Original Article

In vitro Cytotoxicity of Silver Nanoparticles Incorporated in a Soft Silicone Liner

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Abstract

Introduction: Silver nanoparticles (SNPs) have recently been suggested to increase the antimicrobial properties of soft liner materials. However, their safety remains a matter of debate. This study aimed to evaluate the cytotoxicity of Mucopren® soft silicone liner material (Mucopren; Kettenbach, Germany) incorporated in SNPs. **Methods:** The SNPs with over 98% purity were added to Mucopren in 0.5, 1, 2, and 3 weight percentage (wt%) concentrations and manually homogenized. The mixture of the pieces of Mucopren plus SNPs and SNPs alone were placed in 96-well plates containing Dulbecco's Modified Eagle Medium culture, FBS, and antibiotics with L929 fibroblasts. Cell viability and biocompatibility were determined after 1, 2, and 3 days of incubation using the methylthiazol tetrazolium assay. Optical density was read by an ELISA reader at 570 nm and compared to those of positive and negative controls. **Results:** Among Mucopren mixed with different SNPs concentration, the cell toxicity had no significant difference in the same days, and cell toxicity decreased over time (P=0.016). The SNPs alone were less cytotoxic than Mucopren incorporated SNP samples (P>0.05). **Conclusion:** Within the limitations of this study, the addition of 0.5, 1, 2, and 3wt% concentrations of SNPs to Mucopren did not cause a significant change in its cell toxicity in an in vitro condition.

Keywords: Cytotoxicity, Nanoparticle, Silver, Soft Liner

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Introduction

Soft liners are commonly used to improve the quality of injured tissue, support denture structures, and enhance the retention and stability of dentures in patients with thin and sharp alveolar ridges, extensive ridge resorption, severe bony undercuts, genetic or congenital defects of the palate, and ill-fitting dentures (1). However, these materials easily degrade and are susceptible to colonization by microorganisms (2, 3). About 65% of patients using dentures develop denture stomatitis in which *Candida Albicans* is the main responsible (1). It adheres to both denture base and soft liner and causes serious complications, especially in elderly patients (2, 3). The regular replacement of the liner, along with proper oral hygiene and use of antimicrobial agents may be efficient in controlling the colonization of microorganisms. Sodium hypochlorite, chlorhexidine, glutaraldehyde, triclosan, peroxides, and microwave radiation have also shown to be effective for this purpose (4). Silver and silver-containing compounds have long been used as antimicrobial agents with optimal tissue response and low toxicity (5, 6). Silver nitrate is used for the treatment of visceral diseases, salivary gland fistula, and bone abscess, and silver sulfadiazine is also used as a broad-spectrum antibiotic in burns (7). Silver-zeolite has shown antiviral and antifungal properties when added to soft liners (3, 8). Following the introduction of nanotechnology, silver nanoparticles (SNPs) were introduced as the new generation of antimicrobial agents

(7). Due to their excellent antimicrobial properties, SNPs are suitable for wound healing; therefore; they are extensively used in medicine (9,10). The AgNPs have been incorporated into dental biomaterials to prevent or reduce biofilm formation (11). A greater surface to volume ratio and small particle size results in excellent antimicrobial action without affecting the mechanical properties, and this unique property makes these materials the filler of choice in different biomaterials (11, 12). The SNPs added to soft liners may serve as hidden antifungal agents, decreasing microorganism adherence, which is optimal in the oral environment (13). This fungicidal activity of SNPs has been documented to be equal to other antifungal agents, such as amphotericin B and fluconazole (14). Although recent studies have proved the antimicrobial effect of SNPs on denture hard and soft liners (13, 14), the hazards associated with human exposure to these materials have not been investigated to facilitate the risk assessment process. Some authors even posed the possibility of the cytotoxic effect of soft denture lining materials themselves, whereas others considered them quite biocompatible (16-19). Silver seems to be distributed in all organs, especially the intestine and stomach. In the skin, silver induces a blue-grey discoloration termed argyria (20). Another potential route of exposure to SNPs is through the respiratory system. Cytotoxicity and genotoxicity of SNPs in the human lung cancer cell line have been evaluated, and the levels of cytotoxicity were reported following the production of reactive oxygen species and increased release of lactate, causing mitochondrial and DNA damage (21, 22). Therefore, a Tolerable Daily Intake value of 2.5 µg/kg bw/day has been reported for silver (20). The size of nanoparticles is a critical factor in determining inflammatory immune response even when noncytotoxic concentrations are employed (22). In vitro tests for the assessment of cytotoxicity of SNPs have shown that these nanoparticles can pass through the nuclear membrane. This enhances the action of medications containing SNPs as they can target the function of hydrophilic surfactants, such as Tween 80 (23). The interactions between cells and nanoparticles have also been shown to result in DNA damage, cancers, developmental toxicities associated with future growth retardation, deformity, or even fetal death (24). Therefore, the necessity of designing new studies aimed at overcoming the toxicity of SNPs concerning their antimicrobial activities is emphasized. This study aimed to evaluate the cytotoxicity of SNPs added to Mucopren soft silicone liner material (Mucopren; Kettenbach, Germany).

