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Original Research

A novel wound rinsing solution based on nano colloidal silver

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Abstract

Objective(s): The present study aimed to investigate the antiseptic properties of a colloidal nano silver wound rinsing solution to inhibit a wide range of pathogens including bacteria, viruses and fungus present in chronic and acute wounds.
Materials and Methods: The wound rinsing solution named SilvoSept® was prepared using colloidal nano silver suspension. Physicochemical properties, effectiveness against microorganism including *Staphylocoocous aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739 ,*Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404, MRSA , *Mycobacterium spp*. , HSV-1 and H1N1, and biocompatibility tests were carried out according to relevant standards .
Results: X-ray diffraction (XRD) scan was performed on the sample and verify single phase of silver particles in the compound. The size of the silver particles in the solution, measured by dynamic light scattering (DLS) techniqu, ranged 80-90 nm. Transmission electron microscopy (TEM) revealed spherical shape with smooth surface of the silver nanoparticles. SilvoSept® reduced 5 log from the initial count of 10^7 CFU/mL of *Staphylocoocous aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404, MRSA, *Mycobacterium spp*. Further assessments of SilvoSept solution exhibited a significant inhibition on the replication of HSV-1 and H1N1. The biocompatibility studies showed that the solution was non-allergic, non-irritant and noncytotoxic.
Conclusion: Findings of the present study showed that SilvoSept® wound rinsing solution containing nano silver particles is an effective antiseptic solution against a wide spectrum of microorganism. This compound can be a suitable candidate for wound irrigation.

Keywords: Antimicrobial, Nano silver, SilvoSept®

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Introduction

Silver is one of the oldest metals used extensively for its antimicrobial activities throughout history. In recent years with the advent of nanotechnology, various applications of nano silver have emerged and developed. Silver nanoparticles have enormous scientific, technological, and commercial potential due to their unique size and shape dependent properties (1-6).

Among the available metallic nanoparticles, silver and its derivative compounds have been utilized in various nano-based commercial products for their antimicrobial properties. Several studies have suggested that antimicrobial efficiency of nano particle is generally enhanced as its specific surface area increases or its size decreases (7). Variable synthesis methods (8-11) and a wide category of products in this respect has already been available on the market. In medical area, there are wound dressings, contraceptive devices, surgical instruments and bone prostheses all coated or embedded with nano silver particles (12). Several mechanisms have been proposed to explain the inhibitory effects of silver ion/silver metal on bacteria. It is generally believed that heavy metals react with cell membrane proteins by combining with the thiol (-SH) groups, resulting in inactivation of the proteins (13). Recently, different microbiological and chemical assessments have proven that interaction of silver ion with thiol groups plays a pivotal role in bacterial inactivation. Furthermore, it is revealed that bulk silver in an oxygen-charged aqueous media catalyzes the complete destructive oxidation of microorganisms (14). Metallic nanoparticles (Me-NPs), with a high specific surface area and a high fraction of surface atoms, have been studied extensively and demonstrate unique physicochemical characteristics such as catalytic activity, optical and electronic properties, antimicrobial activity, and magnetic properties (14). Taking these into account, it can be expected that the high specific surface area and high fraction of surface atoms of Ag-NPs give it higher antimicrobial activity compared to bulk Ag metal (14).

Clinically, silver has been used mainly in liquid state (silver nitrate) or incorporated in cream (silver sulphadiazine) for the management of burn wounds and the prevention of associated burn sepsis or as nanocrystalline silver dressing for preventing wound adhesion, limiting nosocomial infection, controling bacterial growth and facilitating burnwound care (15).

Continuous increase in resistance to antibiotics in human pathogens leads to the re-emergence of MDR pathogens and parasites. Infections caused by such pathogens require a multiple treatment, containing broad-spectrum antibiotics. Actually, these treatments are less effective, more toxic as well as expensive. Nanotechnology is providing a good platform and noble applications for silver nanoparticles to overcome the drug resistance problems (16).

The present study exhibited that colloidal nano silver wound rinsing solution is capable to inhibit a wide range of pathogens including bacteria, viruses and fungus present in chronic and acute wounds.

Materials and Methods

Preparation of SilvoSept®

SilvoSept® was prepared using colloidal nano silver solution at 20±4 ppm.

