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ACUTE DERMAL TOXICITY AND IRRITABILITY STUDIES OF Ag2Ga NANONEEDLE MEDIATED SILVER FORMULATION AS PER OECD 402 AND 404 PROTOCOLS

B. S. Wakure * and N. M. Bhatia

Bharati Vidyapeeth, College of Pharmacy, Kolhapur - 416004, Maharashtra, India.

Keywords:

Ag2Ga nanoneedles, Acute dermal toxicity, Acute dermal irritation, Corrosion, Topical drug delivery, OECD 402/404

Correspondence to Author:
B. S. Wakure

Research Scholar,
Bharati Vidyapeeth,
College of Pharmacy, Kolhapur - 416004, Maharashtra, India.

E-mail: wakure@gmail.com

ABSTRACT: We have developed Ag2Ga nanoneedles and achieved individual nanoneedles with diameter ranging from 200 to 250 nm with sharp tip and micro scale length required for a drug carrier to deliver drugs to site of application. Nanoneedles are designed to be inserted in human tissue and therefore they have to be compatible with the local environment of both in terms of toxicity and intended function. The variation of toxicity results for various nanostructures were reported in literatures. Hence, it is important to assess the information about toxicity of novel needle shaped nanostructures. Acute toxicity study was performed to examine the acute dermal toxicity of Ag2Ga nanoneedle mediated silver gel formulation on the rat's skin, according to the OECD Guidelines Test no.402. Additionally, acute dermal irritation study was also performed to examine the irritancy potential following single, 1 h and 4 h, semi occluded application of Ag2Ga nanoneedle preparation on the rat's skin according to OECD Guidelines Test no. 404. Acute toxicity study observations showed no skin toxicity or mortality and no vital transformations (statistically significant $P< 0.05$) in the weight of the body among all groups of animal after single dose administration of nanoneedle preparation. The results of acute dermal irritation trials did not show any signs of health hazard related to skin irritation and allergic risks. The Ag2Ga nanoneedles produced a primary irritation index of zero and was classified as a non-irritant to animal skin after administration according to Draize classification scheme.

INTRODUCTION: Nanostructured materials are emerging as diverse constituents in drug delivery, which may assemble innovative strategies to unravel many present untreatable diseases and new strategies to integrate untouchability of essential biological issues [1, 2]. Nevertheless, due to their potentially dangerous effects, there has been a lot of concern for the environment and health. Such biological applications will not be concrete if there is no proper assessment of the potential risks of nanoscale material for humans and for all other biological systems. Nanomaterial has worked extensively for their toxicity [3]. However, the published observations are random and are widely debated. There is a wide-ranging congruence that the great variation of toxicity results base in literatures stems from the application of nanostructures with their wide range of size distribution, high surface area, lengths and chirality's discovered by the current synthesis methods. Information on the toxicity of nano-structures will be meaningless without use of accurate measurements, genuine characterizations, and the use of reliable materials [4].

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In addition, the use of different protocols, cell lines and animal models to assess toxicity and long-term fate of the nanostructures can be a very important reason for discrepancy. Standard experimental protocols should be established, so that it can be compared to laboratory studies. It is important to take care when applying a traditional toxicology course to assess the safety of nanoscale materials such as nanotubes, nanoneedles, nanowires *etc* [1]. Nanoneedles are designed to be inserted in human tissue and therefore they have to be compatible with the local environment of both in terms of toxicity and intent function. The maximum duration of contact with the tissue range from minutes to daylong. Hence if the material is non-toxic in the short-term, malfunctioning due to biological host response *e.g*. bio fouling is not occur.

Regarding toxicity, bio inert materials such as titanium, stainless steel or gold, gallium or biodegradable polymers such as PLGA, can be used with confidence as nanoneedle material. Biocompatibility has not been investigated for nanoneedles, however recent studies show that silver gallium (Ag2Ga) alloy is biocompatible in long term (day to week) use. The Silver-Gallium amalgams were reported for its use as restorative material in dentistry and revealed as best substitute for mercury in dental amalgams [5]. These results indicate that silver gallium nanoneedles inserted in skin for short time, may not cause any harmful toxic reactions.

MATERIALS AND METHODS:

Nanoneedles: Nanomaterial is hollow or solid structure, with diameter in the 1-1000 nm range, and can be filled or loaded with drugs and detection agents. Targeted drug delivery can achieve by attaching targeting moieties on the surface of nanomaterial, to make it useful. The nanomaterial include nanoparticles, lipid core micelles, polymeric micelles, biodegradable polymeric nanoparticles, liposomes, nanopores, nanospheres, nanocapsules, nanotubes, nanowires, nanoshells, nanocantilever, dendrimers, gold nanoparticles, protein nano-particles, Buckminster fullerene and quantum dots.