Materials and Methods

The soft liner selected in this study was Mucopren cold cure silicone long-term liner (Mucopren; Kettenbach, Germany) supplied as two pastes.

Specimens preparation

In a sterile condition, under a cell culture laminar hood, SNPs (Nanoshel; 80-100 nm in size; 99.9% purity) were poured into a ceramic mortar, and the particles were milled to make a homogenous mass. The soft liner base and catalyst were injected into two mixing pads in equal volumes. Each mixing pad, base, and catalyst was separately weighed by a digital scale (0.000 gr, Kern, Germany). Considering the required 0.5, 1, 2, and 3 weight percentages (wt.%) of SNPs, they were added to the base paste under the hood and mixed for 2 min. The mixture was then mixed with the catalyst for another 1 min. To ensure uniform distribution and homogenous dispersion of SNPs, transverse sections were made from each wt.% samples and evaluated under a scanning electron microscope (KYKY-EM3200, KYKY Technology, Shanghai, China) (Figure 1). Then, the specimens were cut in 10 mg pieces (weighted by a digital scale, 0.000 gr, Kern, Germany), exposed to ultraviolet light for half an hour, and placed in sterile tubes to prevent microorganism contamination.

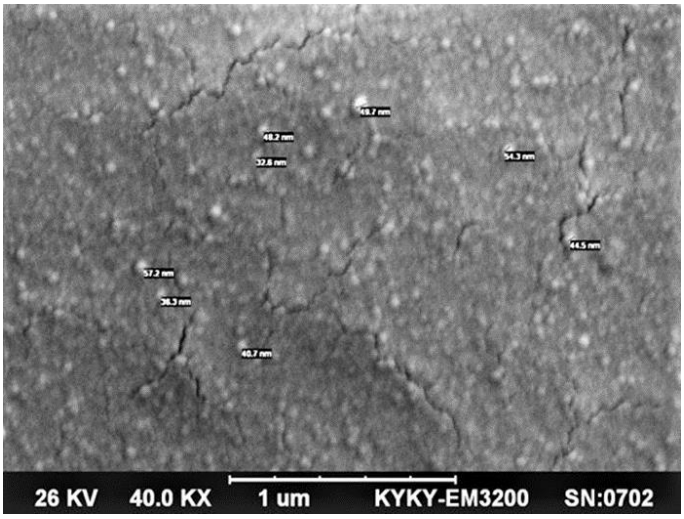


Figure 1. Electron microscopic micrograph of a sample containing silver nanoparticles

Methylthiazol Tetrazolium Test

Cytotoxicity assay was performed according to the International Organization of Standardization (ISO) 10993-5. In this standard, 6 samples were required for each group. The L929 standard fibroblast cell line was obtained from the National Cell Bank of Iran and grown on a 100-mm diameter Petri dish. Fourth passage cells were counted, and 30,000 cells were seeded in wells of a 96-well plate and incubated at 37°C in 5% CO_2. After 24 h, the pieces of each concentration of Mucopren incorporated SNPs specimens were placed in each well together with 100 µl of Dulbecco's Modified Eagle Medium, fetal bovine serum, and antibiotics (i.e., penicillin/streptomycin) (Figure 2). Table I demonstrates the study groups and subgroups. Therefore, the cells were exposed to Mucopren incorporated into different concentrations of SNPs or SNPs alone. It should be mentioned that the amount of SNPs in the SNPs alone group was measured to be the same as the amount of SNPs incorporated in corresponding Mucopren groups. Culture medium without the mixture served as the negative control, and distilled water, which is fatal for the cells, was used as a positive control. Cell viability was assessed by the conversion of 3-(4,5-dimethylthiazol–2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to insoluble formazan (25). The MTT assay was performed at 24, 48, and 72 h following the incubation of cells. Continuing the test does not seem logical due to a reduction in cell metabolism after being confluent, and making subculture with changing the media will return the condition to the first step. The overlaying solution was removed, and the cells were rinsed with phosphate-buffered saline (PBS) (Biowest, France). The MTT solution (Atocel, Austria)