Physicochemical properties

Single phase of nano silver particles in SilvoSept® was investigated through X-Ray Diffraction technique. The XRD scans were performed using an XRD instrument (model: X'Pert Pro MPD, company: PANalytical) (model X PERTPRO).

To determine the size distribution profile of nano silver particles in the SilvoSept®, dynamic light scattering nano silver was measured with Malvern Instrument model ZEN 3600, at wavelength 633 nm. Transmission electron microscopy (TEM)

via electron beam interacting with nano silver in SilvoSept® as passes through, was performed using Zeiss instrument model EM 900, accelerating voltage 50, 80 Kv. SilvoSept® Concentration was measured by atomic absorption spectroscopy using Perkin Elmer instrument.

Effectiveness against microorganism

Quantitative suspension test for the bactericidal activity evaluation of SilvoSept® was performed using *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC6538P as test organisms according to EN 1276:2009. Anti-MRSA (Methicillin-Resistant *Staphylocoocus aureus*) activity and anti-*Mycobacterium* spp. of SilvoSept® was performed according to suspension test method as EN1276: 2009. Also, antifungal property of SilvoSept® using *Candida albicans* ATCC10231 and *Aspergillus niger* ATCC16404 was performed. To evaluate SilvoSept® effect on Herpes simplex Virus 1 (HSV-1), Hep2 cell line (epithelial human cells) was used. Two SilvoSept® dilution of 1:10 and 1:20 were prepared. The test was performed simultaneously with the undiluted SilvoSept® and as well as 1:10 and 1:20 dilutions.

Cell culture preparation

After preparation of a complete cell sheet, trypsin was added to the cell culture flask. After cell layer detachment from the flask, cells were diluted with growth medium, that is, MEM with 10% Fetal Calf Serum (FCS). Cell count was adjusted to 100000 cells in 1 mL of medium. 1 mL of the diluted cells was poured into each cell culture tube and the tubes were positioned in cell culture tube rack with a slope of 5 degrees. The tubes were kept in 36°C incubator for 48 hours so that the cell sheet was completed in each tube.

After 48 hours, to prepare cells for HSV-1 inoculation, the growth medium was changed with maintenance medium (MEM with 2% FCS).

Culture and titration of virus

HSV-1 was inoculated to the cells prepared in the previous step. After observation of Herpes virus Cytopathic Effect (CPE), 0.2 mL aliquotes of the propagated virus were prepared in cryovials and kept in -80°C freezer to be used in the next step. One of the cryovials was used for virus titration. The titer was determined based on $TCID_{50}$ unit (Tissue Culture Infectious Dose 50%) and 100 $TCID_{50}$ was used for the next steps of the test.

The evalution of the SilvoSept® effect on HSV-1.

The evalution of the SilvoSept® effect on HSV-1 in cell culture was conducted in 3 conditions. The virus and three dilutions of SilvoSept® (undiluted, diluted 1:10, diluted 1:20) were simultaneously inoculated onto the cell culture. The virus was inoculated onto cell culture, and after two hours, 3 dilutions were inoculated. Also, 3 dilutions were inoculated onto cell culture, and after two hours, the virus was inoculated. Two tubes of virus control (virus+cell, without SilvoSept®) and (SilvoSept®+cell, without virus) were prepared as positive and negative controls, respectively. To assure test precision and accuracy, each step of inoculation was repeated 10 times.

Using an inverted microscope, the inoculated tubes were checked for HSV CPE every day for 5 days. When the virus control tubes showed 4+ CPE, the results of the other tubes were reported.

The interaction of SilvoSept® with H1N1 influenza A virus was investigated. It was prepared for the Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay, where this test was used to determine the inhibitory activity of SilvoSept® on H1N1 influenza A virus. MDCK cells were used as the infection model. Electron microscopy analysis and flow cytometry assay were used to determine whether SilvoSept® could

reduce H1N1 influenza A virus-induced apoptosis in MDCK cells.

