

RESEARCH ARTICLE

Investigation of acute dermal irritation/corrosion, acute inhalation toxicity and cytotoxicity tests for Nanobiocide®

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ABSTRACT

Objective(s): Nanomaterials, especially silver Nanoparticles (Ag-NPs), are employed in an increasing number of commercial products. This has led to an ever growing exposure of human beings to this substance. The first purpose of the Nano Committee of Food and Drug Administration of The Islamic Republic of Iran (IFDA) is developing guidelines to assess and approve commercial nano-health products for their safety of human applications. Nanobiocide® as a commercial product of stable colloid including 2000 ppm Ag-NPs for surface antimicrobial applications was investigated according to IFDA guidelines in the approval process.

Methods: The first fabrication and characterization method of the product were determined. The human exposure to Nanobiocide® were studied by cytotoxicity assay, dermal irritation and inhalation toxicity assay based on the standard assay.

Results: According to cytotoxicity assay by MTT method the concentration-dependent of cell viability was reduced and Inhibitory concentration-50 was about 1160 ppm. The Draize dermal irritation scoring system (DDIS) showed no irritation to the skin of rabbits. No sign of gross toxicity, adverse pharmacological effect, or abnormal behavior based on inhalation toxicity was observed.

Conclusion: The consideration of toxicity of Nanobiocide® is one of the major key for medical application. The results obtained revealed that the Nanobiocide® may be safe using in domestic and veterinary applications.

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INTRODUCTION

The rapid fabrication of nanotechnology has led to a great rise in the number of products containing nano-sized materials. However, the novel physicochemical properties of materials on the nanoscale confirm the call for appropriate assessment of the possible effects

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to human health. The investigation of the reasonable toxicity of nanoparticles (NPs) plays a significant role for particles having at least two dimensions below 100 nm. Although proof recommends that NPs could have adverse effects on human health [1], the elemental cause/effect relationships are

not even fully realized. In particular, the behavior of nanoparticles inside the cells is still a riddle, and no metabolic and immunological responses induced by these particles are understood so far. Among the nanoparticles, silver nanoparticles (Ag-NPs) have been extensively considered as an antimicrobial agent recently. Silver-impregnated catheters and wound dressings are employed in therapeutic applications. In spite of the wide usage of Ag-NPs in wound dressings, which can cause easy entry into the cells, very few reports on the toxicity of silver nanoparticles are available. For measurement of toxicity of silver nanoparticles, the standard tests were employed. The standard tests including the acute inhalation toxicity, the acute dermal irritation/corrosion and cytotoxicity tests are considered in most cases. For instance, Inhalation toxicity of Ag-NPs was examined by Ji *et al.*, [2]., In the case, they found no major changes in the hematologic and blood biochemical values. No gender specific or histopathologic changes were identified in the 28-day study in rat model. To further assess these findings, a sub chronic 90-day inhalation study was carried out [3]. The investigation of this study showed that the lungs and liver were major target tissues. Lung effects included decreased tidal and minute volume and inflammatory reactions (i.e., mixed inflammatory cell infiltrate and chronic alveolar inflammation). Another study shows that, the genotoxicity, acute oral and dermal toxicity, eye irritation, dermal irritation and corrosion and the skin reaction of commercially manufactured Ag-NPs in relation to the OECD (The Organization for Economic Cooperation and Development) test guidelines and GLP (Good Laboratory Practice). Although, some cytotoxicity was observed, the Ag-NPs were not set up to induce genotoxicity in a bacterial reverse mutation test and chromosomal aberration test. In this work, no abnormal sign or mortality was observed at a dose level of ~ 2000 mg/kg in acute oral and dermal toxicity tests using rat model. Likewise, acute eye and dermal irritation and corrosion tests by means of rabbit model discovered no important clinical sign or mortality and no acute irritation or corrosion reaction for the eyes and skin. In the skin reaction test using guinea pig, one animal (1/20) illustrated distinct or patchy erythema, so Ag-NPs can be classified as a weak skin sensitizer [4].

Surveys have proven that the lungs and liver are major target tissues for prolonged Ag-NPs exposure [4, 5]. The considerable depletion of the

antioxidant glutathione, lessened mitochondrial membrane potential and increased ROS (reactive oxygen species) of silver nanoparticles was studied by Hussain *et al.*, [6]. In this study, Ag-NPs cytotoxicity is likely mediated through oxidative stress in liver cells. The non-cytotoxic doses (b 0.5 µg/ml) of Ag-NPs induced the expression of genes related to cell cycle progression and apoptosis in human hepatoma cells (HepG2) was confirmed by Kawata *et al.*, [7].

