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Assessment of dermal biocompatibility and antimicrobial activity of silver-made nipple cap

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ABSTRACT: Breastfeeding is considered a gold standard for infant development. However, several complications such as nipple fissure, infection and ulceration might be accomplished during nursing, which may lead to early cessation of breastfeeding. For this purpose, silver-made nipple cap SilverNurse® (SN) has been designed to control symptoms of nipple fissure in lactating women by aiming to eliminate antimicrobial resistance compared to the alternative treatment methods. Therefore, aim of the present study is to assess antimicrobial efficacy and dermal safety of silver-made SN in context of biocompatibility with cytotoxicity assay, *in vitro* EpiDerm skin irritation and *in vivo* skin irritation tests. According to the represented results, SN exhibited potent antimicrobial activity on *Staphylococcus aureus, Escherichia coli, Enterococcus hirae,* and *Pseudomonas aeruginosa* bacteria as well as *Candida albicans* fungi. Cytotoxicity by MTT assay showed that prepared extract of SN has a lack of cytotoxicity in tested concentrations. In addition, *in vitro* EpiDerm skin irritation model and *in vivo* skin test demonstrated that SN can be classified as biocompatible and non-irritant for dermal use without any sign of erythema and edema. Therefore, in the present study, it was evaluated that SN can be applied safely in daily comfort fields for lactating women without dermal complications related to infection and skin irritation.

KEYWORDS: Silver; biocompatibility; skin irritation; EpiDerm SIT; antimicrobial activity.

1. INTRODUCTION

Breastfeeding is considered as the gold standard for infant feeding owing to the rich content of breast milk containing nutrients that infants require for their development, well-being and safety [1]. The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommended that infants should be given just breast milk in the first 6 months of infancy and its continuation until 2 years along with supplementary feeding [2]. According to the WHO, only 40% of infants under six months of age are exclusively breastfed around the world. The breast fissure is one of the most common factors reported by women to early cessation of breastfeeding [1]. It can be defined as a painful rupture or ulceration of the nipple skin at the tip and areola around the breast that manifests as a cleft, skin loss, wound, or clinical signs of erythema, edema, and blisters. [3]. The nipple fissure is experienced by 80% to 90% of the breastfeeding women [1]. Beside incorrect breastfeeding and nipple sucking, poor hygiene and the nipple infection with *Staphylococcus aureus* and *Candida albicans* are the widespread causes of the breast fissure [4]. Through the decades, various unproven interventions have been used to prevent or relief breast pain, including topical ointments, solutions, sprays, air and light exposure to the nipple, time-restricted breastfeeding, breastfeeding education, herbal medications as mint extract or tea bag as well as preparations such as yeast protection shells, hydrogel, and adhesive polyethylene films [1, 5, 6]. Since ancient times, silver has been known as a useful and natural measure against a broad range of microorganisms [7]. Despite the fact that its use has declined due to the discovery of new antibiotics, it has lately been re-evaluated following occurrences of antibiotic resistance [3].

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It is well known that silver ions are able to precipitate protoplasm proteins in the bacteria, affect enzymes, interrupt cell division and damage the cellular envelope [7]. SilverNurse® (SilverNurse, Istanbul, Turkey) is a cup-shaped medical device composed of trilaminate silver (99.9%) (Figure 1) and commercial counterpart of Silver Cap®, which has been marketed in Italy since 1998. It is used to relief symptoms in lactating women due to nipple pain in after every breastfeeding session without altering the taste and smell of breast milk and can be simply cleaned with water. Compared to the other commercial equivalents, SilverNurse® (SN) is composed of 925 sterling silver without copper and nickel residue, which restricts release of silver ions and prevents babies from absorbing them [8]. Owing to its unique design and scrape on the inside side, it promotes a good latch to the nipple while keeping the atmosphere wet and hypoxic. According to an observational and prospective study by Marrazzu et al. (2015) it was stated that Silver Cap provided a noteworthy resolution of painful symptoms in lactating women compared with the Silver Cap® non-using control group. In addition, it was suggested that treatment with Silver Cap® was more appreciated by the participants compared to the standard care in the study [3]. Although adverse reactions related to Silver Cap® use have not been reported since 1998, there are no available data regarding to biocompatibility of these devices. It is known that dermal silver exposure has relatively low potential for skin irritation. Moreover, intact skin is considered as an effective barrier for silver uptake, as well [9]. Considering their purpose of usage, biocompatibility tests such as cytocompatibility, hemocompatibility, or tissue compatibility are designed to reflect biocompatible nature of medical devices in the human physiological environment. Hence, biocompatibility tests are crucial for medical devices in order to assess consumer safety [10]. In the present study, we aimed to explore the biocompatibility of handmade-SN including *in vivo* and *in vitro* tests as well as antimicrobial efficacy *in vitro* for the first time in literature according to ISO standards [11–13].

