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SAFETY EVALUATION OF SILVER NANOPARTICLES FOR DERMALAPPLICATION

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ABSTRACT

Silver nanoparticles (AgNPs) are an important class of nanomaterials used as new potent antimicrobial agents for a wide range of medical, Cosmetic, hygienic Products. However, toxicity of AgNPs and impact of their physicochemical characteristics in in- vitro and in- vivo models still need to be comprehensively characterized. The aim of this study was to investigate the Cytotoxicity and Toxicity effect of prepared silver nanoparticles in-vitro and in-vivo models. The invitro cytotoxic effect of biologically synthesized silver nanoparticles against L929 cell lines was assessed by the MTT colorimetric technique. For toxicological evaluation Rats were exposed to two concentration 1% and 3% Silver Nanoparticle ointment following OECD guideline 402 for acute dermal toxicity And Sub-Acute Dermal Toxicity of 3% Silver Nanoparticle ointment was evaluated following OECD guideline 410 in rats and results were analyzed by Pathozyme Blood Chemistry analyzer and histopathology was performed on Liver, Kidney, and skin for sub-acute toxicity. Silver nanoparticles exerted no significant cytotoxic effect toward normal cells L929 even at the highest concentration. There was no mortality during the course of experimentation for acute dermal toxicity and in the Sub Acute Dermal Toxicity Study, no mortality in rats were observed and showed no clinical signs of intoxication after application till the end of the study and no grossabnormalities in any organs were observed in all the group animals and showed normal histopathology. Hence, the silver nanoparticles were found to be safe for dermal application in minimal dose.

INTRODUCTION

Nowadays engineered nanomaterial has attracted a great deal of attention due to its unique chemical, physical and biological properties which are different from those of bulk material with the same composition. Nano materials are likely to become the cornerstone in healthcare and cosmetic products in near future of many industrial sectors.[1] The widespread application of silvernanoparticles is mainly related to the renowned antimicrobial and antiseptic activity of silver.[2] However, public is concerns about their potential adverse effects[3] which has encouraged scientists to focus on their safety profiles too.

Introduction of nanotechnology has always shown improvements in the field of cosmetics and personal care, which resulted in better testing and analytical approaches to the research in the functionality of applied products on the biological level, which ultimately enables the realizationof cosmeceuticals (i.e. the combination of cosmetics and pharmaceuticals).[4] Silver nanoparticlesare known as commercialized nanoparticles, which accounted for over 50% of the global nanomaterial consumer products in 2015. Silver nanoparticles are one of the important materials in various applications including wound healing, food packaging, and cosmetics due to their abilityto damage bacteria cells and weakening the cell membrane.[5] The unique properties of silver nanoparticles attracted the attention of many industries, particularly those in which an antiseptic effect is particularly desirable. The product can be used in dyeing of cosmetic foundations, eye shadows, powders, lipsticks, inks, varnishes or eyebrow pencils. According to Ha et al,[6] the convectional products which consist of metal nanoparticles such as metallic pigments are not considered harmful to human health and may have some health benefits. The authors also reported that because of the presence of silver nanoparticles, the product has got the anti-inflammatory activity which plays the important role in protecting the skin against the adverse effects of the microorganisms. Silver nanoparticles can penetrate deep into the skin and exert bactericidal action,this property can be beneficial in antiacne preparation where the action is desired in lower strata of epidermis.[6]

As the skin is the largest organ of the body it functions as the first-line barrier between the external environment and the internal organs of the human body. Nano silver has for years been of interestto toxicologists. Topically applied NPs can potentially penetrate the skin and access the systemic circulation and exert adverse effects on a systemic scale.[7] However, despite the results of numerous studies, the issue of nano silver toxicity is still unresolved. In most of studies, the size-dependent relations were tested with AgNPs with large differences

between diameters e.g. 10 and100 nm or 20 and 200 nm. There are various studies stating the toxic effect of nanoparticles whichare less than 50 nm. But the result of toxic effects of nano silver particles of 15 and 100 nm, withthe bigger particles (100 nm) exhibited greater toxicity. For this purpose, we selected AgNPs witha bigger size of 100 nm and 200 nm to evaluate its in-vitro toxicity.[8] Further 200nm Silver nanoparticles was evaluated for in-vivo dermal toxicity with the aim to produce safe nanoparticlesfor dermal application. Topical application of nanosilver can induce the benign condition known as argyria, a grey—blue discoloration of the skin caused by deposition of silver particles in the basal laminae of such tissues. This has prompted a limitation on the recommended daily dosage of silver.[3] There is some research papers that showed silver nanoparticles could be found in skin, liver, and spleen of guinea pig after dermal application and result has shown some slight damagesin liver, spleen and skin. A study conducted by Costa et al.[3] suggests mitochondrial dysfunction and impaired energy production as the possible underlying mechanism of neurodegeneration. Repeated administration resulted in significant accumulation in organs with smaller AgNP (<100 nm) being predominantly picked up by the liver while larger particles (>100 nm) mainly accumulated in the spleen. This pattern of particle uptake is regulated by the pore size of capillaryfenestrae (~100 nm). Dose-dependent accumulation has previously been observed in rats following repeated oral doses of AgNP.[3] Due to the lack of knowledge regarding toxic effect ofsilver nanoparticles on various organs of the body especially through dermal application, we decided to evaluate histopathological effects of silver nanoparticles on different organ through dermal application on rats by following OECD guideline.

Experimentation

As described previously in the article[9] Silver nanoparticles were chemically synthesis by reducing silver nitrate by oxalic acid and to detect its conformation and size, it was further characterized for UV-visible spectrophotometer, and transmission electron microscope (TEM). After the confirmation Silver nanoparticles were further studied for cytotoxicity and dermal toxicity.

MATERIALS AND METHODSMATERIALS

Cell line-L929, Culture media- MEM medium with Antibiotics and 10% FBS, 96 well Tissue culture plate, Neubauer's chamber, MTT Reagent (sigma), Phosphate buffer saline, Acidic isopropanol, 96 well Plate reader.

Methods

Cell lines and culture conditions

Cell line-L929 (Mouse Fibroblast cell line) was used in the study. Cultures were maintained in a humidified atmosphere with 5% CO2 at 37 °C. The cells were sub-cultured twice each week, seeding at a density of about 2× 103 cells/ml. Before the analysis of the AgNPs, cells were washed with PBS and fresh medium was added. For final analysis, exponentially growing cells were collected and resuspended in fresh culture medium with 10% FBS.

