase activity	ATPase activity test	not toxic
Biochemical marker: LDH-ase activity	LDH-ase activity test	not toxic

Obtained results define the synthesized AgNP as biosafe according to the all analyzed parameters of biosafety estimation.

The estimation *in vitro* of AgNP' antimicrobial activity against spectrum of pathogen microorganisms isolated from the patients with diagnosis abscesses and phlegmons of various parts of maxillofacial area are presented in Table. 2.

Table 2. Antimicrobial activity of AgNP in compare with traditional commonly used antiseptic Chlorhexidin

Clinical isolates	Strains growth at the presence of chlorhexidin digluconate in the determination medium at concentration 0.5 mg/ml	Strains growth at the presence of AgNP in the determination medium at concentration 0.16 mg/ml by the metal	Strains growth at the presence of AgNP in the determination medium at concentration 0.08 mg/ml by the metal	Control of the strain growth
<i>Staphylococcus aureus</i>	+	I	I	++++
<i>Micrococcus</i>	I	I	I	++++
<i>Escherichia coli</i>	+++	I	I	++++
<i>Pseudomonas aeruginosa</i>	+	I	I	++++
Fungi genus <i>Candida albicans</i>	++	I	I	++++
Yeast Fungi	+++	I	I	++++

"I" - complete growth inhibition of the test strain;
 "++++" - intensive growth of the test strain;
 "+" - there is only a single colony growth on the cup.
 "++" - marked increase growth inhibition compared with control;
 "+++ " - weakly growth inhibition compared with control;

Complete growth inhibition of all clinical isolates has been observed under the influence of AgNP at concentrations 0.16 as well as 0.08 mg/ml by the metal.

In compare with high bactericidal action of the AgNP, chlorhexidine digluconate at concentration 0.5 mg/ml in the determination medium demonstrated antimicrobial effectiveness *in vitro* only for *Micrococcus* strain, while the clinical isolates *E. coli*, *S. aureus*, *P. aeruginosa*, *C. albicans* fungi and *Yeast Fungi* had low sensitivity to this antiseptic drug.

AgNP effectiveness *in vivo* in treatment of purulent-inflammatory diseases of the maxillofacial area has been estimated at the experimental model of laboratory rats' jaw purulent abscess.

On the 4th day after injection of suspension containing clinical isolate of *Staphylococcus aureus* and active carbon powder was observed occurrence of inflammatory processes – abscess with expressed soft tissue swelling of the right submandibular area. The experimental animals in comparison with the control healthy group were sluggish, prone to poor appetite. The skin over the existing infiltrate was tense and hyperemic (Fig. 2).

Figure 2. Submandibular abscess formation at laboratory Wistar rats



Expansion of abscess via external access over the center of infiltration and jet rinsing by experimental substance of AgNP and comparison drug - antiseptic solution Chlorhexidine - was performed on the common practice in maxillofacial surgery technique.

The figure 3 demonstrate effectiveness of the treatment of purulent-inflammatory diseases of maxillofacial area using experimental AgNP substance (Fig. 3, A) and commercial antiseptic solution Chlorhexidine (Fig. 3, B).

Figure 3. Uncovered submandibular abscesses on the 5th day after last rinsing using: A –synthesized 30 nm AgNP substance (concentration of water dispersion 0.8 mg (Ag)/ml); B - commercial antiseptic solution Chlorhexidine (concentration of chlorhexidine digluconate water solution 0.5 mg/ml)