Figure 1. Representative image of SilverNurse®.

2. RESULTS AND DISCUSSION

2.1. Antimicrobial efficacy

Silver ions are known to have a strong antimicrobial effect and used as antimicrobial agents in medicine for decades owing to its growth inhibitory effects on microorganisms [14]. In the present study, it was observed that the SN has antimicrobial effect against gram positive and gram negative bacteria (> 7 log reduction) and to a lesser extent fungal *C. albicans* (>6 log reduction) within 90 minutes (Table 1).

Table 1. Log reduction of test organisms exposed to SN extract.

Test Organisms	Log Reduction
Staphylococcus aureus (ATCC 6538)	7.25
Escherichia coli (ATCC 10536)	7.20
Enterococcus hirae (ATCC 10541)	7.12
Pseudomonas aeruginosa (ATCC 15442)	7.07
Candida albicans (ATCC 10231)	6.11

Previously, it has been shown that silver has remarkable antimicrobial effect on a broadspectrum of microorganisms whether in the form of nanoparticle [15] or coating material [16,17]. Since long-term use of antibiotics are known to be related with antimicrobial-resistance [15], the invention of new antimicrobial

agents has gained importance. Although antibiotic use is considered as compatible with breastfeeding [18], period of breastfeeding might be suggested as relatively long-term for antibiotic use. Therefore, with a potent antimicrobial activity, silver might be considered as an alternative antimicrobial agent without antimicrobial resistance and no antimicrobial transfer to breastfed babies.

2.2. Cytotoxicity by MTT assay

As a part of biocompatibility, cytotoxicity was assessed by MTT assay, which is an imperative pre-screening method with high sensibility and convenience [10]. Our results represented that tested concentrations of SN extracts showed cell viability above 70% compared to control for a 24 h exposure, which indicated that SN did not show a cytotoxicity in L929 cells (Figure 2).

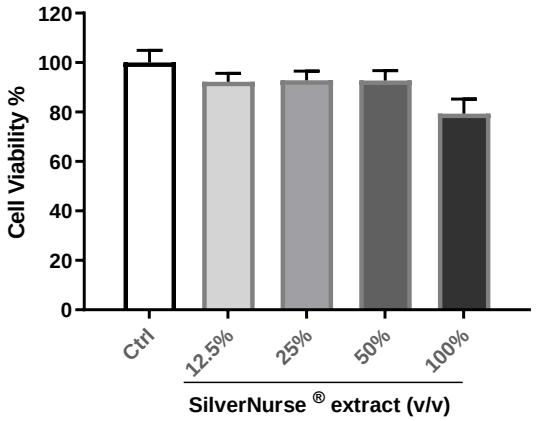

Figure 2. Cell viability of L929 cell line after the 24 h exposure of four different concentrations of SilverNurse® extracts (Ctrl: Control group treated with cell culture medium).

Studies in literature mainly held cytotoxicity of silver as in the form of nanoparticle [19,20] or silver-coated implants [21] instead of pure silver made biomedical device. Therefore, as far as we knew, represented study is the first report evaluated cytotoxicity of silver- nipple cap in context of biocompatibility.