The purpose of this study is to investigate the acute dermal irritation/corrosion, acute inhalation toxicity and cytotoxicity tests for the complex "Nanobiocide®" in vivo experiments. To make clear that the health hazards associated with silver nanoparticles (Ag-NPs), we investigated the genotoxicity, acute oral and dermal toxicity, eye irritation, dermal irritation and erosion and the skin reaction of registered "Nanobiocide®" according to the standard test guidelines and GLP.

MATERIALS AND METHOD

Preparation and characterization of Nanobiocide®

There are a range of physical and chemical method significantly used for the synthesis of silver nanoparticles, such as reduction in solution, [8] chemical and photochemical reactions in reverse micelles, [9] thermal decomposition of metal compounds, [10] radiation assisted, [11] electrochemical, [12] sonochemical, [13] microwave assisted process [14].

Most frequently preparation of silver nanoparticles is performed by chemical reduction method. Nanobiocide®2000 was fabricated based on a chemical method (Fabricated by NanoPoshesh Felez and was registered for confirmation). The starting point of the synthesis is the production of a silver acetate ($\text{AgC}_2\text{H}_3\text{O}_2$) solution. The preparation of silver nanoparticles in briefly, 3.1 gr silver acetate was added into 1000ml deionized water. The mixture was stirred while 7.5 gr sodium borohydride was added drop by drop wisely into the solution until the color's solution turned to light brown. The capping agent including triethanolamine lauryl ether sulfate (TLES), Cocoamidopropyl betaine (CAPB) and guar hydroxypropyltrimonium chloride (GHPTC) were employed to make the nanoparticle stable in the medium. All reagents were purchased from Merck and are analytical grade. The Nanobiocide®2000 was characterized, and the main characteristics are summarized in Table 1.

Table 1. Ag-NPs characterization

Condition	Method	Result
Nanobiocide [®] 2000	X-ray diffraction	Approximately 10 nm (Scherrer Equation)
Solution	TEM	Approximately 10 nm Spherical (Fig. 1) 46 nm
Solution	DLS	PdI= 0.403 Zeta Potential= -38.6 mV
Solution	ICP	Ag= 2048 ppm

Cytotoxicity assay

In fresh media, The cells were resuspended and were treated with 0.5 mg/ml solution of 3-(4,5-dimethylthiazolyI-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich. St. Louis, MO, USA: 100 µl/well) at 37°C for 3 h. The cell viability was determined by the Trypan blue exclusion method and was found at about 95%. Lymphocytes were incubated for 5 days at 37°C in RPMI (Roswell Park Memorial Institute) media with various concentrations of Nanobiocide[®] (25, 50, 100, 150 and 200 µg/ml, equivalent to 250, 500, 1000, 1500 and 2000 ppm). The lymphocytes were gained by centrifugation. Human blood was obtained from 3 healthy male volunteers between 20 and 30 years of age (non-smokers and not under any medications), after their approval.

MTT assay was carried out to assess the effect on mitochondrial succinate dehydrogenase activity. Cells with treatment concentrations of Nanobiocide[®] (0, 25, 50, 100, 150 and 200 µg/ml) were seeded onto 96-well culture plates (at 1×10^5 cells per well). At the end of exposure, cell culture medium was removed, and the cells washed with PBS twice to eliminate excess nanoparticles and media. The number of viable cells was examined by uptake of MTT. Optical density (OD) was read at 570 nm. The interference of nanoparticle in the assays was discarded by maintaining sample blanks for all the concentrations tested. The obtained OD values from the sample blanks were decreased while compared to the OD values after the assessment. The achieved values after measurement have been submitted as a percentage compared to control values. All experiments were performed at least in triplicate on three divide occasions.

