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Investigation of Systemic Toxic Effects of Nanobiosilver Use in Rodent Models

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Abstract
This study aims to investigate the toxicity of biosilver particles on the rodent model. The study used 10 Guinea pigs for the sensitization test (Guinea pig maximization test) and 80 8-to-12-week-old BALB-C mice for systemic toxicity researches. The mice used in the study were divided into 6 groups: acute, subacute, subchronic experimental groups and the control groups thereof. The experimental animals were given NanoBioSilver intraperitoneal doses of 50 ml/kg, including a single dose for the acute systemic toxicity test and 7 repetitive doses for NanoBioSilver, subacute and subchronic toxicity tests. At the end of the study, liver tissues were sampled from animals, which were then examined histopathologically. Throughout the study, no significant changes were observed in clinical findings of the groups. Also, no significant changes were found in vital tissues of the study groups. In conclusion, biosilver particles were found to have no acute, subacute and subchronic toxic effects on the rodent model.

Keywords: Acute, biosilver, subacute, subchronic, toxicity.

INTRODUCTION

The concept of technology, which is as old as the history of humanity itself, continues to deeply affect the societies through its gradual development. The technology has been developing very rapidly especially in recent times, which makes it difficult to follow those developments on an individual and social basis. Against the backdrop of these developments, Nanoscience and Nanotechnology (N&N) are now regarded as an important technological field that will mark the 21st century (Özer, 2008).

Nanoscience is a discipline which examines the unique behaviors and properties of the materials ranging from 1 to 100 nanometers (one billionth of a meter) in size (Sarsar et al. 2014).

Nanomaterials are designed with a view to make use of the unique properties of the nanoscale. Typically, nanoparticles have a greater chemical reaction, biological activation and catalytic property compared to particles with the same chemical structure but a larger surface area. Yet, even though it is desired to make use of those new properties, changing the size of the materials on this scale may bring about toxicological risks (Garnett and Kallinteri, 2006; Limbach et al. 2007; Nel et al. 2006; Bergin and Witzmann, 2013).

Nanotechnology has achieved tremendous progress in determining the potential negative effects of the biological effects of nanomaterials. Though, an aspect which has not yet been satisfactorily explored so far is physicochemical properties of nanomaterials are changed by physiological environment. This raises new confusion about the solid phase nanomaterials (Braydich-Stolle et al. 2014).

The viability tests, morphological observation and oxidative stress-generating capacity provide indications about the toxicity-generating mechanism of nanomaterials (Schrand et al. 2012). This study aims to investigate the systemic toxicity of biosilver particles on the rodent model.

MATERIALS AND METHODS

Experimental Animals and Grouping

Ethical approval for this study was obtained from the Local Ethics Committee of Kırıkkale University (Decision No: 2018-52). In systemic toxicity tests, 80 8-to-12-week-old mice were used. The experimental animals were grouped as follows: 5 female & 5 male mice in acute toxicity test group, 10 female & 10 male mice in each of the subacute and subchronic toxicity test groups. The mice used in the experiment were selected randomly, marked individually and encaged 5 days before the application.

As experimental animal, the sensitization experiments, however, employed five male and five male albino guinea pigs weighing 300 g to 500 g and dermatologically free of any disease.

The control groups were composed of BALB-C mice having the same characteristics and number with those in biosilver groups. Those groups were administered the same dose of 0.9% sodium chloride (NaCl) instead of biosilver. Also, the control group of the sensitization test was composed of healthy young Guinea pigs, including three males and two females.

Biosilver Synthesis and Extraction

A total of 100 mg silver nitrate (Merk) was dissolved in pure water and diluted to 100 ppm. A 10-ppm solution of ascorbic acid & pure water was prepared. The root and the stem of the *Cotinus Coggygria* plant were boiled in 6g/L for 6 minutes. The liquid part was evaporated in the rotary evaporator and thus extracted. Then, 100 ml of 100 ppm silver solution, 10 mg of *Cotinus* plant extract, 100 ml of ascorbic acid, 13.4 mg of 0.1M solution $NaHPO_4$ and 180 ml of 0.15 molar NaCl- solution were mixed in a beaker. And then, the pH of the solution was adjusted to 7.8 using NaOH. The resulting solution was mixed in magnetic stirrer at 38.6 ° C for 28 hours using a magnetic stirring bar.

As specified in the standard test protocols TS EN ISO 10993-11 & 10993-12, since biosilver cannot be applied directly to experimental animals, the biosilver was kept in 37 °C for 72 hours before the extraction procedure was performed. According to the section 10.3.1 of the said protocol, the extraction was prepared at a rate of 3 cm^2/ml due to the structure of the biosilver used.

