The Healing Effect of Four Different Silver Complexes on Full-thickness Skin Burns in a Rat Model

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Abstract. Aim: This study was carried out to investigate the effect of four different silver substances (S1, S2, S3, and S4) on burn wound healing in a rat model. Materials and Methods: One hundred and eighty Wistar rats were used. Animals were randomized into six groups to receive no treatment (CG, control group), and local application of the solvent of silver substances (SG, solvent group), as well as of the four silver substances (EG1-EG4 groups for substances S1-S4, respectively). On days 0, 3, 6, 12, 21, and 31 following burn wound infliction, the size and healing progress of each wound were recorded and evaluated by means of clinical evaluation, planimetry and histological examination. Results: According to our findings lower infection rates, as well as significantly accelerated wound healing and faster re-epithelialization were recorded in EG1, EG2, and EG4 compared to the other groups. Discussion: The use of S1, S2, and S4 substances proved to be an effective treatment of burn wounds that ensured better outcomes compared to the control and solvent groups, as well as with the use of S3 substance. Nevertheless, they failed to produce short-term healing of the full-thickness burn. Further research is required to examine the possibility of speeding the treatment of full-thickness burns by these complexes in order to reduce healing time to acceptable limits and prevent the need for surgery.

Burns destroy the skin barrier against invading bacteria, rendering the burn wound itself the primary source of local and systemic infectious complications, which are a major source of morbidity and mortality in patients with burns (1, 2). As a result, several topical antimicrobial agents have been developed to minimize proliferation of bacteria and other microorganisms. The aim of topical treatment depends on the depth of the burn, as for superficial burns, it is to optimize re-epithelialization, whereas for deep burns it is to minimize microbial proliferation until burn wound grafting (3).

The introduction and use of topical antimicrobial agents has resulted in significant reduction in burn-associated morbidity and mortality. However, no single agent is effective against all microorganisms, since each agent has its own advantages and disadvantages. Without doubt, topical antimicrobial therapy has significantly diminished the occurrence of invasive burn wound sepsis; however, there is an ongoing search for other compounds and agents to prevent or control burn wound infection (4).

Silver has a long history as an antimicrobial agent, and there are several reviews referring to the history of its use. In 1968, Fox introduced 1% silver sulfadiazine cream and ever since many silver complexes have been developed for burn treatment (cited in 5). The mechanism of action of silver is not totally clear, and several different mechanisms are speculated to be involved (5, 6). Topical silver treatment has many advantages, including a wide antimicrobial spectrum, low toxicity, and minimal pain on application. However, there are some complications related to silver use, but they are rare. Furthermore, there are certain concerns regarding the potential adverse effects of silver products on wound healing, and specifically the development of a sloughy layer impairing good clinical wound assessment, and potential silver cytotoxicity towards keratinocytes and fibroblasts (7-13). This is not a problem for deep burns which require surgery anyway, but it is an adverse effect for intermediate burns where delayed wound healing often results in extensive scarring.

Today a wide variety of slow-release silver dressings are available that have been developed to overcome issues of cytotoxicity and layer formation over the burnt area (5). On this basis, the objective of this study was to evaluate the
healing effect of four different silver complexes with different ligands in a Wistar rat model of full-thickness burn.

Materials and Methods

Chemicals. Three different silver (I) halide (chloride or iodide) complexes (S1, S2 and S3) with heterocyclic thioamide 5-chloro-2-mercapto-benzothiazole (CMBZT, C$_7$H$_5$CIN$_2$S) and tri(p-tolyl)phosphine (TPTP, C$_{21}$H$_{21}$P) of formulae \([\text{AgCl(CMBZT)(TPTP)}_2] \cdot (\text{MeOH})\) (S1), \([\text{AgCl(TPTP)}_3] \cdot (0.5\cdot\text{H}_2\text{O})\) (S2), \([\text{AgI(TPTP)}_3]\) (S3), and water-soluble silver (I) (as monovalent) cluster of formula \([\text{Ag}_6(\mu_3-\text{HMNA})(\mu_3-\text{MNA})_2] \cdot [(\text{Et}_3\text{NH})_2 \cdot \text{DMSO})_2 \cdot \text{H}_2\text{O})\) (S4) (where H$_2$MNA is 2-mercapto-nicotinic acid, C$_6$H$_5$NO$_2$S) were synthesized and fully characterized according to procedures already reported (14, 15). The analytical data of the aforementioned complexes: melting point, elemental analysis, nuclear magnetic resonance spectroscopy of $^1$H and $^{13}$C, Fourier transform-infrared spectroscopy, and ultraviolet-visible spectroscopy, were identical to those previously reported (14, 15). The structural formulae of the aforementioned complexes are presented in Figure 1.

Each complex, as powder, was suspended in glyceryl trioctanoate (T9126; Sigma-Aldrich, St. Louis, MO, USA) by stirring for 4 h on a magnetic stirrer in order to form a homogeneous solution, giving a final concentration of 2×10$^{-4}$ M. All solutions were kept at 4°C throughout experimentation. Solutions were applied immediately to the burn surfaces.