Table I. Study groups and subgroups

Group number	Group	Code	Subgroup
1	Mucopren	-	-
2	Mucopren plus SNPs	A	Mucopren plus 0.5% SNPs
		B	Mucopren plus 1% SNPs
		C	Mucopren plus 2% SNPs
		D	Mucopren plus 3% SNPs
3	SNPs	A	0.5% SNPs
		B	1% SNPs
		C	2% SNPs
		D	3% SNPs
4	Positive and negative controls	-	-

SNPs: Silver nanoparticles

with 5 mg/mL concentration in PBS was prepared and diluted 1:10 with a culture medium and added to each well. The plates were incubated at 37°C for 3 hours in 98% humidity and 5% CO_2. During this time, viable cells with active metabolism converted the yellow MTT salt to insoluble formazan crystals using the mitochondrial succinate dehydrogenase enzyme (Figure 2). These crystals were seen under a light microscope. After ensuring the formation of formazan crystals under an inverted microscope, the overlaying medium was gently removed and dimethyl sulfoxide was added to wells to dissolve the formazan crystals to form a purple solution. Optical density, which had a direct association with the metabolic activity of cells, was read by an ELISA reader (Metertech M965 Microplate reader, Korea) at 570 nm wavelength. Cell viability (compared to the positive and negative controls and expressed in percentage) was calculated and reported at different time points.

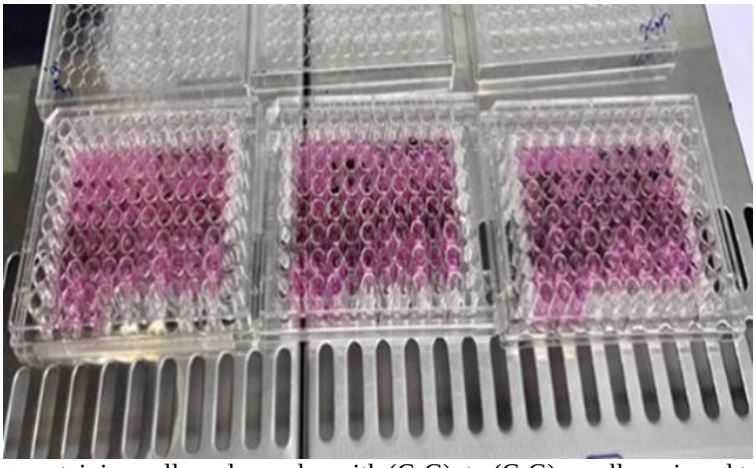


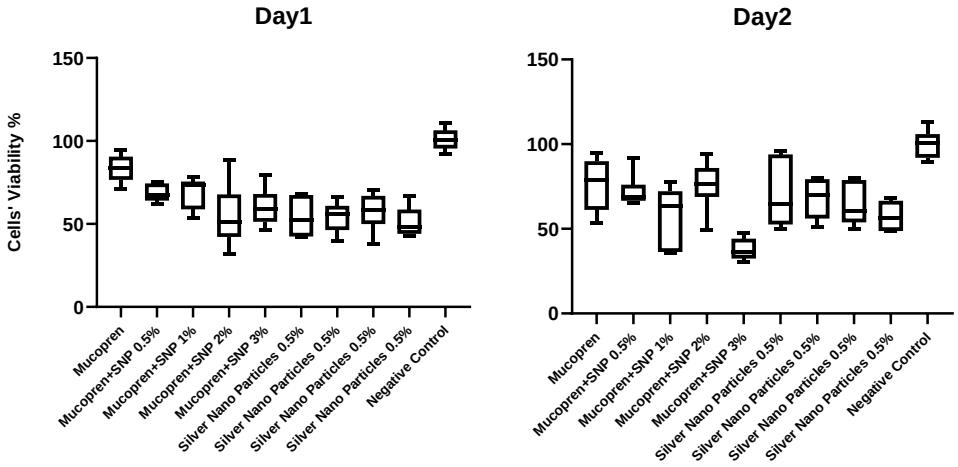
Figure 2. 96-well plate containing cells and samples with $(C\text{-}G)_2$ to $(C\text{-}G)_{11}$ wells assigned to test groups and other wells assigned to negative and positive control groups

Statistical Analysis

The SPSS software (version 22, SPSS Inc., IL, USA) was used to assess the data of the MTT test with the analysis of variance (ANOVA) and Tukey's HSD paired comparison test. The results were expressed as the mean ±standard deviation of viable cells for different experimental groups. P-value less than 0.05 was considered statistically significant.