Biocompatibility tests

Irritation and delayed-type hypersensitivity test Irritation and delayed-type hypersensitivity test was carried out on SilvoSept® according to ISO10993-10 using 4 white male New Zealander rabbits which were 2154-2262 g at the beginning of the test. Approximately 24 hours before test, the fur was removed from an area about 240 cm^2 wide by clipping and shaving the dorsal and flank zones of the animals. An area of the back, about 6 cm^2 wide, was designated for the application of the test sample. 25×25 mm of SilvoSept® was applied directly to the skin on cranial site of each rabbit. The application sites were covered with non-occlusive dressing and wrapped with a semi-occlusive bandage. The patches were removed 4 hours after the application and for repeated skin irritation test. Observations for skin sensitivity and irritation test about general conditions of the animals were verified daily. Reactions were evaluated following patch removal and were evaluated again at 24, 48, 72 hours after exposure. Skin irritation was scored and recorded according to the table 1.

Table 1. Grading values for skin irritation scores.

Erythema and scar formation	**Grade**
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness with slight scar formation; injuries in depth)	4
Oedema formation	**Grade**
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

In vitro cytotoxicity test

In vitro cytotoxicity according to ISO10993-5 was performed on SilvoSept®. The test was performed using Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids (NEAAs), HEPES, and Hank's balanced salt solution (HBSS). Antibiotics and L-glutamine were obtained from Gibco Invitrogen (Life Technologies, Paisley, UK). Filter inserts was provided from Nunc (Denmark). Cell Proliferation Reagent MTT solution was purchased from Roche Diagnostics (Roche, Germany). Standard compound methotrxate were purchased from Sigma–Aldrich. Methotrexate stock solutions were prepared in dimethyl sulfoxide (DMSO).

SilvoSept® was appropriately soaked in DMSO or put in filter insert in order to expose its different concentrations to the cells. Cell cultures include Caco-2, T47D, HT29 and NIH-3T3 cell line were prepared from Pasteur Institute (Tehran, Iran). The cells were grown in either DMEM containing 4.5 g/l glucose or RPMI 1640 and supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells growing as a monolayer were kept at 37°C in a humidified incubator in air containing 5% CO_2. For MTT assay, cells were seeded at 0.8-1 × 104 cells/well density in 24-well microplates.

After 72 h, SilvoSept® cytotoxicity test was performed in the concentration range of 635 to 10160 µg/ml of SilvoSept®, using four cell lines: T47D ER+ (breast carcinoma), Caco2 (colon carcinoma), HT29 (colon carcinoma) and NIH-3T3 (normal cell line fibroblast). Control cultures were maintained in DMEM or RPMI 1640 similar to the treated cultures.

Cytotoxic effects on the growth and viability of cells were determined using tetrazolium dye (MTT; 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide) assay as described by Mossman (1983). 3 × 103 cells/ml were plated in 96-microwell plates. MTT solution was prepared at 5 mg/ml in PBS, filter sterilized and stored in the dark at

4°C for a maximum of 1 month. MTT reagent (20 µl) was added to each 100 µl of culture. After incubation for 3 h at 37°C the formed water insoluble formazan dye was solubilized by addition of 100 µl acidified isopropanol to the culture wells. The plates were further incubated for 20 min at room temperature, and optical densities (OD) of the wells were determined using an Anthos 2020 (Salzburg, Austria) ELISA microplate reader at a test wavelength of 570 nm and a reference wavelength of 690 nm. Each plate contained 'blank' background control wells containing an appropriate volume of media but no cells. All experiments were performed at least three times, with three wells for each concentration of SilvoSept®. The control cells were grown under the same conditions without compound addition. Cell survival (% of control) was calculated relative to untreated control cells.

Statistical analysis

Statistical analyses were performed with Student's paired t-test. ANOVA test with Hoc post-test were used between groups. P values <0.05 were considered to be significant. The values presented are means ± SD.

Results

Physicochemical properties

The structure of nano silver particles in SilvoSept® was investigated using X-ray diffraction (XRD) analysis. Typical XRD patterns of the sample, are shown in the Fig. 1.

Table 2 shows the experimentally obtained X-ray diffraction angle and the standard diffraction angle (17) of Ag specimen.

The size of the nano silver in this solution, measured using Dynamic light scattering (DLS) were 80-90 nm (Fig. 2).

A TEM scan of SilvoSept® is presented in the Fig.3. The silver nano particles are spherical in shape with a smooth surface morphology. Atomic absorption measured SilvoSept® concentration at 21 ppm.