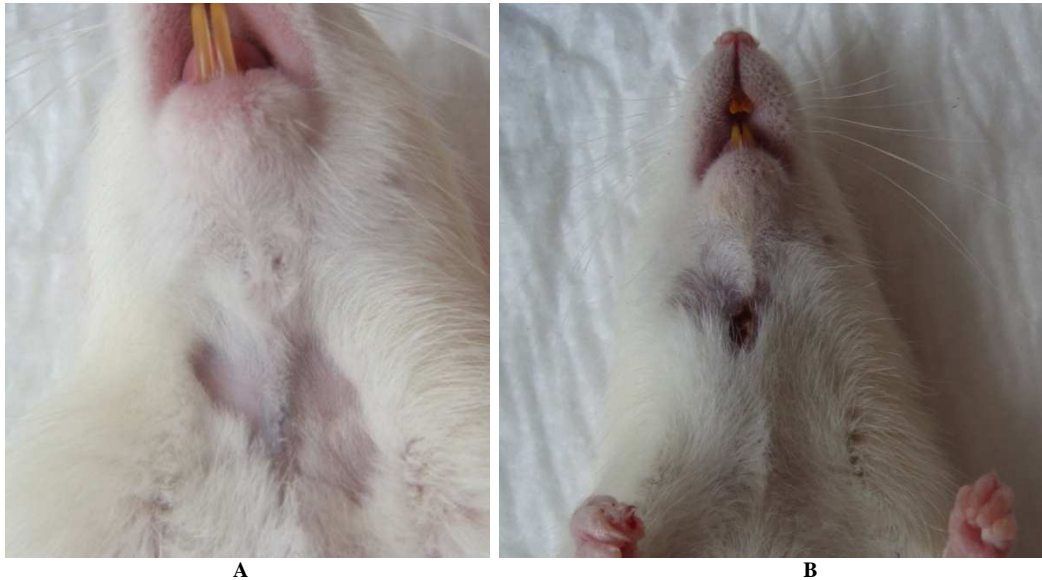
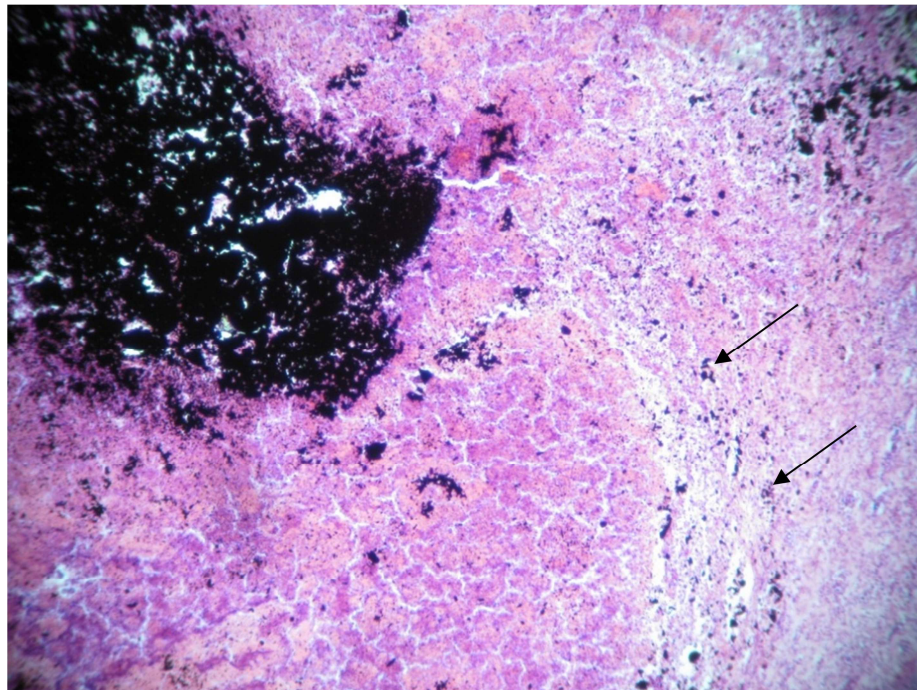


Figure 4. Microphotography of abscess after treatment by Chlorhexidine. Hematoxylin-eosin dye, $\times 200$



Obtained results of *in vivo* studies revealed high effectiveness of the synthesized AgNP in treatment of the experimental abscesses in compare with Chlorhexidine efficacy.