Procedure

Cell viability was determined by the MTT assay based on the cleavage of the tetrazolium salt by metabolically active cells to form a water-insoluble formazan dye. The cells were plated separately at the concentration of 1×105 cells/ml were added in 96 well plate (cell count was taken on Neubauer's chamber). Then the plate was incubated at 37 ^{0}C in a CO_2 incubator for24 hrs. After 24 hrs. incubation plate was observed under inverted microscope and 2µg/ml, 4µg/ml, 6µg/ml 8µg/ml, 10µg/ml 50µg/ml and 100µg/ml Concentrations of sterile test sample was prepared. Then 100 µl from each concentration was added in triplicate. After this, plate was incubated at 37 ^{0}C in a CO_2 incubator for24 hrs. On next day after 24 hrs incubation plate was observed under inverted microscope. And test sample was removed and 100 µl fresh DMEM medium was added. Then 10µl of 5mg/ml MTT reagent was added in each well. The plate was wrapped in aluminum foil and incubated for 4hrs in CO_2 incubator. After 4hrs incubation plate was removed and observed under inverted microscope and photographs were taken. The entire medium was removed by flicking the plate and 200 µl of acidic isopropanol was added in each well. After 1hr Spectrophotometrically absorbance of the purple blue formazan dye was measured at 492nm on 96 well Plate reader. The average of the viable cells was calculated. A graph was plotted between the percentage cell viability and concentrations of Silver nanoparticles (dilutions).

Experimental animals and housing condition

In the present study total 50 experimental Sprague Dawley Rats were used. 5 male and 5 femaleswere used for acute dermal toxicity. Both male and females ages were 14 to 15 weeks and their body weight were between 192 – 262 gm. For sub-acute dermal toxicity 20 males and 20 femaleswere used and their ages both males and females were 15 to 18 weeks with the body weight between 170 – 250 gm. Each rat was housed in stainless steel cages and allowed to adapt to the conditions of the animal house for 14 days before the experiments.

The environmental condition was maintained at Room temperature between 22+30 ^{0}C, relative humidity 50-60 % and illumination cycle set to 12 hours light and 12 hours dark. Accommodation for acute dermal toxicity, three rats in one cage and Two Rats in another cage per group was housed in stainless steel cages, with food and water bottle, and bedding of clean paddy husk. Pelleted feed supplied by Supplier were given as diet. Potable water passed through 'Aquaguard' water filter was provided ad libitum in glass bottles with stainless steel sipper tubes. The study was performed according to the OECD guideline no: 402 and 410 for acute and sub-acute dermal toxicity. The study was performed at APT Research Foundation, Pune.

Acute Dermal Toxicity

The objective of the present study was to evaluate the Acute Dermal Toxicity of 1% and 3% SilverNanoparticle ointment following OECD guideline 402 in rats.

Rats were divided into the following groups.

Group 1: 5 M + 5 F (Test 1 -1% ointment)

Group 2: 5 M + 5 F (Test 2 -3% ointment)

The test was conducted as per OECD Guidelines for testing of Chemicals, 402.

The test material was applied at a dose of 2000 mg/kg to rats, 05 male and 05 female. Approximately 24 hours before the experimentation, fur on the dorsal area of the trunk was removed with the help of electric clippers, exposing an area of about 10 % total body surface area. The test material was applied and a collar was placed around the neck to prevent ingestion of the test material for 24 hours. At the end of 24 hours the collar was removed and the unabsorbed material washed. Water was allowed ad libitum. At the end of the 14 days observation period the surviving experimental animals was sacrificed, gross necropsy was performed and all animals wereexamined carefully.

Observations

Clinical observations: Toxic symptoms and mortality was recorded at ½, 1, 2, 4, 6 and 24 hoursand later twice a day upto 14 days to determine their general health, behavior and moribund condition. Any abnormality was observed during this period and was recorded and the subsequentprogress monitored.

Body Weight Change: Animals were weighed individually on the day the test substance

administered and weekly thereafter and prior to sacrifice.

Necropsy: A gross necropsy was performed on all animals that were sacrificed at the terminationof the test.

Sub-Acute Dermal Toxicity

The objective of the study was to evaluate the Sub-Acute Dermal Toxicity of 3% Silver Nanoparticle ointment following OECD guideline 410 in rats.

Study Design

Rats were divided into the following groups.

Group 1: 5 M + 5 F (control)

Group 2: 5 M + 5 F (Satellite control)

Group 3: 5 M + 5 F (Test)

Group 4: 5 M + 5 F (Satellite test)

The test was conducted as per OECD Guidelines for testing of Chemicals, 410, adopted 12th May1981.

The 3% test material was applied at a dose of 1.0 mg/kg to rats. Approximately 24 hours before the experimentation, fur on the dorsal area of the trunk was removed with the help of electric clippers, exposing an area of about 10 % total body surface area. The test material was applied daily for 28 days. Water was allowed ad libitum. At the end of the 28 days observation period the surviving experimental animals was sacrificed, gross necropsy was performed and all animals wereexamined carefully. The satellite groups were kept for further 14 days without application i.e. fortotal 42 days.

Observations

Clinical observations: Toxic symptoms and mortality was recorded at ½, 1, 2, 4, 6 and 24 hoursand later daily thereafter up to 28 days to determine their general health, behavior and moribund condition. Any abnormality was observed during this period and was recorded and the subsequentprogress monitored.

Body Weight Change: Animals were weighed individually on the day the test substance administered and weekly thereafter and prior to sacrifice.

Necropsy: A gross necropsy was performed on all animals that were sacrificed at the terminationof the test.

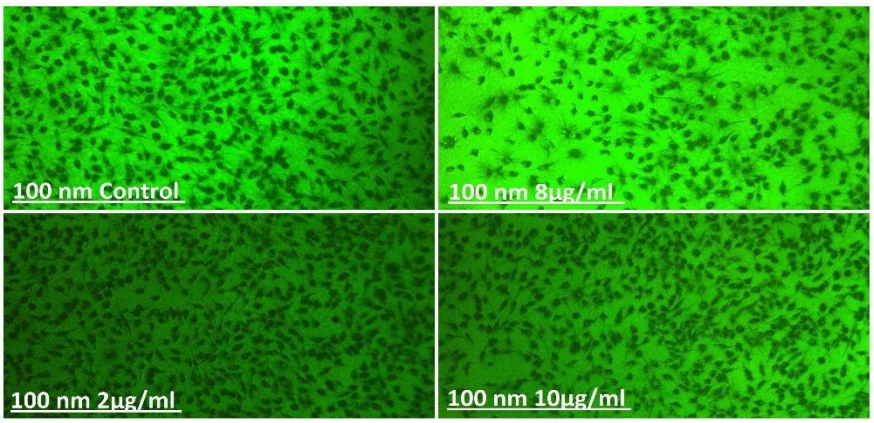
Blood Chemistry: Blood Samples were collected separately in tubes for clinical chemistry on the day of sacrifice of the animals. Samples were analyzed by Pathozyme Blood Chemistry analyzer.