2.3. *In vitro* skin irritation test

Skin irritancy testing is one of the biocompatibility tests recommended for all medical devices along with the cytotoxicity. Within this concept, a validated test method, EpiDerm SIT (MatTek, Ashland, USA), was used to assess dermal irritation potential of SN as an essential component of the safety evaluation [22]. According to testing results, SN extract did not induce skin irritation since the assessed tissue viability did not decline below 50% due to exposure of prepared extract. On the other hand, assay protocol accomplished the acceptance criterion which the relative viability of 5% SDS exposed tissues below 20% (Figure 3). Hence, it might be suggested that SN did not induce dermal irritation and it can be safely used by lactating women.

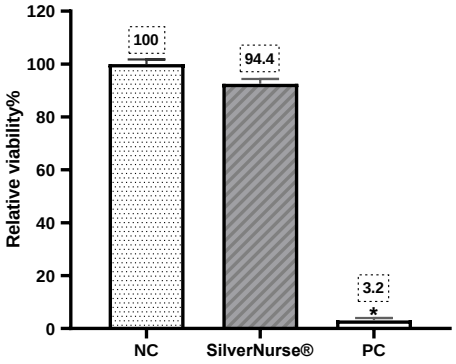

Figure 3. Relative cell viability (%) after exposure of SN extract in Epiderm SIT *in vitro* irritation test.

NC: Negative control (DPBS), PC: Positive control (5% SDS), SN: SilverNurse extract (100%, v/v). The significant differences between NC and PC was defined with *p<0.0001.

2.4. *In vivo* skin irritation

The skin is the largest organ of human body and an important route of exposure for environmental toxicants [23]. Hence, *in vivo* dermal irritation potential of SN has been assessed for a comparative observation on intact skin. According to our results, no significant body weight changes, abnormal clinical signs or mortalities were observed in the experimental animals (data not shown). In addition, negligible erythema without edema formation was observed 1, 24, 48 or 72 h after exposure to the SN. The negative control group also showed no signs on the skin during the experimental period. The primary irritation index for SN was 0, and none of the animals exhibited any skin damage during 72 h (Table 2). Thus, *in vivo* skin irritation test conducted proved that SN is non-irritant to skin, where the results are comparable both *in vivo* and *in vitro*.

Table 2. Skin irritation test of SN using male New Zealand White Rabbits.

		The primary irritation index		
	Animal no.	**1**	**2**	**3**
Samples	**SN**	0	0	0
	Negative control	0	0	0
	Positive control	1.66	2	1.33

SN: Silvernurse® extract; negative control: NaCl 0.9% (w/v); positive control:Sodium dodecylsulfate 10% (w/v).

Previously, dermal silver exposure as silver-nanoparticle has been reported to not induce gross irritation in porcine skin. On the other hand, microscopic and ultrastructural observations showed areas of focal inflammation in the upper stratum corneum layers of the skin [24]. In contrast, Miyani et al. (2016) suggested that silver-nanoparticles may have limited ability to penetrate skin beyond the stratum corneum and thus were unable to induce dermal irritation [25]. These controversies in the findings might be attributed to the different characteristics of nanoparticles such as size and coating, which alters skin penetration and absorption [23,25]. Therefore, studies assessed skin irritation potential of silver as in the form of nanoparticle might not reflect dermal biocompatibility of a pure-silver made medical device. Aside from nanoparticles, silver salts such as silver sulfadiazine and silver nitrate have also been reported as noteworthy wound-healing agents and used in topical formulations [26,27]. Similar to previously mentioned studies, a silver sulfadiazine loaded gel was lack of skin irritation potential and did not lead to erythema and edema in acute dermal irritation test [28]. As far as we know, the present study is the first one which assess dermal irritation of a pure-silver made nipple cap within the frame of biocompatibility. Hence, we suggest that Silvernurse® can be safely used by lactating mother without complications related to skin irritancy.

2.5. Statistical analysis

The results obtained were statistically evaluated using the GraphPad Prism 8.0 (GraphPad Software, La Jolla-CA, USA) software and $p<0.05$ was considered as significant. One-way analysis of variance (one-way ANOVA) was used to define the statistical difference between groups.