Nanoneedles are hollow, tubular or conical needles in the nanometer size range. They are composed of non-toxic, biodegradable and eco-friendly components like silicon or boron-nitrate. Nanoneedles allow the conjugation of drug molecule on its large external surfaces for drug delivery. Nanoneedles serve as a powerful new tool for quantitative examination of biological processes in cell nucleus or cytoplasm; and for studying the biophysical properties at the molecular levels in living cells [6, 7].

Preparation of Nanoneedle: The Ag2Ga nano-needles were prepared by the way of physical techniques like nanofabrication and sputtering at Nauganeedles, K.Y., USA. By the process of nanofabrication, we have achieved the nanoneedles with tip diameter ranging from 200 - 250 nm and desired micro scale length for (trans) dermal drug delivery of model drug molecules.

Nanoneedle Mediated Silver Formulation: For topical delivery of silver molecules, we have formulated nanoneedle mediated silver gel preparation. The formulation was prepared by mixing 3:1 Ag2Ga-Chitosan nanoneedles preparation and gelatine dissolved in 2M citric acid. The 2M citric acid was produced by dispersing 42 gm citric acid in 100 ml distilled water. Initially, gelatine solution was prepared separately by dissolving in 2M citric acid. Finally, nanoneedles were put into gelatine solution, silver nanoparticles have been added in it and ultimate preparation thus obtained would have the concentration of 20 µg/gm of silver. The PEG-200 was employed in formulation as a crosslinking agent. The formulation has the ability to apply topically due to its gel like consistencies. The final composition of various formulations on the basis of varying concentration of ingredients were given in **Table 1**.

TABLE 1: COMPOSITION OF NANONEEDLE SILVER GEL FORMULATION

S. no	Formulation	Chitosan parts	Gelatine parts	Silver parts
1	I	1	1	20 µg/gm
2	II	1	3	20 µg/gm
3	III	3	1	20 µg/gm
4	IV	3	3	20 µg/gm

Acute Dermal Toxicity:

Objective: Acute toxicity study was performed to examine the acute dermal toxicity of Ag2Ga nanoneedle mediated silver formulation on the rat's skin, according to the Organization for Economic Co-operation and Development (OECD) Guidelines 402 [8].

Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a test substance. Dose is the amount of test substance applied and is expressed as weight of test substance per unit weight of test animal *(e.g*. mg/kg). The LD_{50} (median lethal dose), dermal, is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of treated animals when applied to the skin. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Study Protocol: All procedures described were reviewed and approved by the Institutional Animal Ethical Committee of Laboratory Animal Research Services (LARS), Reliance Life Sciences, Rabale, Navi Mumbai - 400701, Maharashtra, India vide approval IAEC.17/15 dated 08 June 2015.

Assay Substance: The assay substance evaluated in this study is the final product ready for transdermal delivery of drugs facilitated by nanoneedle, that is, a preparation containing the silver gallium nanoneedles with silver or insulin drugs conjugated on its surface. Chitosan and PEG were employed in preparation as polymers.

Selection of Animal Species: The study used healthy young adult female albino rats. Nulliparous and non-pregnant female were selected. At the commencement of it's dosing, young adult rats (at least 8-10 weeks old) with a size which facilitates the conduct of the test (200-300 g) and with healthy, intact skin were employed.

Housing and Feeding Conditions: The temperature in the experimental animal room should be 22 ºC (±3 ºC). Relative humidity should be at least 30% and not more than 70%. For feeding, conventional laboratory diets were used with an unlimited supply of drinking water.

Preparation of Animals: The animals were acclimatized to the laboratory conditions for at least five days prior to the start of the study. Animals are randomly selected for use in studies and marked for providing a personal identification. On the day before administration of the test chemical, all fur was removed from the dorsal/flank area of the test animals (*i.e*. at least 10% of the total body surface area) by close clipping. Care must be taken to avoid abrading the skin, which could alter its permeability. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

Methods: For acute dermal toxicity, a test substance is applied to no less than 10% of the area of the skin of rats, followed by 14 days of observation. Death of the animals is used to determine an LD_{50} value. This method provides information about the health hazard likely to occur from a short-term exposure to solid or liquid test substance by the dermal route.

Dose and Treatment Group: For each dose, at least 5 animals were used. The test substance is applied to the skin (not less than 10 percent of the body surface area) in graduated doses to several groups of experimental animals, one dose being used per group. Three dose levels of 200 mg/kg, 1000 mg/kg and 2000 mg/kg of body weight were used, to produce a dose-response curve. A limit test of at least 2000 mg/kg was made.