Table 2. Experimental and standard diffraction angles of Ag specimen.

Experimental diffraction angle (2θ in degrees)	Standardl diffraction angle (2θ in degrees)
42	44.3

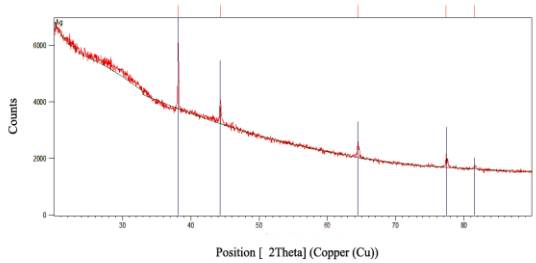

Figure 1. X-ray diffraction pattern of Ag nano particles.

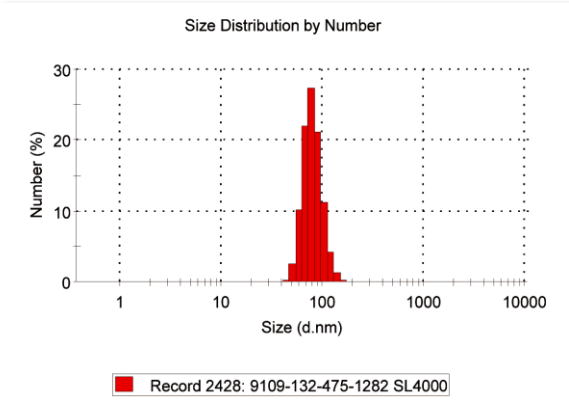

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Figure 2. Size distribution of nanosilver particles in SilvoSept®.

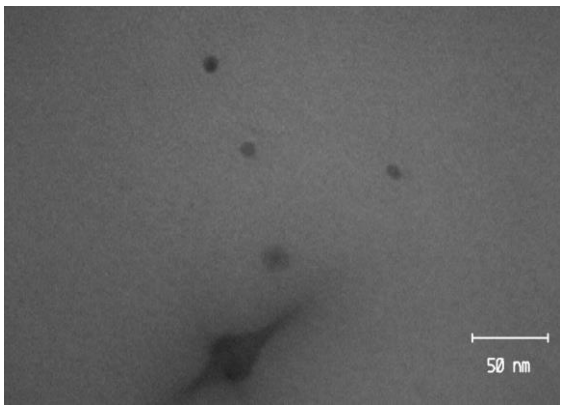

Figure 3. A TEM image of SilvoSept®.

Effectiveness against microorganism

Antibacterial and antifungal activity of SilvoSept® is summerised in Table 3. The results of anti-MRSA and Mycobacterium spp. show 5 log reduction from the initial count of 10^7 CFU/mL at 2 h (Table 4).

From the results presented in table 5 it can be deduced that SilvoSept® can inhibit the replication of HSV-1 in all inoculation conditions(inoculation imultaneously/ SilvoSept® two h after virus/virus two h after SilvoSept®) even when they are 1:20 diluted, therefore SilvoSept® can inactivate HSV-1 turbulance.

This study demonstrates that SilvoSept® have anti-H1N1 influenza A virus activities according to table 6.

Table 3. Antibacterial and antifungal activity of SilvoSept®.

Test	Result
Staphylococcus aureus ATCC 6538P	5 log reduction from the initial count of 10^7 CFU/mL at 1 min
Pseudomonas aeruginosa ATCC 9027	
Escherichia coli ATCC 8739	
Candida albicans ATCC 10231	
Aspergillus niger ATCC 16404	4 log reduction from the initial count of 10^6 CFU/mL at 5 min

Biocompatibility tests

On the basis of the results of skin irritation and sensitization test, SilvoSept® did not cause any irritant effect on skin. Tables 7 summarizes the results for each rabbit.

Table 4. Anti-MRSA and Mycobacterium spp of SilvoSept®.

Test	Result
Anti-MRSA activity: Methicillin-resistant	5 log reduction from the initial count of 10^7 CFU/mL at 2 hours
aphylococcus aureus 1 Methicillin-resistant	
aphylococcus aureus 2 Methicillin-resistant	
aphylococcus aureus 3	
Mycobacterium spp.	