3. CONCLUSION

Silver-made medical device Silvernurse® might be classified as cytocompatible and non-irritant for dermal use according to the human skin equivalent *in vitro* model EpiDerm-SIT and *in vivo* skin irritation tests. This study suggested that the developed product can be proposed for application in biomedical and daily comfort fields for lactating women with no harmful effects in terms of dermal irritation with a potent antimicrobial activity.

4. MATERIALS AND METHODS

4.1. *In vitro* antimicrobial efficacy

TSE EN 1275 and TSE EN 1276 methods were used to test the antimicrobial effect of the sample against *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Enterococcus hirae* (ATCC 10541), *Pseudomonas aeruginosa* (ATCC 15442) and *Candida albicans* (ATCC 10231) [12,13].

For the preparation of test microorganisms, 10 mL of Nutrient Broth (for bacteria) and Sabouraud 2% Dextrose Broth (for fungi) were transferred into sterile test tubes and were marked for *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Enterococcus hirae* (ATCC 10541), *Pseudomonas aeruginosa* (ATCC

15442) and *Candida albicans* (ATCC 10231). The tubes were incubated for 18-20 hours at 37 °C in a shaker incubator with speed set for 95-120 g. The next day, tubes were centrifuged at 1409 g for 20 min at 4°C. After the centrifugation, obtained supernatant was discarded and the pellet was re-suspended with 10 mL of phosphate buffer saline (PBS) and re-centrifuged. Then, the supernatant was discarded and the pellet was resuspended with 10 mL of PBS for the second time. The final inoculum concentration was adjusted by PBS to achieve a final concentration of 1.5 x 108 cfu/mL. Following, ten-fold serial dilutions of the inoculum were made and plated by a sterile swab to verify the final concentration.

For the sample preparation, 8 grams of the test product were weighted and transferred into a sterile test tube. One mL of the bacterial or fungal inoculum and 1 mL of the bovine serum albumin as an interfering substance were added into a separate tube and mixed for 10 seconds. Then, the mixture was transferred to the tube containing of test material and mixed. After 90 minutes contact time, 1 mL of bacteria (or fungi) - product mix was transferred into a tube with 9 mL Dey- Engley neutralizing broth and mixed vigorously. Then, appropriate dilutions were immediately made with Letheen broth.

For the bacterial count 1 mL from each dilution was inoculated in microbial count Agar in dublicate, spreaded with sterile swab and the plates were incubated at 37°C for 24 hours. For fungal count, 1 mL from each dilution was inoculated in malt extract agar in duplicate, spreaded with sterile swab and the plates were incubated at 30°C for 48 hours. As a positive control, 8 mL of sterile deionized water, 1 mL bacteria (or fungi) and 1 mL albumin suspension were used.

4.2. Cytotoxicity by MTT assay

Cytotoxicity profile of medical devices should be evaluated before marketing as a part of hazard characterization [29]. For this purpose, cytotoxicity profile of SN was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test on L929 mice fibroblast cells (ATCC, USA). Briefly, the cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), which is supplemented with 10% fetal bovine serum, 1% penicillin (10.000 units/mL) and streptomycin (10.000 µg/mL) (Gibco, USA) at 37°C in humidified atmosphere of 5% CO_2. SN was extracted according to TS EN ISO 10993-12 with cell culture medium for 24±2 h at 37±1°C as a final concentration of 0.2 g/mL [11]. The seeded L929 cells in a 96-well plate were exposed to serially diluted doses of SN extract (12.5-100; v/v) by using DMEM. After 24 h of exposure, 0.5 mg/mL MTT was added to wells and incubated for 2 h at 37°C. Following the incubation, the microplate-well contents were discarded and 100 µL of isopropanol was added to the each well. Following, the absorbance of the produced purple-colored formazan was detected at 570 nm spectrophotometrically (Thermo Multiskan Spectrum, Finland). The absorbance of control group was considered as 100%. The percentage of cell viability was calculated according to the equation given below:

$$Cell\ Viability\ (\%) = (OD570_{TS})/(Mean\ of\ OD570_{ctrl}) \times 100\% \qquad [Eq.\ 1]$$

$OD570_{TS}$ is the OD of the test substance (TS) and $OD570_{ctrl}$ is the OD of the medium control.