Observation: All animals should normally be observed for at least 14 days. During the first day the animals should be observed frequently and then the observations should be made daily. Observations obligated to count alternations in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Body Weight: The changes in body weights are main sign of clinical toxicity of substance administered. Hence, individual weights of animals were recorded on 1, 7 and 14 days of administration of the product. At the end of the test surviving animals are weighed and then humanely killed.

Statistical Analysis: The body weights were processed statistically by t-test for mean comparison with $p < 0.05$.

Acute Dermal Irritation/Corrosion Study:

Objective: This study was performed to examine the irritancy potential following single, 1 h and 4 h, semi occluded application of Ag2Ga nanoneedle

preparation on the rat's skin according to (OECD) Guidelines Test no.404.

Selection of Animal Species: The study employed young adult rats (at least 8 - 10 weeks old) with weight range of 200 - 300 gm and with healthy, intact skin were preferred with gross observation.

Housing and Feeding Conditions: After an acclimatization period of at least 5 days each animal was given a number for proper identification and written with marker pen on their tail. The temperature and relative humidity were set to achieve limits of 22 ºC (±3 ºC) and 30% to 70% respectively. Lighting should be artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Methods: The acute dermal irritation study was performed using rats according to the Organization for Economic Co-operation and Development (OECD) Guidelines 404 [9]. The concentrations of the nanoneedle in the test substances were the maximum allowable for administration. Nano-needle preparation of 500 mg was applied in dermal irritation study. On the day before the test each animal was clipped free of fur from the dorsal/ flank area using electrical clipper. Care was taken to avoid abrading the skin and only animal with healthy intact skin by gross observation were selected for the study.

One animal was initially treated. Three suitable sites were selected on the back of animal. At each test site the preparation was applied and sites were covered with a 6 cm^2 cotton gauze patch. Access by the animal to the patch and ingestion of the test substance was prevented by wrapping the animals in an elasticated corset for the duration of the 1 and 4 h exposure periods. The first patch is withdrawn after three min. If no serious skin reaction is observed, a second patch is applied at a different site and removed after one h. If at this level the observations show that the exposure can be extended humanely for four h, then third patches are applied and removed after four h, and the reaction is graded.

After considering the skin reactions produced in the first animals, two additional animals were treated with preparation in likewise manner. The patches were removed at each time points 1 h and 4 h after application. Any residual test material was removed by gentle swabbing with cotton soaked in spirit. Approximately one h following the removal of patches, and 24, 48 and 72 h interval, the test sites were observed for evidence of primary irritation and graded according to scale prescribed by OECD guidelines Test no.402 and presented in **Table 2**. To determine the reversibility of effects, the animals should be observed up to 14 days after removal of the patches.

TABLE 2: GRADING OF SKIN REACTIONS

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4
Oedema Formation	
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 area of exposure)	4
Maximum possible: 4	

RESULTS AND DISCUSSION:

Acute Toxicity Study: A study of acute toxicity is designed to detect the relative toxicity of a substance by the dermal route of exposure and determination of a dermal LD. This study serves as a basis for classification of toxicity and selection of the dose for long term toxicity studies. It is an initial step in establishing a dosage regimen in sub chronic and other studies and may provide information on dermal adsorption and the mechanism of toxic action of a substance by this route.

General Sign and Behavior of the Rats: In all animals groups, no detectable changes of the somatomotor activity or behavioral patterns were observed. There were no alterations of the respiratory, circulatory, or central and autonomous nervous systems, and no problems were detected on the skin, eyes or mucous membranes.

The toxic effects of silver gallium nanoneedle products on rat's skin are demonstrated in **Table 3** and **4**, individually. No toxic signs or mortality

were seen in any animal under experimentation, which made due up to 14 days after administration of preparation once on the 1st day at single measurements level of 200, 1000 and 2000 mg/kg body weight. All animals were observed initially for 6 h and pursued by 12 h after application of preparation.

In the treated groups, during the initial 6 h fast pulse was seen and this may be because of the anxiety of treatment.

TABLE 3: MORTALITY RATE OF RATS AFTER APPLICATION OF NANONEEDLE PREPARATION FOR THE ACUTE DERMAL TOXICITY STUDY

Group	Number of dead rats	Number of rats per group (n=5)	Mortality rate*(%)
G1	0	5	0
G2	0	5	0
G3	0	5	0

*Mortality rate is number of dead rats divided by total number of rats per group (n=5), G1: Dose 200 mg/kg bw, G2: Dose 1000 mg/kg bw and G3: Dose 2000 mg/kg bw.

TABLE 4: BEHAVIORAL PATTERNS AND GENERAL APPEARANCE OF RATS

Abnormal sign	Treatment Groups G1-G3	
	6 h	12 h
Skin and fur	No change	No change
Behavioral pattern	Tachycardia	No change
Mucous membrane	No change	No change
Salivation	No change	No change
Sleep	No change	No change
Diarrhea	No change	No change
Coma	No	No
Tremors	No	No

G1: Dose 200 mg/kg bw, G2: Dose 1000 mg/kg bw and G3: Dose 2000 mg/kg bw.