Dermal irritation assay

Six males, white albino rabbits, in the range of 2-3 kg body weight were employed in the present study. The animals utilized were nulliparous and non-pregnant. They were kept individually in

the standard stainless steel cages and sterilized paddy husk as bedding. The bedding material was changed daily and water bottles were changed on alternate days as well. The rabbits were acclimated for a minimum period of five days in the controlled environment (temperature: 20 ± 3 °C; relative humidity: $50 \pm 20\%$ and light: 12 h light/dark cycle) and ad libitum water and standard rabbit pellet food. These animals were distributed randomly into two groups: Normal control (0.5 ml of vehicle) and test-dose group (0.5 ml of target dose, 2000 ppm). The fuzz was removed by closely clipping to an area of about 6 cm² on both sides of the dorsal surface of the trunk of the animals, about 24 h before the analysis. Care was taken to prevent scraping the skin, and only healthy animals with intact skin were employed. The materials were applied to the clipped skin area on the left side of animals and the areas were covered with a gauze patch that was held in place with non-irritating tape. The right untreated side was reserved as a control area. The patch was loosely held in contact with the skin by means of an appropriate semi-occlusive dressing. Restrainer (neck collar) was used to avoid the ingestion of the test substance from the application site. The initial test was performed by one animal. The residual substance was washed.

The skin reaction was subjectively examined and scored at the first hour patch removal and continued at 1, 2, 3, 7 and 14 days. The Guideline 404 of OECD (Acute Dermal Irritation/Corrosion) was utilized for above test.

Inhalation toxicity assay

Nine-week-old male and female Sprague-Dawley rats were purchased and acclimated for 1 week before commencing the experiments. The rats were housed in five mesh cages (five rats per cage each was placed in an isolated chamber) at room temperature (23 ± 2 °C) and humidity ($55\% \pm 7\%$) with a 12-hour light/dark cycle during the acclimation and experimental time. The rats were

Table 2. Draize dermal irritation scoring system (DDISS)

Erythema and Eschar Formation	Value	Edema Formation	Value
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight edema (barely perceptible)	1
Well-defined erythema	2	Slight edema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate edema (raised approximately 1 mm)	3
Severe erythema (beet- redness) to slight, eschar formation (injuries in depth)	4	Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

fed by filtering water ad libitum. The 10-week-old rats, weighing approximately 320g for the males and 225g for the females, were then separated into two groups (five rats in each group/sex): fresh-air control, test-dose group (target dose, 2000 ppm). The animals were exposed to silver nanoparticles for 4 hours and then observed for 2 weeks following the OECD test guideline 403 for acute inhalation toxicity. Afterward, the rats were daily analyzed for any evidence of exposure-related effects, including respiratory, dermal, behavioral, nasal, or genitourinary changes suggestive of irritation. The rats were not fed during the exposure time (4 h). The body weights were evaluated at 5 steps: beginning of study, animal grouping, 7 and 14 days, after the 4 h inhalation exposure and before necropsy (Table 4-6).

RESULTS AND DISCUSSION

The MTT assay was employed for measuring cytotoxicity of the mitochondria dehydrogenase. The measurement shows that the concentration-dependent of cell viability was reduced (above 25 and 50 µg/ml was significantly considered). In

Table 3. Evaluation of primary skin irritation index

Evaluation	Score
Non Irritant	0.0
Negligible Irritant	0.1-0.4
Slight Irritant	0.41-1.9
Moderate Irritant	2.0-4.9
Severe Irritant	5.0-8.0

the present paper, the cytotoxicity was obvious in MTT and the results obtained revealed that the half maximal Inhibitory concentration-50 (IC50) was about 1160 ppm. The results obtained from MTT assay were shown as mean ± SD in Fig. 2.

The DDISS was shown in Table 2. The sample of Nanobiocide®2000 was found to be “nonirritant” (Evaluation of primary skin irritation index was shown in Table 3) to the skin of rabbits while compared with the normal control sample. Moreover, there were no signs of gross toxicity, adverse pharmacological effect, or abnormal behavior. No gross abnormalities were

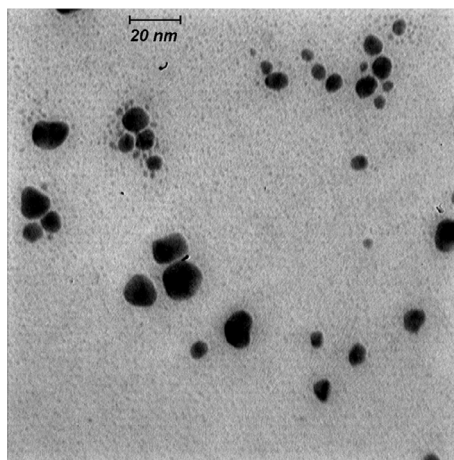


Fig 1. TEM image of Nanobiocide®

Table 4. Individual body weights of fresh air control group

Animal No.	Sex	Body weight		
		Initial	Day 7	Day 14
1	M	317	357	401
2	M	329	366	392
3	M	310	349	398
4	M	326	364	411
5	M	308	349	395
6	F	234	238	269
7	F	232	239	277
8	F	240	261	285
9	F	219	234	280
10	F	215	244	269