Application Method of Biosilver and Determination of Dose Level

The application method and dosage of the biosilver to be applied to the experimental animals were determined based on the standard test protocol TS EN ISO10993-11 (Table 1.). In calculating the right dose (ml/kg body weight) according to this protocol, the animal species, body weight/surface area and the physical & chemical and biological properties of the test sample were considered. Taking into account those factors, biosilver was injected to the mice intraperitoneally at a dose of 50 ml/kg, being once in acute toxicity tests and for 7 days in subacute and subchronic tests.

Sensitization Test (Guinea pig maximization experiment)

Preparation of the Experimental Group

The experimental group included only healthy guinea pigs with firm skins. Before the testing procedure, the application sites of the animals were shaved, and 0.1 ml was injected per application site for intradermal injection.

Intradermal induction phase

The shaved region between the two scapulae was divided into sites A, B and C, and each animal received an intradermal injection of Freund's complete adjuvant (sc-24018, Santa Cruz) with physiological saline, biosilver (undiluted extract) and biosilver at 50:50 (volume ratio), Freund's complete adjuvant with physiological saline (50%) emulsion, being 0.1 ml each, respectively.

Local induction phase

Seven days after the intradermal induction phase was achieved, a local application was performed to each animal with test samples impregnated in a gas cloth of approximately 8 cm^2. The skin was pretreated with 10% sodium dodecyl sulfate (Merck, 151-21-3) to avoid irritation 48 hours before local application. The local application was terminated after 48 hours.

Challenge phase

At 14 days after completion of the topical induction phase, challenge all test and control animals with the test sample. Administer the test sample and a vehicle control by topical application to sites that were not treated during the induction stage, such as the upper flank of each animal, using appropriate patches or chambers soaked in the test sample at the concentration selected in for site C. Dilutions of this concentration may also be applied to other untreated sites in a similar manner. Secure with an occlusive dressing. Remove the dressings and patches after 24 h.

Table 1. Application method and dosage of the biosilver to be applied to experimental animals

Species	Subcutaneous ml/kg	Intramuscular ml/kg	Intraperitoneal ml/kg	By probe By feeding ml/kg	Intravenous ml/kg
Rat	20	1	20	50	40
Mouse	50	2	50	50	50
Rabbit	10	1	20	20	10
Dog	2	1	20	20	10
Monkey	5	1	20	15	10

Observation of Skin Reactions

Within 24 to 48 hours after dressings were removed, the appearance of the competitive skin regions of both control animals and test animals and the skin reactions, under good lighting, were assessed according to the Magnusson and Kligman scale (Table 2.).

Table 2. Magnusson and Kligman Scale (TS EN ISO 10993-10, 2014).

Patch test reaction	Grade scale
No visible changes	0
Discrete or patched erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

RESULTS

Toxicity Test Results

As a result of systemic acute, subacute and subchronic toxicity tests of the NanoBioSilver, the testing periods required for all three stages were followed up in accordance with the clinical observation criteria in the table specified in TS EN ISO10993-10. Accordingly, the results were systematically evaluated in experimental animals which received NanoBioSilver throughout the testing period.

In terms of the respiratory system, no such findings as abdominal breathing, difficulty breathing, breathing rhythm, breathing stopped or cyanosis were found.

During the experimental period, no such findings as loss of righting, partial or complete disappearance of sensory ability, loss of voluntary motor movements and sensitivity, a pathological condition characterized by hypertonia that makes it impossible to move, imbalance, fatigue, flailing, sweating, etc. were found in the motor activities of the animals.

Also, there were no conjunctivitis, exophthalmus, increased or decreased lacrimation, inflammation of the iris, opacity of the eye, myiasis or mydriasis in experimental animals whose ocular signs were followed. In the wake of the cardiovascular follow-ups performed following applications in the experimental animals that were administered biosilver at the specified test period and doses, there occurred no impaired heart rhythm (arrhythmia or bradycardia), increased heart rhythm (tachycardia) and vasodilatation or vasoconstriction in the vein walls.

Following the administration of biosilver to the intraperitoneal space, no increased or decreased salivation, soft stool, diarrhea, vomiting or diuresis were found in the digestive system.

Live Weight Results

All along the testing period, the weights of experimental animals were followed up, and the weight change rates, liver weights and liver index values are presented below (Figure 1-4). The food and water consumption were found normal in all groups. No out-of-limit weight variation was observed in any mouse between the start and end time of the testing procedure. The liver weights of the experimental animals were also found to fall within normal limits (4 to 6%).