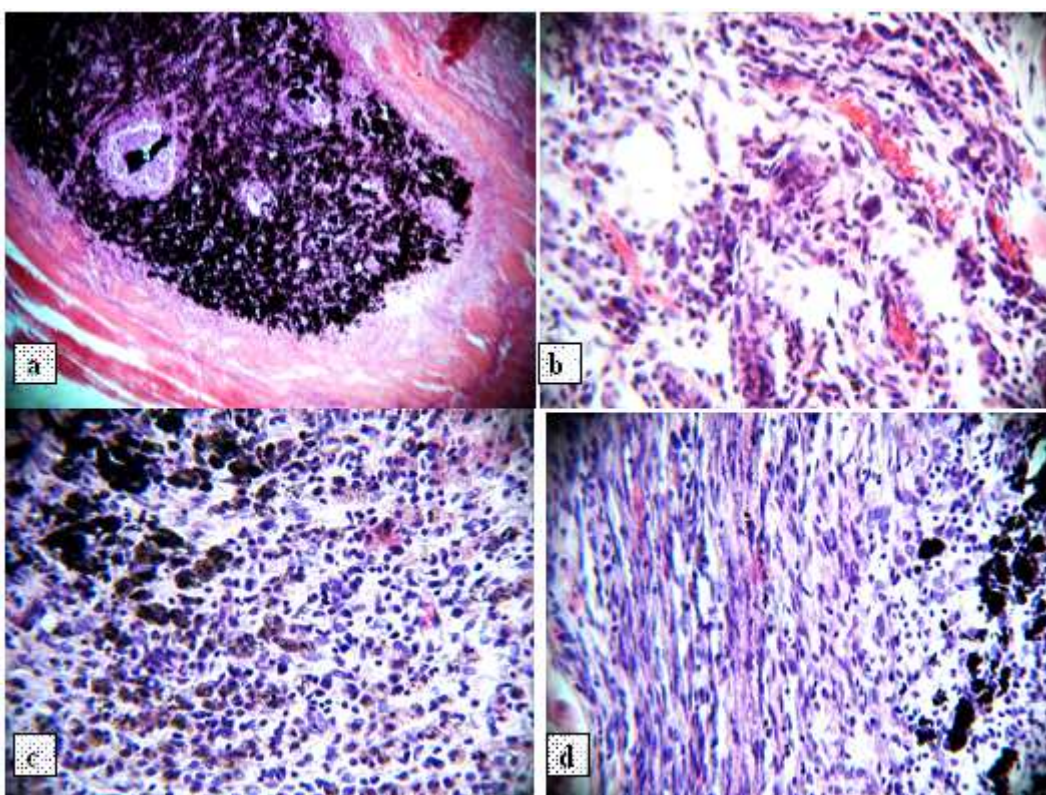
Visual high effective treatment of the experimental abscesses under the influence of the AgNP in compare with Chlorhexidine effectiveness has been confirmed by the data of histological analysis of the tissues' parts of mandible area with lesion.

After treatment of abscess by Chlorhexidine it has been observed the accumulation of foreign bodies inside the space of abscess, which is surrounded by destroyed leucocytes. Only small part of foreign bodies has been located in macrophages (Fig. 4, arrows).

Data of histological analysis indicated, that after treatment of abscess by traditional antiseptic Chlorhexidine the inflammation process was still active.

After treatment of abscesses using AgNP the absence of oedema in the areas adjacent to abscess has been observed. Accumulated foreign bodies have been divided by blood vessels and fibroblasts, which germinated to the central part of abscess (Fig. 5).

Figure 5. Microphotography of abscess after treatment by the synthesized spherical 30 nm AgNP: a - accumulation of foreign bodies have small area; b - blood vessels network in the areas adjacent to abscess; c - foreign bodies in macrophages; d - fibroblasts and collagen fibers in the connective tissue capsule adjacent to abscess; Hematoxylin-eosin dye, a - $\times 200$, b, c, d - $\times 400$



The data reveal high antimicrobial activity, high anti-inflammatory activeness as well as stimulation of regeneration process after treatment of abscess by the synthesized spherical 30 nm AgNP in compare with influence of traditional antiseptic Chlorhexidine.

The obtained results of *in vitro* and *in vivo* studies were the basis for selecting of AgNP water dispersion concentration as an active pharmaceutical ingredient in dosage forms - gel, ointment, surgical wound irrigation solution.

In the composition of the studied irrigation solutions AgNP have been used in concentrations 0,8 and 0,08 mg/ml, in compositions of ointments and gels – 2,0 and 0,2 mg/g, because in semi-solid dosage forms a basis affects on release of active substance and therefore requires usage of higher concentrations of active pharmaceutical ingredient [12]. As a thickener in gels carbopol has been used, ointments has been developed on the emulsion w/o basis, for preparing surgical wound irrigation solution purified water has been used.

Development of AgNP drugs in various dosage forms will expand the range of innovative drugs for the treatment of purulent-inflammatory diseases in the practice of maxillofacial surgery and dental implantology.

CONCLUSION

1. Sterile monodisperse spherical AgNP with average size 30 nm have been synthesized by the method of chemical condensation in water medium.
2. Synthesized AgNP have been characterized as biosafe using parameters of cytotoxicity, genotoxicity, mutagenicity and biochemical markers ATPase and LDHase activities. It indicates the possibility of their potential medical application.
3. It has been revealed AgNP' high antimicrobial activity *in vitro* against spectrum of pathogen microorganisms isolated from the patients with diagnosis abscesses and phlegmons of various parts of maxillofacial area.
4. AgNP high antimicrobial activity, anti-inflammatory activeness and stimulation of regeneration process in compare with treatment by traditional antiseptic Chlorhexidine have been defined *in vivo* on the model of abscesses of Wistar rats' submandibular area.
5. Compositions and technology of different dosage forms: gel, ointment and surgical wound irrigation solution based on AgNP have been developed, which can be effective for treatment of purulent-inflammatory diseases of the maxillofacial area.

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