Histology: A small piece of liver, kidney and skin were collected 24 h after the last administration. The portion of organs were fixed in 10% neutral buffered formalin solution, dehydrated in a graded series of ethanol and xylene solutions, and embedded in paraffin. Sections were cut with a microtome, deparaffinized, rehydrated in a graded series of ethanols, and stained with hematoxylinand eosin.

RESULTS

Cytotoxic effects of silver nanoparticle on normal L929 cell lines

Despite of its potent antibacterial activity and wide variety biological applications, the use of Silver nanoparticles as therapeutic agent is limited because of their potential for cytotoxic activity against mammalian cells. The *in vitro* cytotoxic effects of silver nanoparticles were screened against normal L929 mouse fibroblast cell lines by means of MTT assay which relies on the fact that the metabolically active cells reduce MTT to purple colour formazan. Thus, the intensity of the dye was read at 492 nm which is directly proportional to the number of viable cells shown in figure 1and 2 of both 100 and 200 nm. Silver nanoparticles exerted no significant cytotoxic effect toward normal cells L929 even at the highest concentration 100 µg/ml, for both 100 and 200 nm particles,showing cell viability above 70 % shown in Graph 1. The IC_{50} value of this assay was calculated at 9.1 and 9.3 µg/ml for 100 and 200 nm respectively.



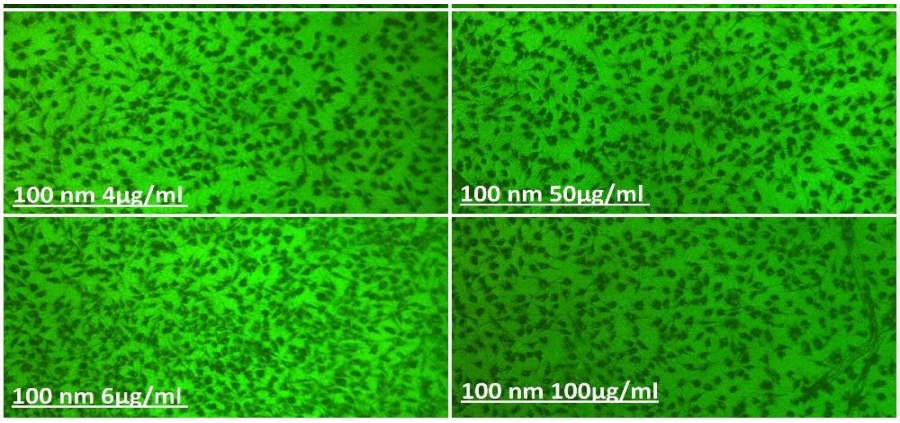


Figure 1: microscopic image of Cell line-L929 (Mouse Fibroblast cell line) after MTT incubationcontaining 100nm silver nanoparticles.

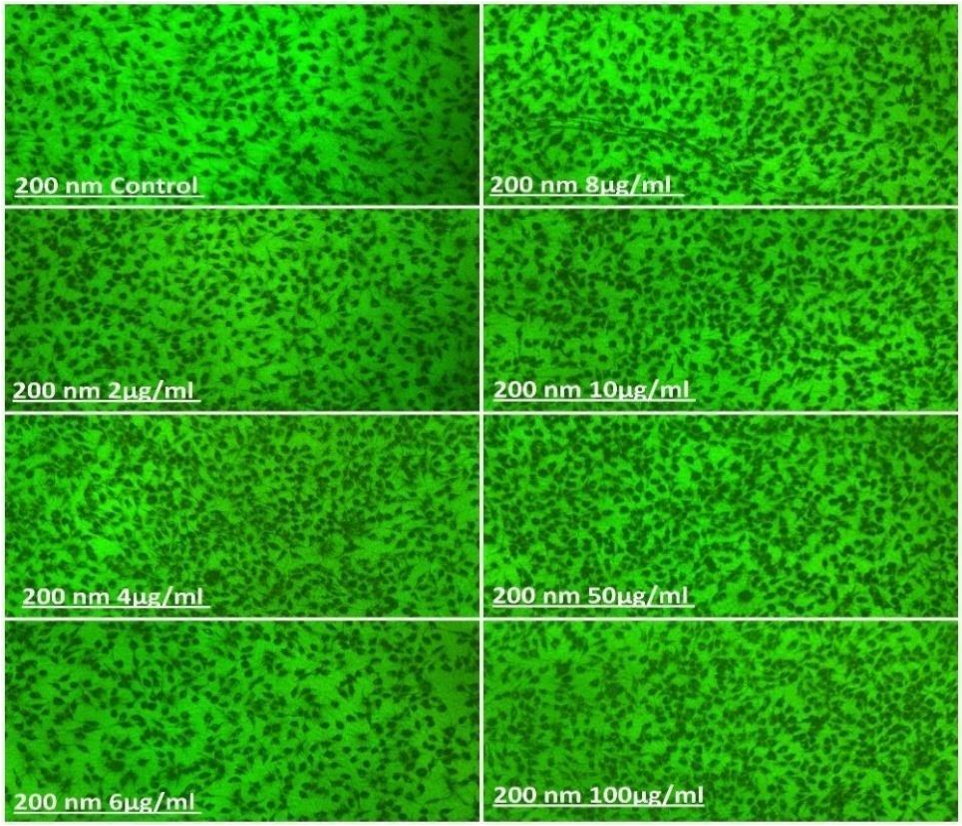
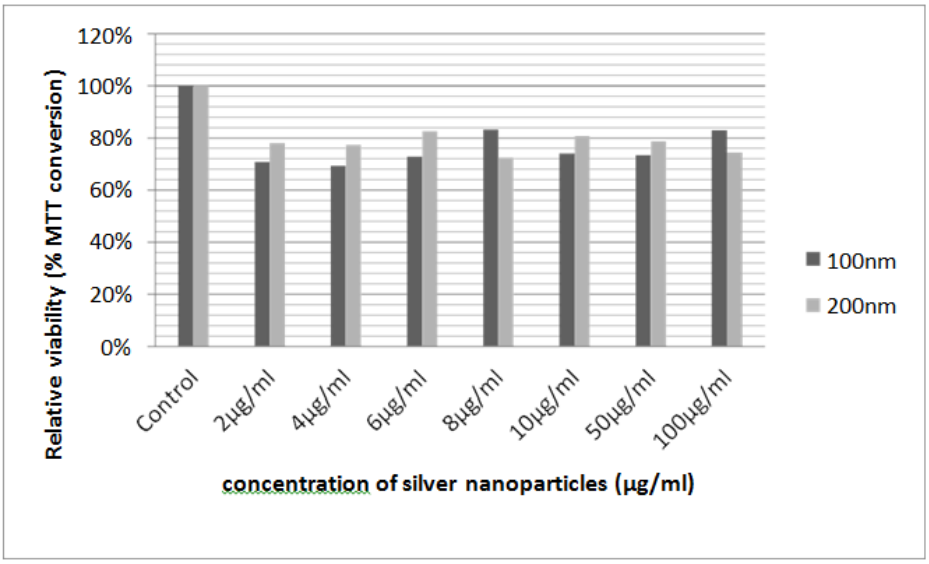