Body Weight: Table 5 shows the measured body weight of animals throughout the 14 days of observation and presented in **Fig. 1**.

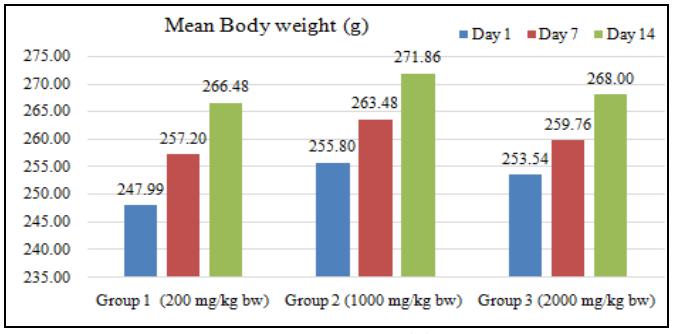

FIG. 1: MEAN BODY WEIGHT (g) OF RATS IN ALL GROUPS Data collected were recorded and presented as mean ± standard deviation of mean. G1: Group with dose of 200 mg/kg, G2: Dose 1000 mg/kg and G3: Dose 2000 mg/kg bw.

There were statistically significant differences for all animal groups. Differences were found between days 1 and 14, but not between days 1 and 7, or 7 and 14. It should be emphasized that although in some cases there were no statistically significant differences, a steady weight gain was observed between successive measurements that matches the expected weight gain. No vital transformations in the weight of the body were observed.

TABLE 5: BODY WEIGHT OF THE RATS THROUGHOUT THE 14 - DAY OBSERVATION PERIOD (MEAN ± STANDARD DEVIATION) (G)

Group	A no	Body weight (g)		
		1 day	7 day	14 day
G1	Average	247.99	257.20	266.48
	SD	10.29	9.71	9.01
	P value	0.18	0.16	0.02
G2	Average	255.80	263.48	271.86
	SD	8.17	7.81	9.25
	P value	0.17	0.16	0.02
G3	Average	253.54	259.76	268.00
	SD	6.77	7.08	6.89
	P value	0.19	0.10	0.01

*Statistical significance. (P < 0.05), G1: Group with dose of 200 mg/kg bw, G2: Dose 1000 mg/kg bw and G3: Dose 2000 mg/kg bw.

Macroscopic Findings: The organs of the animals and their skin showed no macroscopic alterations whatsoever. Therefore, no samples were taken for histopathological processing.

Conclusion: Under the conditions of the assay, nanoneedle preparation showed no skin toxicity after a topical single administration in rats.

Acute Dermal Irritation Study: The scores for erythema and oedema at the 24 and 72-h readings were totalled for the three-test animal (12 values) and this total was divided by six to give the primary irritation index of the test sample. The test material was classified according to the scheme devised by Draize J H 10 and presented in **Table 6**.

TABLE 6: PRIMARY IRRITATION INDEX FOR CLASSIFICATION OF IRRITANCY

Primary Irritation Index	Classification of Irritancy
0	Non-irritant
>0 to 2	Mild irritant
>2 to 5	Moderate irritant
>5 to 8	Severe irritant

Four hours Exposure Period: The individual scores for erythema/eschar and oedema are presented in **Table 7**.

No dermal responses, including erythema/eschar or edema, were found in rats treated with nanoneedles.

No erythema or oedema was observed after the challenge with nanoneedles in the nanoneedle-treated rats.

TABLE 7: INDIVIDUAL SKIN REACTIONS FOLLOWING 4h EXPOSURE

Skin reaction	Observation Time	Animal no			Total
		1	2	3	
Erythema/ Eschar	1 h	0	0	1	(1)
Formation	24 h	0	0	0	0
	48 h	0	1	0	(1)
	72 h	0	0	0	0
Oedema	1 h	0	0	0	(0)
Formation	24 h	0	0	0	0
	48 h	0	0	0	(0)
	72 h	0	0	0	0
Sum of 24 and 72 h readings (S)		0			
Primary irritation index (S/6)		0/6=0			
Classification		Non-irritant			

CONCLUSION: Neither irritation nor corrosion was caused on the rat's skin. The Ag2Ga nanoneedles produced a primary irritation index of 0.0 and was classified as a non-irritant to rat's skin after administration according to Draize classification scheme.

This information on the toxicological effects of nanoneedles is still limited. However, the results of dermatological trials did not show any signs of health hazard related to skin irritation and allergic risks.

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