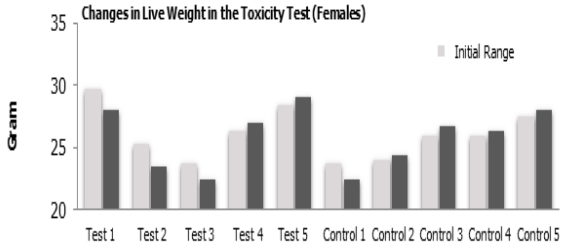

Figure 1. Changes in Live Weight in the Toxicity Test (Females)

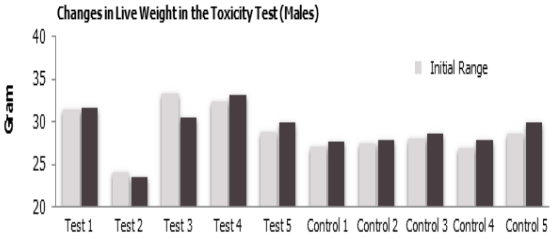

Figure 2. Changes in Live Weight in the Toxicity Test (Males)

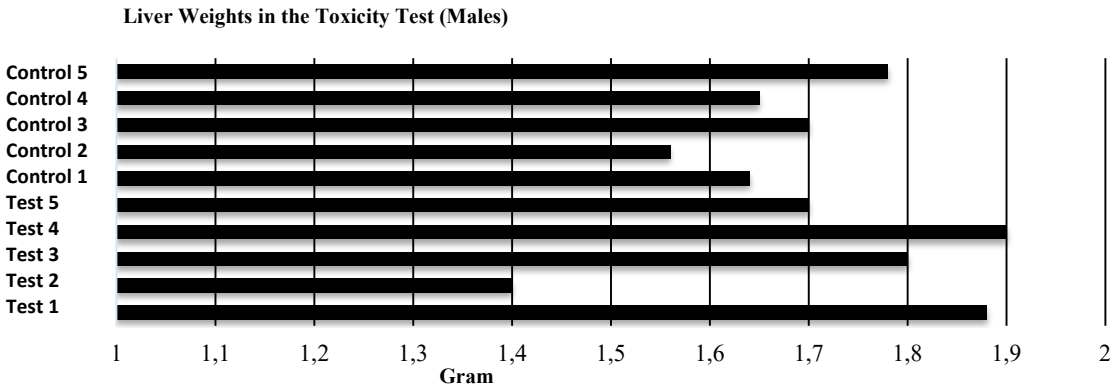

Figure 4. Liver Weights in the Toxicity Test (Males)

Necropsy and Histopathological Results

As a result of the tests performed in terms of acute (7 days), subacute (28 days) and subchronic (90 days) toxicities to determine the systemic toxicity of biosilver, all of the experimental animals were examined postmortem histopathologically.

Of the experimental animals with no pathological condition found in the external examination; the adrenal glands, lymph, all gross lesions, aorta, heart, bone marrow (femur, rib or sternum), brain (representative sites including cerebrum, cerebellum and pons), cecum, colon, duodenum, ileum, jejunum esophagus, eyes, gall bladder, kidneys, liver, lungs and bronchi as well as mammary glands in females and epididymis in males were examined at necropsy and histopathologically, which revealed no findings.

Sensitization Test (Guinea pig maximization experiment) Results

A Sensitization Test (Guinea pig maximization test) was performed using the tested nanosilver, and the test was ended at the end of the 27-day observation period. At the end of the 27-day observation period, the nanosilver tested was evaluated as "0.35 (no visible change)" according to the grade scale presented in the procedure TS EN ISO 10993-10 "Table 2 Magnusson and Kligman Scale". Table 3 presents the values observed in male and female animals according to the Magnusson and Kligman scale throughout the study.

Table 3. Values observed according to Magnusson and Kligman Scale.

Test Animals		24th hour after the dressing was removed	48th hour after the dressing was removed	Individual Average	Average	Result
Test Male	1	1	0	0.5	**0.4**	**0.35**
	2	1	0	0.5		
	3	0	0	0		
	4	1	0	0.5		
	5	0	1	0.5		
Test Female	1	0	0	0	**0.3**	
	2	0	0	0		
	3	1	0	0.5		
	4	0	1	0.5		
	5	0	1	0.5		
Control	1	1	0	0.5	**0.3**	**0.3**
	2	0	0	0		
	3	1	0	0.5		
	4	0	0	0		
	5	1	0	0.5		

DISCUSSION

Despite the fact that silver has been known for so many years as a powerful antibacterial agent with antifungal effects, it has revived with its biocidal effects in the form of suspension and nanoparticles in the recent years (Navarro et al. 2008).