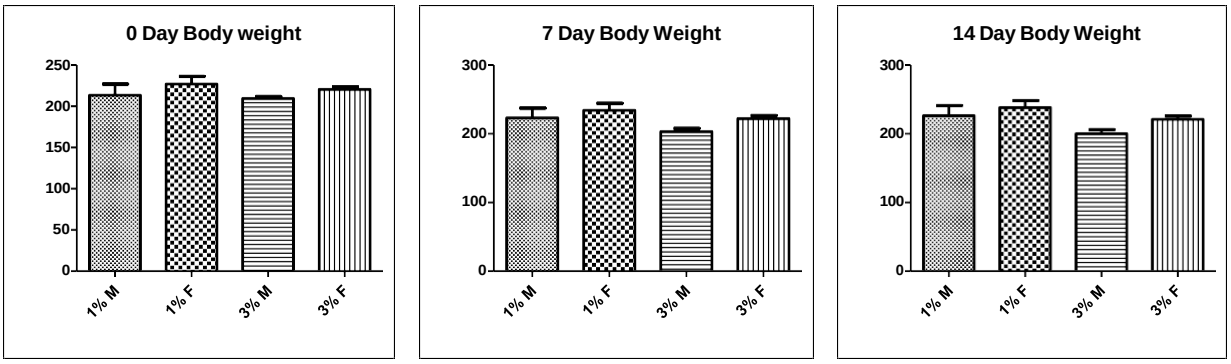
Figure 2: microscopic image of Cell line-L929 (Mouse Fibroblast cell line) after MTT incubationcontaining 200nm silver nanoparticles.



Graph: 1. the percent of viability measured by MTT assay on L929 cells after treatment with different silver nanoparticles concentration for 24 h. An OD value of (unexposed cells) control cells was taken as 100% viability (0% cytotoxicity).

Acute Dermal Toxicity

The results of body weight changes in the acute dermal toxicity study were as follows.



Graph: 2. Body weight from 0 day to 14 days.

Body weight was measured on Day 0, Day 7 and Day 14 wherein, there were no statisticallysignificant changes in the body weight gain in test animals as compared between the groups.

There was 5.7% increase in the Body weights of 1% Ointment treated males and 4.6 % increase inthe Body weights of 1% Ointment treated females.

However, there was 3.78% decrease in the Body weights of 3% Ointment treated Males and only0.18% increase in the Body weights of 3% Ointment treated females, which indicates mild

toxicity.

Irritation score index per Day.

Table: 1. Irritation score index per DaySurvival and clinical sign.

Group	Ani.No	Sex	Marking	1	2	3	4	5	6	7	8	9	10
1%	1	M	H	0	0	0	0	0	0	0	0	0	0
	2	M	T	0	0	0	0	0	0	0	0	0	0
	3	M	HT	0	0	0	0	0	0	0	0	0	0
	4	M	RF	0	0	0	0	0	0	0	0	0	0
	5	M	W	0	0	0	0	0	0	0	0	0	0
1%	6	F	RH	0	0	0	0	0	0	0	0	0	0
	7	F	RLS	0	0	0	0	0	0	0	0	0	0
	8	F	LF	0	0	0	0	0	0	0	0	0	0
	9	F	LH	0	0	0	0	0	0	0	0	0	0
	10	F	W	0	0	0	0	0	0	0	0	0	0
3%	11	M	H	0	1	0	0	0	0	0	0	0	0
	12	M	T	0	1	0	0	0	0	0	0	0	0
	13	M	RH	0	1	0	0	0	0	0	0	0	0
	14	M	RLS	0	1	0	0	0	0	0	0	0	0
	15	M	W	0	1	0	0	0	0	0	0	0	0
3%	16	F	HT	0	0	0	0	0	0	0	0	0	0
	17	F	H-RH	0	0	0	0	0	0	0	0	0	0
	18	F	LH	0	0	0	0	0	0	0	0	0	0
	19	F	FLS	0	0	0	0	0	0	0	0	0	0

There was no mortality during the course of experimentation and there were no signs of erythema or oedema observed on the skin surface of 1% ointment treated male and female rats, it can be concluded that 1% ointment is safe for use. A slight redness was observed on the 3% ointment treated Male rats which were not prevalent in 3% ointment treated female rats.

Based on the above results it can be concluded that the 1% ointment caused no irritation and can be considered as safe for use.

On the other hand, 3% Ointment caused reduction in the body weight during 14 days study durationand caused Mild irritation (redness) during 24-hour post application.

However repeated dose of application of the test material is required to assess the safety of the testmaterials and thus was decided to perform sub-acute dermal toxicity further.

Results for sub-acute dermal toxicity

Clinical Findings for sub-acute dermal toxicity

Group I – Control: All the animals appeared normal and showed no clinical signs of toxicity till the end of the study.

Group II – Satellite control: All the animals appeared normal and showed no clinical signs of toxicity till the end of the study.

Group III – Test 1.00 mg/kg: No Mortality was seen. All the animals appeared normal and showedno clinical signs of toxicity till the end of the study.