Figures 4 to 7 show SilvoSept® effect on the viability of these cell lines. As it is shown in the figures, SilvoSept® does not show significant cytotoxicity comparing control and other groups.

The experiments for cytotoxicity determination of SilvoSept® were performed on four cell lines HT29 (colon carcinoma with fast proliferation), Caco2 (colon carcinoma with slow proliferation but able to be resistant to several compounds), T47D (estrogen dependent breast carcinoma) and non- human and non-carcinoma cell line (NIH-3T3 mouse fibroblast). Maximum 1% of total medium in 24 well dishes (10 µl) were used as blank. Because of limitation of SilvoSept® treatment the mentioned concentration is the maximum concentration which can be tested and is below the real concentration that may be exposed to the body.

Selection of cell lines is based on their features.

Table 5. SilvoSept® can inhibit the replication of HSV-1.

Number of tests / Cell culture tube contained	1	2	3	4	5	6	7	8	9	10
Virus control (virus+cell, without SilvoSept®)	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Negative Control 1: SilvoSept® undiluted+cell	T	T	T	T	T	T	T	T	T	T
Negative Control 2: SilvoSept® 1:10+cell	T	T	T	T	T	T	T	T	T	T
Negative Control 3: SilvoSept® 1:20+cell	-	-	-	-	-	-	-	-	-	-
Simultaneously:										
SilvoSept® undiluted+100$TCID_{50}$HSV-1	T	T	T	T	T	T	T	T	T	T
SilvoSept® 1:10+100$TCID_{50}$HSV-1	T	T	T	T	T	T	T	T	T	T
SilvoSept® 1:20+100$TCID_{50}$HSV-1	1+	-	-	-	1+	-	-	-	-	1+
First virus, 2 hours later SilvoSept®										
SilvoSept® undiluted+100$TCID_{50}$HSV-1	T	T	T	T	T	T	T	T	T	T
SilvoSept® 1:10+100$TCID_{50}$HSV-1	T	T	T	T	T	T	T	T	T	T
SilvoSept® 1:20+100$TCID_{50}$HSV-1	-	-	-	-	-	-	-	-	-	-

Turbidity; +: Positive, HSV-1 replication; -: Negative, inhibition of HSV-1 replication

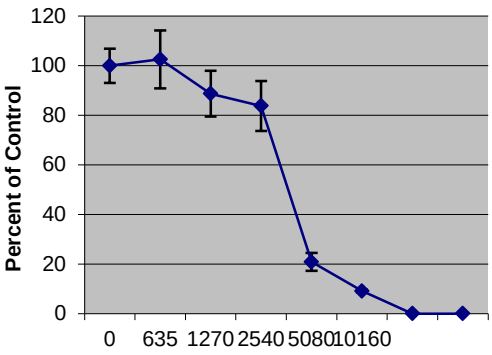

Figure 4. The effect of SilvoSept on the proliferation of 3T3 cells (mean±SD, n=12).

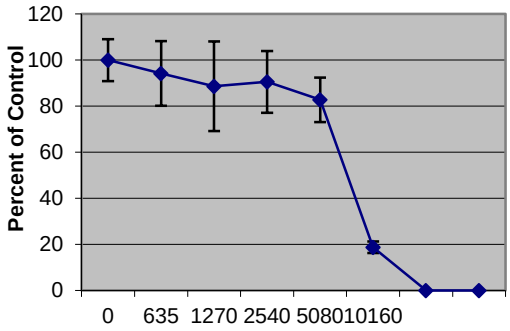

Figure 5. The effect of SilvoSept on the proliferation of Caco2 cells (mean±SD, n=12).

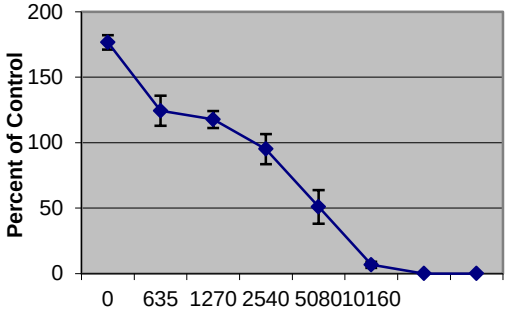

Figure 6. The effect of SilvoSept on the proliferation of HT29 cells (mean±SD, n=12).