In their study, Drake and Hazelwood, (2005) reports that toxicity occurs in humans when exposed to very high doses of a form of silver, which is usually biologically available, that since metallic silver products generate very poorly solute or soluble silver ions, exposure to them will not pose any risk to human health, however, when exposed to extremely high doses of silver nitrate, this may lead to a decrease in blood pressure, diarrhea, irritation in the stomach and a decrease in respiratory rate, and that in low doses, but in case of long-term exposure, some of the chronic symptoms would include degeneration in the liver and kidney and ulceration in the stomach. This study, however, revealed that no toxic effects were observed when the mice were intraperitoneally exposed to nanosilver at a dose of 50 ml/kg in acute, subacute and subchronic test procedures.

In their experimental study in which silver nitrate was given to rodents intravenously or by drinking water, Berry et al. (1995) confirmed low nephrotoxicity of silver in the urinary tract, and observed silver deposits in glomerular basement membranes, arteriolar endothelium and elastic laminae with no obvious structural harm. In contrast, no pathological findings were found in the kidneys at the end of the study.

Jiang et al. (2017) reported that nanomaterials such as nanostructured surfaces, nanoparticles and nanocomposites represent new suitable sources for future therapeutics designed for cardiovascular diseases and that nanomaterials can effectively increase the desired cellular responses within the cardiovascular system, leading to increased potential for clinical use, with their unique physiochemical properties and special properties such as surface energy and surface topographies. In parallel with the results presented by Jiang et al. (2017), the present study also revealed that the silver product administered intraperitoneally to the mice did not cause any cardiovascular pathological disorders in animals.

The healing process of skin wounds includes proliferation and reshaping of tissue. It stimulates inflammation of injury and ensures release of pro-inflammatory cytokines. The proliferation leads to formation of granulation tissue and angiogenesis, which are supported by macrophages. During the re-formation of the tissue, damaged tissue is removed, and the extracellular matrix is rearranged; this last process is controlled by various matrix metalloproteinases (MMPs) and tissue inhibitors. The nanosilver treatment has been found to be beneficial in wound healing since the fact that the healing process is accelerated with short-term inflammation. A thermal harm animal model using male BALB/C mice, bandages coated with nanosilver (14 nm) reduced inflammation and stopped bacterial growth and caused faster healing with reduced scar compared to the control mice (Cameron et al. 2018). The results from sensitization tests performed in this study are in line with this information and prove that the nanosilver product does not cause sensitization on the skin.

However, information on toxic effects of nanosilver in respiratory system of rodents is still too limited. There are evidences that subchronic inhalation of AgNPs causes dose-dependent, mild pulmonary inflammation and changes in pulmonary function, and inhaled AgNPs can enter the systemic circulation to be distributed to extrapulmonary organs such as the liver and brain. On the other hand, minimal or no toxicity has been reported in studies using low inhalation doses (Seiffert et al. 2016). This study, however, shows that no clinical changes were found in respiratory functions of the experimental animals as a result of acute, subacute and subchronic intraperitoneal tests performed.

In the digestive system, NP stability, dissolution and release of potentially toxic ions partly depend on liquid pH, composition and exposure time. The intestinal mucus layer is a complex network containing highly branched glycoproteins, lipids, cellular and serum macromolecules, and constitutes the first barrier for NPs swallowed. The electrical charge of the surface plays a highly important role; the neutral or positive surface charge prevents the mucosa from sticking, supports penetration and prevents the passage

of negative hydrophilic and lipophilic compounds. In their study performed in vitro on the human digestive study performed in vitro on the human digestive system, Walczak et al. (2012) conducted a study showing that 60 nm silver nanoparticles reduce the interaction and aggregation of chlorine ions after digestion of silver ions.

CONCLUSION

To correctly determine the right doses for the intake of nanosilver ions in mammalian digestive systems in in-vivo studies, in-vivo and in-vitro tests should be performed more frequently.

Nine out of ten studies conducted using oral exposure to AgNP in rodents has evaluated the tissue distribution and/or toxicity in healthy animals and focused on modulating effects on inflammation in a colitis model.

The evidences obtained from those studies generally reveal that AGNP is unlikely to have negative effects on host tissues caused by its acute or subchronic oral administration. Moreover, evidences often suggest that the orally-taken AgNP has a low level of tissue distribution and bioavailability.

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