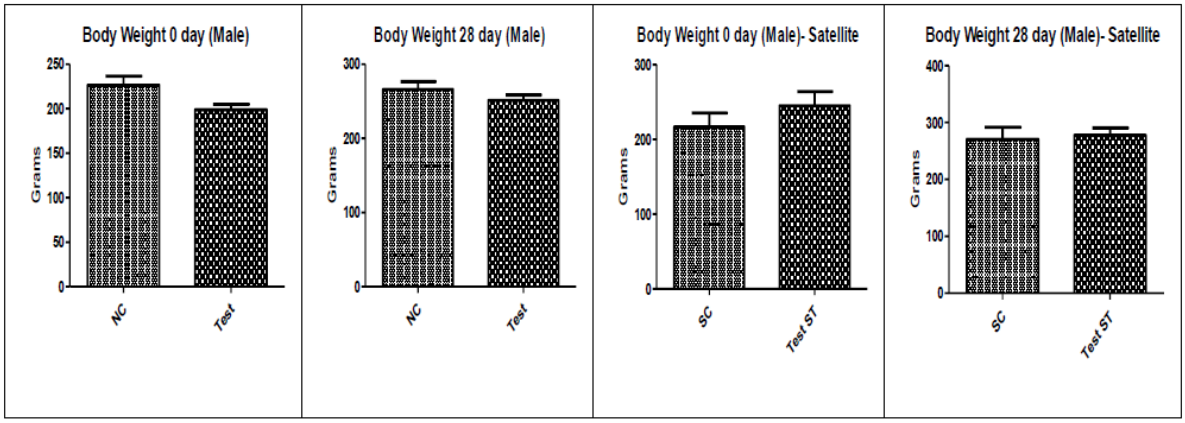
Group IV – Satellite Test: No Mortality was seen. All the animals appeared normal and showed no clinical signs of toxicity till the end of the study.

Mortality rate

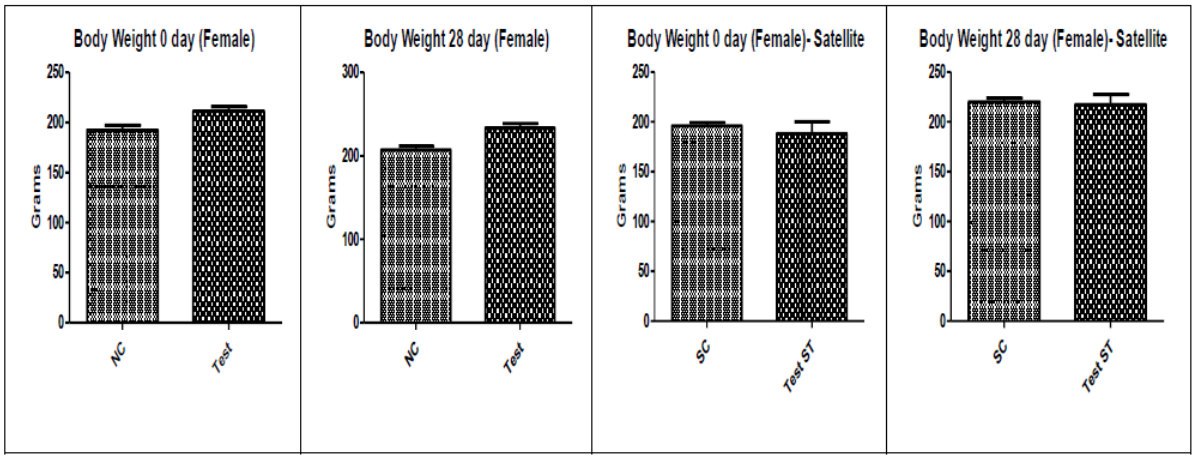
No mortality was recorded during application of 3% silver nanoparticles ointment in preliminary study. Thus, it was practically considered as nontoxic agent in the acute dermal toxicity study.

Body Weight

There was no statistically significant change in the body weight gain in Test dose group and Satellite Test dose group when compared with the control group and Satellite control group animals respectively when analyzed by One Way ANOVA test by using GraphPad Prism 5.



Graph: 3. Body weight changes of male group during 0 day and 28 day.



Graph: 4. Body weight changes of female group during 0 day and 28 day.

Hematological Data

There were no statistically significant changes in the hematological parameters – Hb, HCT, WBC, RBC, Platelets in all the groups when analyzed by One Way ANOVA test by using GraphPad Prism 5.

Sex: Male

Table: 2. Group Mean Hematology Data. (Where, n=5)Sex: Female.

Group		WBC (Thousand/ cmm)	RBC (Million/ cmm)	HGB	HCT (%)	PLT (Thousand/ cmm)
I. Control	Mean	6.2	8.9	15	46.7	639.6
	±SD	1	1.1	2.1	6.5	123.1
II. Satellite Control	Mean	7.5	8.2	13.5	42.2	677.4
	±SD	3.6	0.4	1.5	4.6	106.5
III. Test	Mean	8.7	9.8	16.7	51.5	490.8
	±SD	2.8	0.8	2	5.5	184.7
IV. Satellite Test	Mean	12.5	8.8	14.1	47	630.6
	±SD	1.7	0.4	0.4	2.1	69.3

Table: 3. Group Mean Hematology Data (where, n=5).

Group		WBC (Thousand/ cmm)	RBC (Million /cmm)	HGB	HCT (%)	PLT (Thousand /cmm)
I. Control	Mean	9.9	9	14.9	46.1	630.4
	±SD	1.9	1.4	2.6	7	186
II. Satellite Control	Mean	7.5	8.8	14.6	45.8	431.4
	±SD	1.2	1.6	2.8	8	173.2
III. Test	Mean	6.5	9.5	16.4	51.3	322.4
	±SD	1.6	1.9	3.6	10.9	137.2
IV. Satellite Test	Mean	7.4	9.3	15.8	48.9	618
	±SD	1.4	0.8	1.6	4.9	126.1

WBC= White Blood Cells, RBC= Red Blood Cells, HGB= Hemoglobin, HCT= Hematocrit PLT= Platelets.

Blood Chemistry

There was no statistically significant increase or decrease in the blood chemistry parameters - Alkaline Phosphatase, Total Proteins, Glucose, SGPT, SGOT, Urea, Creatinine, cholesterol and Triglycerides levels in all the groups when analyzed by One Way ANOVA test by using Graph PadPrism 5.

Sex: Male

Table: 4. Group Mean Blood Chemistry Data (where, n=5)Sex: Female.

Group		GLC	TP	ALB	UREA	CHOL	TGL	SGOT	SGPT	ALP
		mg/dL	gm/dL	gm/dL	mg/dL	mg/dL	mg/dL	IU/L	IU/L	
I. Control	Mean	84.8	7	3.9	28.5	1.1	60.4	183.2	83.2	34.8
	SD	8.3	0.3	0.2	5.6	0.3	5.3	40.4	10.5	9.7
II. Satellite Control	Mean	91.6	7.5	3.9	27.5	0.9	70.8	160.8	110.6	50.2
	SD	9.4	0.4	0.4	2.8	0.2	10.1	21.7	9.7	11.1
III. Test	Mean	90.6	7.3	3.7	32.7	1	66.4	146.6	98.6	49.4
	SD	8	1	0.3	6.9	0.1	6	57.6	18.5	8.3
IV. Satellite Test	Mean	90.2	7.8	4	30.8	1	70.6	166	110.8	53.2
	SD	7.7	0.6	0.4	5.6	0.2	9.1	33.2	9.7	6.1

Table: 5. Group Mean Blood Chemistry Data (where, n=5).