Caco2 cells carries several features of columnar epithelium and HT29 shows the same feature but with high proliferation rate make the cells susceptible to the several cytotoxic materials with effects on cell cycle procedure.

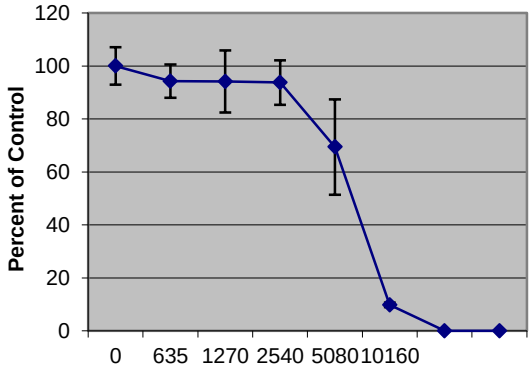

Figure 7. The effect of SilvoSept on the proliferation of T47D cells (mean±SD, n=12).

T47D breast carcinoma cells represent hormone dependent cells which may be affected by cytotoxic materials
which show hormonal effect.
The last cell line is normal cell line with primary cell features.
3T3 may represent the primary cells and results obtained with this cell line reported in several studies are different from cancer cell lines.

Discussion

The XRD assessments of the nano silver particles in SilvoSept® show a single sharp peak indicating nano structure and single phase of the silver. These particles are dispersed uniformely in solution that causes a sharp peak.

Table 6. Anti-H1N1 influenza A virus activities of SilvoSept®.

Concentration of SilvoSept® added to viral suspension (ppm)	Optical absorption mean± SD
4	0.003±0.001
2	0.084±0.005
1	0.280±0.014
0.5	0.543±0.018
0.25	0.547±0.002
0.125	0.553±0.002
0.06	0.565±0.002
0.03	0.565±0.002
0	0.565±0.002

Table 7. Skin irritation test results.

Animal number	Observation +4 hrs	Observation +24 hrs	Observation +48 hrs	Observation +72 hrs	Negative control	Positive control
1	-	Er- Ed-	Er- Ed-	Er- Ed-	Er- Ed-	Er++++ Ed++++
2	-	Er- Ed-	Er- Ed-	Er- Ed-	Er- Ed-	Er++++ Ed++++
3	-	Er- Ed-	Er- Ed-	Er- Ed-	Er- Ed-	Er++++ Ed++++
4	-	Er- Ed-	Er- Ed-	Er- Ed-	Er- Ed-	Er++++ Ed++++

Er= erythema Ed=Edema

The distinct particles in image revealed their stability in the solution. In addition, the TEM scans show the relative high level of uniformity in the size and shape of the silver nano particles.

Antimicrobial properties of SilvoSept® revealed its effectiveness on common pathogens which can be found in all types of wound.

This study demonstrated that silver nanoparticles have anti-H1N1 influenza A and HSV-1 virus activities. The inhibitory effects of silver nanoparticles on influenza A and HSV-1 virus may be a novel clinical strategy for the prevention of these viruses infection during the early dissemination stage of the virus.

Skin irritation test confirmed that SilvoSept® can be used on wounds without any side effects.

the cytotoxicity tests demonstrated that the products has no cell toxicity.

In conclusion, considering the non-irritant, non-cytotoxic properties of SilvoSept® as well as high antibacterial efficiency on a wide spectrum of microorganism, it can be used as a novel antiseptic agent on a various wounds. There are several ways for synthesis of nano silver and hypothesis for antibacterial mechanism. Three types of antimicrobial mechanisms include: 1) In Gram negative bacteria Plasmolysis can be occurred due to nano silver particles reaction with bacterial cell wall. After this reaction, cytoplasm of bacteria separated from bacterial cell wall. 2) nano silver particles inhibit the synthesis of bacterial cell wall. 3) Nanosilver particles may induce metabolic disturbance due to the affinity of silver for disulfide bond in bacterial enzymes (18-20).

Conclusion

Findings of the present study showed that SilvoSept® wound rinsing solution containing nano silver particles is an effective antiseptic solution against a wide spectrum of microorganism. This compound can be a suitable candidate for wound irrigation.

Acknowledgments

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