4.3. *In vitro* skin irritation test

The reconstructed 3D human epidermal model EpiDerm (EPI-200, MatTek, Ashland, MA/USA) validated by European Union Reference Laboratory for alternatives to animal testing-EURL ECVAM was used in order to determine skin irritation of medical devices [30]. For this purpose, SN was extracted according to TS EN ISO 10993-12 with sterile 0.9% isotonic for 72±2 h at 37±1°C as a final concentration 0.2 g/mL. According to manufacturer instructions, tissues were topically exposed to 30 µL of the undiluted extract of SN, negative control (NC), and positive control (PC) for 60 min at 37°C. Sterile Dulbecco's phosphate-buffered saline (DPBS), pH 7.4 and 5% SDS (w/v) were used as NC and PC, respectively. Following SN exposure, tissues were gently washed with sterile DPBS and remaining DPBS was removed by the help of gauze pad. After washing process, the tissue inserts were transferred to another microplate containing fresh assay medium for a 24 h incubation period. Following the incubation, medium was discarded and MTT solution was added to each tissue insert for 3 h. Purple colored-formazan color by cellular mitochondria was extracted with 2 mL/tissue of isopropanol and the optical density of purple chromophore was determined at 570 nm. The prediction of skin irritation potential was determined by the alteration of cell viability of the tissues exposed to SN extract in comparison to NC. The relative cell viability below 50% was accepted as "irritant substance" according to EpiDerm SIT protocol and assessed by using the given formula below:

$$Relative\ viability\ of\ test\ sample\ (\%) = [OD_{TS}/Mean\ of\ OD_{NC}] \times 100 \quad \text{[Eq. 2]}$$

OD_{TS} is the OD of the test sample (TS) and OD_{NC} is the OD of the NC.

4.4. *In vivo* skin irritation test

New Zealand albino rabbits (two Male, one Female) were obtained from the Yeditepe University Medical School Experimental Research Center (YUDETAM) and housed at controlled room temperature (21±1°C) with a 12:12 h light-dark cycle and given free access to food and water. The experimental protocol was approved by the Ethic Committee of Yeditepe University (approval number 2020/07-1). *In vivo* skin irritation test was performed according to ISO 10993–10 (2010). SN was extracted according to TS EN ISO 10993-12 with sterile 0.9% isotonic saline for 72±2 h at 37±1°C and the ratio between the test material and the sterile 0.9% isotonic saline solution was 0.2 g/mL. Briefly, the backs of the experimental animals were shaved on both sides of the spine for application and observation of all test sites (approximately 10 cm × 15 cm). Four hours later, 0.5 mL of SN extract and negative control (0.9% isotonic saline) were applied directly to the test area located on both sides of the spine with a sufficient distance between cranial and caudal ends. The application sites were covered with sterile gauze bandages and all the application area was wrapped with the elastic bandage. At the end of the contact time (4 hours), the bandages were open and the experimental areas were observed. Samples were evaluated by assessing rubor, scar formation and edema reactions according to *in vivo* skin irritation test ISO 10993–10 guideline by irritation scoring system (0: no erythema or no edema; 1: barely perceptible erythema or edema; 2: well-defined erythema or slight edema; 3: moderate to severe erythema or moderate edema; and 4: severe erythema or edema) at grading intervals of 24, 48, and 72 h [31]. The primary irritation index was recorded according to reaction scores (Table 3).

Table 3. Evaluation of scoring results.

Average Score	Evaluation Category
0-0.4	Negligible
0.5-1.9	Light
2-4.9	Medium
5-8	Advanced

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Conflict of interest statement: The authors declare that they do not involve in SilverNurse Medikal Kozmetik İnşaat Sanayi ve Ticaret Limited Şirketi with a financial or non-financial interest in the subject matter or materials discussed in this manuscript and have the permission from SilverNurse Medikal Kozmetik İnşaat Sanayi ve Ticaret Limited Şirketi for the testing of SilverNurse® for the research and publication of this article. The charge for the tests was paid by the producer.

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