Group		GLC	TP	ALB	UREA	CHOL	TGL	SGOT	SGPT	ALP
		mg/dL	gm/dL	gm/dL	mg/dL	mg/dL	mg/dL	IU/L	IU/L	
I. Control	Mean	90.8	8.4	4.2	34.4	0.9	70.8	188.4	91.4	40.6
	SD	8.3	0.5	0.3	5.1	0.1	8	46.8	16.1	10
II. Satellite Control	Mean	93	8.3	3.6	26.3	1	78.4	170	95.4	57.2
	SD	10.8	1	0.2	3.5	0.3	7.6	24.4	16.4	8.7
III. Test	Mean	87.8	7.8	5.3	34.6	1	61.8	201.6	93.2	40.8
	SD	5.8	1.2	0.8	5	0.2	7.9	45	17.3	9.3
IV. Satellite Test	Mean	90.2	7.8	4	30.8	1	70.6	166	110.8	53.2
	SD	7.7	0.6	0.4	5.6	0.2	9.1	33.2	9.7	6.1

GLC= Glucose TGL= Triglyceride

TP= Total Protein SGOT= Serum glutamic oxaloacetic transaminase

ALB= Albumin SGPT= Serum glutamic-pyruvic transaminase

CHOL= Cholesterol ALP= Alkaline Phosphatase

Organ Weights

There was no statistically significant increase or decrease in the absolute weights of all the organsin all the groups when analyzed by One Way ANOVA test by using GraphPad Prism

5.

Sex: Male

Table: 6. Group Mean Absolute Organ Weight Data (g) (where, n=5)Sex: Female.

Group		Adrenals	Heart	Kidneys	Liver	Spleen	Lungs	Testes
I. Control	Mean	0.079	0.8	1.671	8.192	0.879	1.464	1.612
	+-SD	0.008	0.083	0.168	1.899	0.256	0.188	0.393
II. Satellite Control	Mean	0.071	0.799	1.798	9.118	0.716	1.494	2.223
	+-SD	0.007	0.037	0.511	2.241	0.095	0.286	0.477
III. Test	Mean	0.074	0.732	1.714	7.804	0.756	1.555	2.116
	+-SD	0.005	0.128	0.222	1.482	0.152	0.198	0.157
IV. Satellite Test	Mean	0.095	0.927	1.829	9.27	0.782	1.761	2.179
	+-SD	0.034	0.047	0.195	1.165	0.073	0.136	0.123

Table: 7. Group Mean Absolute Organ Weight Data (g) (where, n=5).

Group		Adrenals	Heart	Kidneys	Liver	Spleen	Lungs	Ovaries
I. Control	Mean	0.066	0.77	1.529	6.857	0.644	1.638	0.146
	+-SD	0.011	0.068	0.256	0.789	0.107	0.185	0.012
II. Satellite Control	Mean	0.096	0.8	1.304	7.164	0.708	1.615	0.148
	+-SD	0.013	0.088	0.381	0.697	0.087	0.229	0.023
III. Test	Mean	0.074	0.68	1.549	7.71	0.749	1.523	0.179
	+-SD	0.005	0.257	0.193	0.659	0.082	0.21	0.013
IV. Satellite Test	Mean	0.096	0.8	1.656	7.277	0.717	1.595	0.172
	+-SD	0.014	0.052	0.333	1.692	0.207	0.215	0.017

Histopathology Study

The histopathology of kidneys tissues showed Normal Histological features of renal parenchyma comprised of glomeruli and renal tubules. Focal cellular swelling of few tubules was noted in Satellite control, test group, and satellite test group which were minimal. Intact proximal and distalconvoluted tubules with presence of tubular epithelium and normal cellular features of nucleus and cytoplasm. Absence of any inflammatory or pathological features in the tissue section was observed, overall nothing abnormal detected.

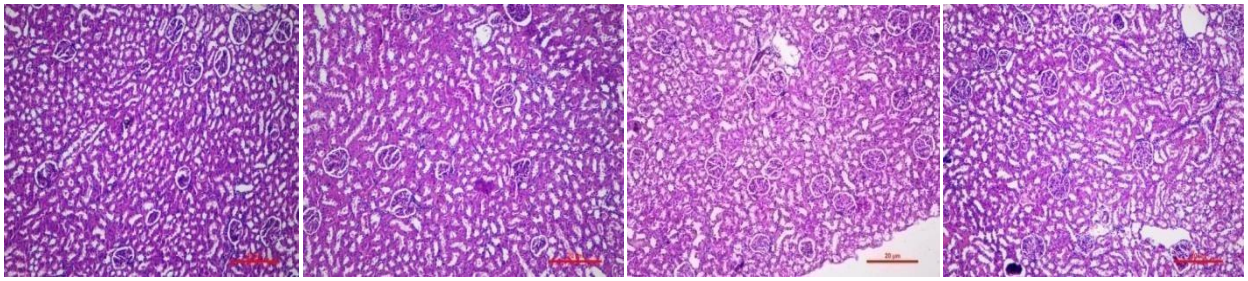


Figure: 3. A) Normal control, B) Satellite control, C) Test group, D) Satellite test.

The histopathology of Liver tissues showed Normal Histopathological appearance of hepatocytes, central vein and portal triad was observed. Hepatocytes appeared arranged in hepatic cords with normal cellular features with respect to size, shape, nucleus and cytoplasm.

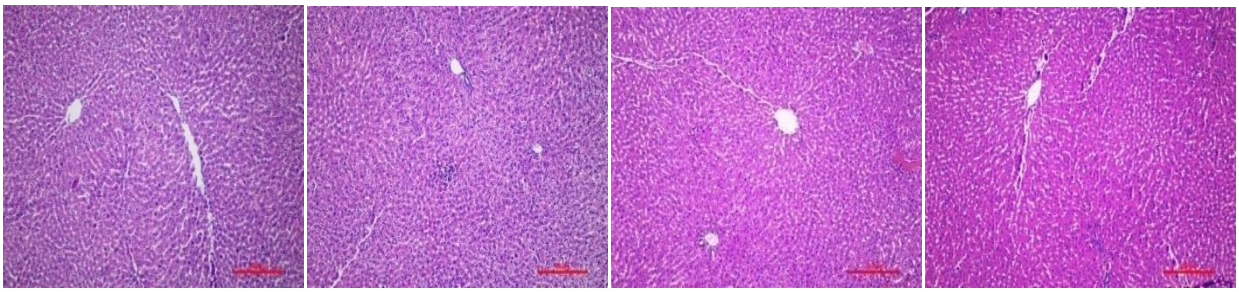


Figure: 4. A) Normal control, B) Satellite control, C) Test group, D) Satellite test.