Results

The highest percentage of cell viability on the first, second, and third days of incubation was noted in groups 1, 3C, and 3D, respectively. Table II and Figure 3 describe the percentage of cell viability at different experiment days. One-way ANOVA revealed a significant difference at 1, 2, and 3 days in different groups (P=0.04, P<0.0001, P<0.0001 respectively). Also, the results of pairwise comparisons by Tukey's test showed that by the increase in the concentration of SNPs, cell viability decreased. However, no significant difference was noted between Mucopren and Mucopren plus SNPs in terms of cell viability (P>0.05). Cell viability values were also higher in SNP concentrations without Mucopren. According to two-way ANOVA, the effect of material (i.e., the combination of different concentrations of Mucopren incorporated SNPs) and storage time on the incubator and their interaction effect on the percentage of cell viability were also statistically significant (P<0.001 for all 3 days). Accordingly, cell viability increased over time, and in most groups of Mucopren incorporated SNPs, cell viability increased on the third day, compared to that on the first day.



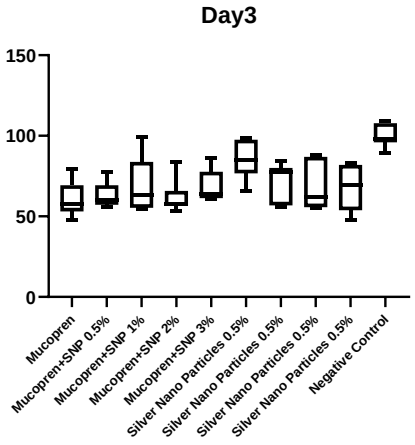


Figure 3. Results of cell viability in different groups in comparison to those in control group after 1, 2, and 3 days of incubation

Table II. Percentage of cell viability in different groups in comparison to those for positive and negative controls after 1, 2, and 3 days of incubation (n=6)

Group	Day	Mean	Standard deviation	Minimum	Maximum
Mucopren	1	66.85	16.17	78.78	94.82
(1)	2	69.72	23.42	34.07	94.51
	3	60.66	11.09	47.83	79.57
Mucopren + 0.5% SNPs	1	58.55	14.07	34.07	75.56
(2A)	2	65.45	19.67	30.77	92.06
	3	56.32	16.53	26.62	77.61
Mucopren + 1% SNPs	1	68.82	9.65	53.27	78.02
(2B)	2	57.85	17.79	35.65	77.97
	3	69.01	17.27	54.35	99.35
Mucopren + 2% SNPs	1	54.8	19.14	31.87	88.37
(2C)	2	75.86	14.76	49.45	94.14
	3	61.72	11.07	53.39	83.91
Mucopren + 3% SNPs	1	60.18	11.27	46.24	79.37
(2D)	2	37.79	6.53	30.28	47.74
	3	68.5	10.16	60.65	86.3
0.5% SNPs	1	42.4	17.69	18.38	67.06
(3A)	2	70.34	19.8	49.82	96.21
	3	85.35	12.07	65.74	98.7
1% SNPs	1	59.19	19.47	39.69	96.19
(3B)	2	79.63	10.39	66.67	97.8
	3	80.05	14.37	55.52	98.7
2% SNPs	1	65.75	16.75	38.12	88.07

(3C)	2	88.97	6.99	78.51	99.76
	3	86.67	15.79	55.88	98.48
3% SNPs	1	56.01	12.48	42.62	74.49
(3D)	2	83.99	18.59	55.71	98.78
	3	87.79	15.15	59.17	99.13