Table 5. Individual body weights of test group

Animal No.	Sex	Body weight		
		Initial	Day 7	Day 14
11	M	321	356	392
12	M	326	361	410
13	M	330	369	415
14	M	317	354	396
15	M	313	353	401
16	F	220	245	272
17	F	208	228	261
18	F	218	237	269
19	F	238	241	275
20	F	240	272	291

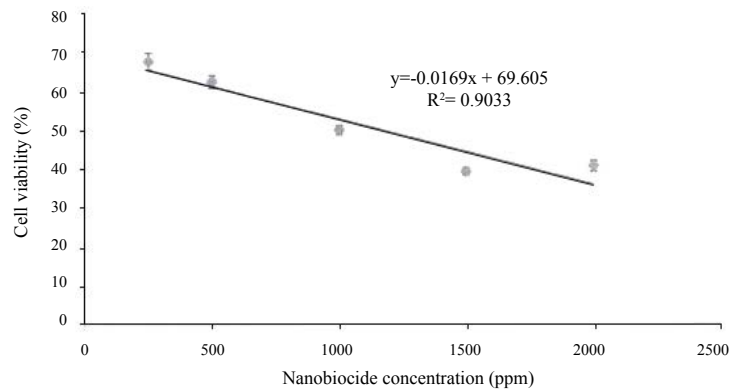


Fig 2. MTT cell viability assay, cytotoxicity induced in Nanobiocide® treated human lymphocyte cells

observed for any of the animals when necropsied at the conclusion of 14-day observation period. The histological image of the test group dermis, which shows normal epidermal cells, organized bundles of collagen and serous goblet cells (Fig. 3).

The animals survived inhalation exposure to the tested compound and gained weight through the observation period. In the maximum dose (2000 ppm Nanobiocide®), the animals showed active and healthy reaction over the whole 14-day

observation phase. No signs of gross toxicity, adverse pharmacological effect, or abnormal behavior were observed. No gross abnormalities were noted for any of the animals when necropsied at the end of 14-day period.

CONCLUSION

Ag-NPs have found as a fundamental class of nanomaterials for a large range of industrial and medical applications such as cosmetics [15],

Table 6. Individual cage-side observations

Animal No.	Findings	Day of occurrence
Males 1-5	Active and healthy	CR-14 ¹
Females 5-10	Active and healthy	CR-14 ¹
males 11-15	Active and healthy	CR-14 ¹
Females 16-20	Active and healthy	CR-14 ¹

¹CR-removal from the exposure tube

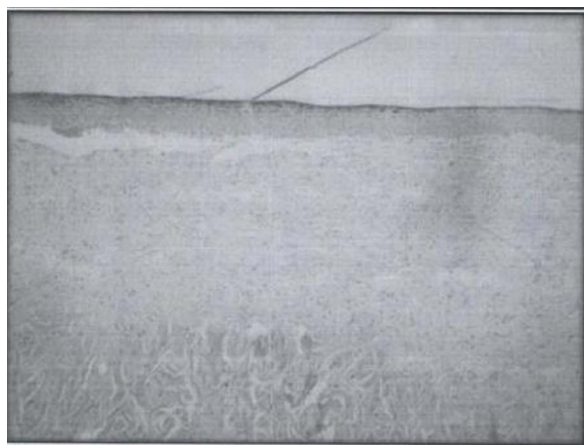


Fig 3. Histological image of test group dermis which shows normal epidermal cells, organized bundles of collagen and serous goblet cells

drug delivery systems [16], therapeutics [17] and biosensors [18]. Nanobiocide®, the stable colloid including 2000 ppm Ag-NPs was fabricated to employ as a surface antimicrobial agent using in domestic and veterinary applications. Understanding of toxicity of Ag-NPs is one of the significant key for human health. The results of this work explain non-toxicity of the interaction of Ag-NP in In-vivo and In-vitro systems. To support this conclusion, chronic and sub-chronic inhalation and dermal toxicity are suggested to carry out.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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