The histopathology of skin tissues showed Normal Dermis and Epidermis layer of skin. Normal histomorphology of hair follicles and sebaceous glands with adequate collagen in the subcutaneouslayer. Absence of any inflammatory lesions was observed.

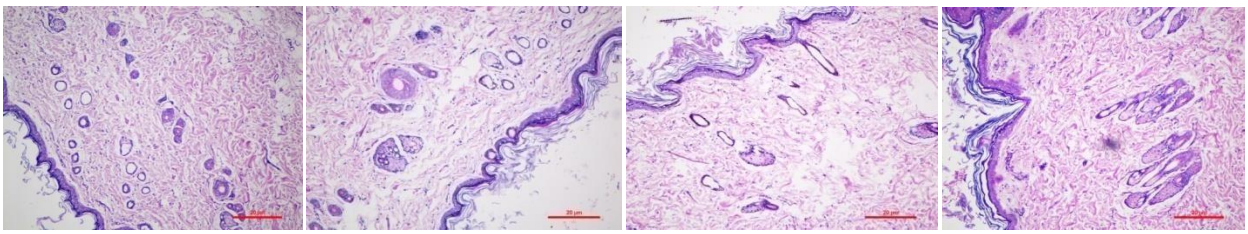


Figure: 5. A) Normal control, B) Satellite control, C) Test group, D) Satellite test.

DISCUSSION

Nanoparticles present in cosmetics products could be a risk factors for toxicological processes. Especially silver nanoparticles which are mostly used because of its potent antibacterial activity and wide biological application including medical devices, it is conceivable that the human body directly or indirectly is exposed to nanoparticles through various routes, including inhalation, injection, and dermal penetration.[10] Most of the information available on toxicity mechanism of silver nanoparticles is based on in-virto and in-vivo studies, but very less data is available on in- vivo studies especially via dermal route. There are many studies done which focus on the toxicityof silver nanoparticles, giving less important towards its benefits of therapeutic application. Therefore it was necessary to focus on the particles size which could be nontoxic and give its benefits which was the major concern of the study. Thus, we decided to focus to the particle size ranging from 100 and 200

nm which could be nontoxic and give the benefits of silver nanoparticles. We tried in the present study to determine the toxicity of silver nanoparticles via in-vitro and in-vivo both.

The mechanism of toxicity of nanoparticles mostly depends on properties such as surface area, size and shape, capping agent, surface charge, purity, structural distortion, and bioavailability. Study done on A549 cells which were exposed to silver particles (20-200nm) for 24 hours, indicated that agglomeration, particle size, and cell type are critical factors for cytotoxicity.[11] Accumulation of smaller silver nanoparticles in cell are said to cause cytotoxicity. Smaller silver nanoparticles (5-28 nm) can produce greater amount of hydrogen peroxide and also induce greaterinflammasome formation.[12] Similarly the potential toxicity of silver nanoparticels on skin tissue was evaluated where this particle was introduced to an immortalized human keratinocyte cell line which was considered as a suitable model for evaluating the toxicological potential of nanomaterials that can cause damage in-vitro.[11] In case of fibroblast, a study was performed to characterize the resistance of CCL-153 and RTgill-W1 cells which were exposed to silver nanoparticles (10-100nm). The result confirmed that silver nanoparticles with smaller size have the ability to enter cells easily thereby decreasing the cell attachment and thus lowering the resistance values.[13] Interestingly another study showed that silver nanoparticles of average size 6 nm were nontoxic to mouse fibroblast line (NIH-3T3) and HaCaTs.[15] Thus it is evident that several studies have reported both beneficial and toxic effect of silver nanoparticles and it was therefore necessary to evaluated 100-200 nm silver nanoparticles cytotoxicity. It is expected that the biokinetics of nanoparticles, usally measured as the rate of nanoparticles uptake, intercellular distribution and exocytosis is contribute tremendously to their toxicity, and thus it was important to perform a cell viability MTT assay to evaluate the possible toxicological effect of silver nanoparticles on L929 cell. Silver nanoparticles in present study exerted no such significant cytotoxic effect toward normal cells L929 even at the highest concentration 100 μg/ml, for both 100 and 200 nm particles, showing cell viability above 70 % with the IC_{50} value of 9.1 and 9.3 μg/ml for 100 and 200 nm respectively. Thus, it can be said toxicity can be dose depend and timedepend manner. Cytotoxicity studies are often limited by the fact that the interference of nanoparticles with cell viability assay remains unexplored in most cases. As MTT assay is just anti-proliferative assay. It does not indicate whether cells are undergoing apoptosis or necrosis butat least we can confirm about viability of cells.

In the second part of this study we focused on acute and sub-acute dermal toxicity. As most of

the data available on toxicity of silver nanoparticles are on in-vitro studies with limited information on in-vivo studies. Many consider silver to be more toxic than other metals toxicity especially when it is in nano form having different toxicity mechanism compare to dissolved silver.[15] Particles which are very small have the ability to enter, translocate within, and damage living organisms, resulting primarily from their small size, which allows them to penetrate physiologicalbarriers, and has the ability to travel within the circulatory systems.[16] Mostly this route of exposure occurs mainly through cosmetics, sunscreens, textiles and clothing imbedded with silvernanoparticles. Nano particles are known to be absorbed by the skin. Skin which is broken, or skinwhich is cut and with wounds, may give rise to easier and direct absorption of nanoparticles into the blood stream and translocation in the body. The fate and effects of these particles on, and within the skin and human needs to be understood and need detail explanation. Other problems still to be investigated include the interference with resident microflora on the skin.[17] Earlier studies have shown that nano-forms of different particles are more toxic than their micro-counterparts after acute exposure via the oral route.[18] Most of the studies on silver nanoparticles are limited to inhalation or oral administration. Therefore, we tried to focus on dermal application. In acute dermal toxicity of ointment containing 1% and 3% of silver nanoparticles in the present study showed contrasting result to the result reported by Anoushe Raesian.[19] Ointment containing 1% of silver nanoparticles was found to be safe with no signs of erythema or oedema observed on theskin surface. Whereas, 3% Ointment caused reduction in the body weight during 14 days study duration and caused mild irritation (redness) during 24 hour post application and thus we decidedto follow the repeated dose of application of the test material to assess the safety of the test materials and thus was decided to perform sub-acute dermal toxicity further.