SNPs: Silver nanoparticles

Discussion

This study assessed the cell viability and biocompatibility of SNPs with over 98% purity in 0.5, 1, 2, and 3wt.% concentrations alone and in combination with Mucopren soft silicone liner material via the MTT assay. The results showed significant differences in cell viability between the groups after 1, 2, and 3 days of incubation. With an increase in the concentration of SNPs, cell viability decreased, while it increased over time. No significant difference was noted in the biocompatibility of Mucopren alone and in combination with different percentages of SNPs. Therefore, the addition of SNPs to Mucopren in our study did not significantly change the biocompatibility. Kurt et al. (26) evaluated the cytotoxicity of denture base materials containing SNPs and showed that the biocompatibility of cells did not decrease significantly when exposed to denture base materials containing 0.25% SNPs. They also concluded that SNPs did not have a toxic effect when combined with polymethyl methacrylate denture base material. The effect of time on the percentage of cell viability was significant in some and insignificant in some other groups. In Mucopren plus 1, 2, and 3wt% SNPs and all concentrations of SNPs alone, the percentage of viable cells on the third day increased, compared to that reported on the first day. It has been reported that toxic compounds are released on the first day of exposure and broken down over time or chemically react with some other compounds in the culture medium and though change the cytotoxicity of the environment (16). Previous studies have shown that the biocompatibility of polymethyl methacrylate increases over time (16, 27, 28). It should be noted that results of biocompatibility may also be attributed to different study designs, incubation conditions, or type of used kit (29). To evaluate cytotoxicity, biocompatibility tests must be performed using the most suitable type of cells to better simulate mutual interactions between cells and materials as the type of cell line selected can significantly affect the results (30). Considering their common applications (31, 32), L929 fibroblasts were used in the present study. Based on the results of the MTT assay, in most Mucopren and SNP groups, cell viability increased on the third day, compared to that on the first day. This finding could be due to the fact that MTT kits can only measure cell viability by assessing mitochondrial metabolism. Besides, in the early hours of exposure of cells to Mucopren incorporated SNPs, the nanoparticles are separated and consequently have the highest toxicity. The particles aggregate over time, and those percentages of cells that remain viable start to proliferate. This proliferation results in the consumption of glucose in the environment and acid production, which results in the further accumulation of SNPs and further reduction in cytotoxicity (33). Based on the results of this study, SNPs alone showed lower cytotoxicity than Mucopren incorporated SNPs. Therefore, the authors hypnotized that perhaps this toxicity is due to the release of certain compounds from the Mucopren and their reaction with SNPs. This finding, of course, needs further investigation. Baker et al. (34) measured the number of toxic compounds in the saliva of patients with the denture and showed that polymethyl methacrylate denture base materials release toxic compounds for up to 1 week after using dentures. Therefore, tissue irritation immediately after the use of dentures may be due to the release of specific materials, and it has been recommended to immerse the denture in water for some time before its placement (35). Denture liners are generally considered biocompatible. Atay et al. (18) assessed the cytotoxicity of soft and hard denture liners and reported that all soft and hard liner materials except for GC tissue conditioner (GC Corporation, Tokyo, Japan) had optimal biocompatibility. According to ISO 10993-5, the cell viability of more than 70% is considered to be a requirement of nontoxicity (18). Song et al. have studied the cell cytotoxicity of some soft liners. They studied the cell toxicity by exposing cells to soft denture lining materials extract in a culture medium after 24-hour storage of samples in distilled water. They expressed the cell viability of 90% and more as nontoxic and within the range of 60% to 90% as slightly toxic. In their method of cell exposure toward soft liners extracts, the cell viability of Mucopren itself on the first day was 86.4±10.3 (compared to that reported for the control group); therefore, it is considered slightly toxic yet acceptable in accordance with the standard. In our study, the cells were directly exposed to the materials without water storage to provide an environment more likely to the clinical

application of soft liners. Although cell viability in Mucopren even decreased more by this method, it still remained in a slightly toxic range. As the results of the cell viability of Mucopren incorporated into different concentrations of SNPs were not significantly different from Mucopren itself, it is logical to consider these groups acceptable as Mucupren. Denture soft liners in direct contact with the connective tissue cells in the absence of epithelium can cause allergic reactions, such as burning sensation in the mouth or redness and pain in the gingival. Vesicles and oral ulcers have also been reported following the use of these materials (36, 37). Phthalates and other aromatic esters of carboxylic acid are used as plasticizers in the composition of acrylic soft liners. When immersed in water, these plasticizers release from the liner (38). In addition, Munksgaard reported that the mean release of dibutyl phthalate on the first day is about 11-32 times more than the daily acceptable threshold for an adult (39). Park et al. (40) assessed the short-term cytotoxicity of different soft liners using the agar method and showed that despite the decrease in cytotoxicity over time, these materials should be used for short periods to prevent their cytotoxic effects. The highest release of monomers and additives often occurs during the first days (41, 42); however, a 3-day incubation period considered short-term. The long-term cytotoxicity result of Mucopren incorporated SNPs is still needed to be evaluated. Moreover, the results might have been different in the clinical settings due to the oral conditions, presence of saliva, and rinse of the oral cavity. Aside from the cytotoxicity, the morphology of cells should also be evaluated to determine the mechanism of cytotoxicity. Furthermore, the effects of the addition of SNPs on the mechanical properties of materials and color change should be evaluated. Thus, it is required to carry out further studies on the properties of SNPs in combination with soft liners.

Conclusion

Within the limitations of this study, the addition of 0.5, 1, 2, and 3wt% concentrations of SNPs to Mucopren did not cause a significant change in its cell toxicity in an in vitro condition.

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Conflict of Interest

The authors declare no competing interests with regards to the authorship and/or publication of this article.

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