As there was no mortality recorded during application of 3% silver nanoparticles ointment in preliminary study, we practically considered it as nontoxic agent in the acute dermal toxicity studyand were further studied for sub-acute dermal toxicity to evaluate the toxicity if any in detail. Recently many researchers claimed that there is no effect on body and organ weight in the study done for oral toxicity nanosilver for 28 days in rats. Also the study which is done on the mice fororal administration of 1 mg/kg body weight of 22 nm, 42 nm, 71 nm, and 323 nm silver nanoparticles for 14 days did not result in any side effect on body weight.[20] In our study we usedthe dermal application to evaluate possible systemic toxic effects of Ag-NP for 200 nm and showed similar result with no clinical sign for toxicity during the 28-days exposure also, no significant organ-weight changes were observed in either the male or

female rats after 28 days, except for theexamination of heart, there were a slight difference in the weight of heart in satellite male which was minimal for ($p<0.05$).

Hematological profile and biochemical parameters such as blood cells count and enzymes activityare mostly evaluated to provide useful information for diagnosing the state of health in patient and animals. Our results on clinical chemistry and hematology differ from the inhalation studies and oral study.[21],[22]

In present study there were no abnormal findings at hematological parameters, glucose content, creatinine and uric acid levels in silver nanoparticles ointment treated-rats. The unchanged levelsof creatinine and urea in the present study indicated normal renal functions in the silver nanoparticle ointment treated rats. Some minimal histopathology was noted in the Kidney, with focal cellular swelling of few tubules was noted in Satellite control, test group, and satellite test group but showed normal Histological features of renal parenchyma comprised of glomeruli and renal tubules. Intact proximal and distal convoluted tubules with presence of tubular epithelium and normal cellular features of nucleus and cytoplasm. Noticeably there was absence of any inflammatory or pathological features in the tissue section.

One of the studies suggests that repeated use of silver nanoparticles orally may cause a mild to moderate toxicity, as indicated by time, size and dose dependent toxic responses in liver tissues. In one of the studies it was reported that silver nanoparticle (size range 71-1300.5 nm) cause inflammatory mediator expression in liver histology. Large size particle does not penetrate the cell membrane effectively but pressurizes the area where it abnormally accumulates. Rather than causing intracytotoxicity it causes teratogenic effects at tissue level by hampering nutrition.[23] Interestingly in present dermal study of silver nanoparticles having average of 200nm showed, notoxic effect and showed the normal histopathology of liver with normal appearance of hepatocytes, central vein and portal triad when observed. Hepatocytes appeared arranged in hepatic cords withnormal cellular features with respect to size, shape, nucleus and cytoplasm. Also, there was no decrease or increase in blood chemistry result of alkaline phosphatase (ALP), serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) in the liver enzyme levels in serum of 200 nm silver nanoparticle ointment treated rats.

A silver nanoparticle causes toxic responses according to three main mechanism oxidative

stress, DNA damage, and cytokine induction. It is also said that the presence of silver ion-reactive, thiol- containing compounds are predisposing factors in silver nanoparticles toxicity.[24] Study has also demonstrated that nanoparticles are incapable of penetrating beyond the most superficial layers, corresponding to a depth of 2–3 μm, of the stratum corneum (the outermost, 15–20 μm skin layer)when tested against the nanoparticles size 20-200nm. Overall, there results demonstrate objectively and semi-quantitatively that nanoparticles used tropically cannot penetrate beyond thesuperficial layers of the barrier, and are highly unlikely, therefore, to reach the viable cells of the epidermis or beyond even partially damaged skin[25] Whereas there are some study that says thatintracellular dissolution of silver nanoparticles occurs about 50 times faster than in water.[26] Veryfew studies are done on the nanoparticles bigger than 100 nm in dermal exposure manner. The present study evaluates the toxicity of silver nanoparticles via dermal application. The histopathology of skin tissues showed normal Dermis and Epidermis layer of skin. Normal histomorphology of hair follicles and sebaceous glands with adequate collagen in the subcutaneouslayer. Absence of any inflammatory lesions was observed. Also, no significant difference was observed in heamatology study. Thus, we can say that silver nanoparticles more than 200 nm are safe to use in dermal application and do not show any adverse effect on skin when used at the minimal dose. It is always recommended to use slightly larger nanoparticles as smaller nanoparticles tends to show greater risk of toxicity and specially for dermal use particle larger than50 nm can show its effective result without any toxicity.

CONCLUSION

Silver nanoparticles and silver ions both can produce ROS and cause oxidative stress in cells at different levels: molecules, organelles, and the entire cell. Silver nanoparticles induce stronger oxidative damage to cell membrane and organelles, including lysosomes, mitochondria, and the nucleus, directly resulting in apoptosis or necrosis. In present study the cytomorphological changes of silver nanoparticles on L929 cell lines at different concentrations showed higher viability and the difference between control is less than 3 % for higher concentration and can almost be considernontoxic to cell. The IC_{50} value 9.1 and 9.3 μg/ml predicts that the 100 - 200 nm silver nanoparticles prove to be a promising drug for dermal application.

The toxicity potential of silver nanoparticles was evaluated in this study and it can be concluded that 0.1 mg/kg of silver nanoparticles dose in the dermal application which contain

3 % of silver nanoparticles in the ointment are safe to use. Overall it can be said that the minimum dose used for dermal application can be useful as in general any drug which are used on high dose and for longer duration can have a side effect and this goes the same with silver nanoparticles.

All most all study done on Silver nanoparticles focuses on smaller particle size and due to the smaller particle size its toxicity also increases as these particles have the ability to reach the bloodbrain barrier, into the blood vessels and get deposit in the different organ of the body. Silver nanoparticle that are mostly used as acne preparation, as an anti-aging preparation and other cosmetic preparation that are mostly used on dermal level should contain the particle size larger than 50 nm, as the intracellular space of the outer most layer of the skin ranges from 50 – 100 nm[27] and thus if particle size which are larger than 50 nm can show its effect on dermal level without penetrating the deeper layer of the skin to reach the blood vessel. In case of silver nanoparticles in the range up to 200 nm, containing minimum active concentration can show its active effect without any toxicity.

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