Animals and treatment. One hundred and eighty female Wistar rats (University of Ioannina, Animal Facility, Ioannina, Epirus, Greece), aged 5 months and weighing 195-240 g, were used. The animals were caged at controlled room temperature (20±2°C) and lighting (12 h light/12 h dark), and fed with standard pellet diet and tap water ad libitum. The animals were handled with humane care in accordance with the National Institutes of Health guidelines and the European Union Directive for the Care and Use of Laboratory Animals (Greek presidential decree no. 160 1991 implementation of the EEC Directive 86/609/EEC) and according to permission number 20EEP02.

Burn wound model. A reproducible full-thickness burn was inflicted on the back of each animal with the use of a rectangular steel stamp, 1 cm thick, measuring 4 cm$^2$. Before creation of burn wounds, animals were anesthetized with intraperitoneal administration of midazolame (6.8 mg/kg body wt) and ketamine (2.3 mg/kg body wt). Hair was removed from the dorsum of the rats and the burn wound was inflicted after heating the stamp to 250°C by placing it for 5 seconds vertically on the depilated skin area with pressure only applied by gravity, a modified method which was based on previous studies (16, 17), and on preliminary experiments we conducted in order to inflict a full-thickness burn wound. The percentage surface area of the burn wound inflicted as compared with the total body surface area (TBSA) of the animals was also calculated with the use of an already reported formula (18).

Experimental design. Animals were then randomly divided according to the silver complex that was applied to the burn wound into the following groups of 30 rats each: Control group (CG): no application of any treatment; solvent group (SG): solvent application (glyceryl trioctanoate); experimental groups 1-4 (EG1-EG4); application of solution of S1-S4. The same volume (0.3 ml) of each solution, as well as of the solvent, was applied locally with a brush to the burn wounds on a daily basis according to the experimental grouping. The daily dose of substances S1, S2, S3, and S4 was 0.161 mg/kg body weight, 0.174 mg/kg body weight, 0.187 mg/kg body weight, and 0.187 mg/kg body weight, respectively. Wounds were not cleaned before application of the silver solutions and were left exposed to the air, without any secondary dressing. Animals were not anaesthetized on daily basis, in order to exclude any other medication from the therapeutic management of the burns.

Animals were observed for 1 h after the application of the solutions in order to prevent them from licking the wounded site. Moreover, the animals were observed to ensure that the solutions remained on the wounded site and to determine if the solutions stained the area.

The size of burn wounds was measured on days 0, 6, 12, 21, and 31 with the use of a high-precision planimeter (HAFF planimeter No 313; Gebruder Haff GMBH, Pfronten, Bavaria, Germany) after tracing their borders on plastic film. All wounds were also photographed at each of the aforementioned time intervals with a digital camera (Olympus, Tokyo, Honshu, Japan).

Macroscopic evaluation. The burn wounds were macroscopically evaluated on days 0, 3, 6, 12, 21, and 31 for the following parameters: i: Presence of necrotic eschar (skin sloughing); ii: haemorrhage; and iii: purulent discharge, as an indication of infection.

Histology. Six animals from each group were sacrificed on post burn days 3, 6, 12, 21, and 31 in order to evaluate the process of burn wound healing. A triangular-shaped specimen centred over the burn wound, including the whole thickness of the wound, as well as the panniculus carnosus, and extending to the surrounding healthy tissue was harvested from the sacrificed animals. The specimens were fixed in 4% neutral buffered formalin, paraffin embedded, cut into 5 μm-thick sections, and stained with haematoxylin-eosin.

Wound healing was semi-quantitatively evaluated (19), using several parameters as a sequence of events on a scale ranging from 1 to 5: Stage 1: Necrosis and denaturation of skin and subcutaneous fat accompanied by the presence of acute inflammatory cells and oedema, as a result of the full thickness burn injury inflicted. Stage 2: Appearance of immature granulation tissue characterized by presence of abundant capillary vessels with swollen endothelial cells within an oedematous loose stroma. Stage 3: Presence of mature granulation tissue with fewer capillaries and more abundant fibroblasts. Stage 4: Almost complete re-epithelialization accompanied by abundant mature fibroblasts and collagen fibrils in the dermis and subcutaneous tissue. Stage 5: Presence of well-developed scar, completely re-epithelialized.

Statistical analysis. Data are expressed as the mean±S.D. The statistical significance between data means was determined by Student’s t-test and two-way analysis of variance (ANOVA) was used for statistical evaluation of differences between groups (SPSS version 16.0, Statistical Package for the Social Sciences software; SPSS, Chicago, IL, USA). $p$-Values of less than 0.05 were considered to be statistically significant.

Results

Clinical - macroscopic findings. No animal deaths or severe complications were recorded through our experiments. All macroscopic findings were recorded and are depicted in Figure 2.
The main findings of macroscopic evaluation can be summarized as follows: On days 0, 3, and 6, macroscopic findings were almost identical in all groups. In the course of time, faster fall of burn eschar was noticed in SG, EG1, EG2, and EG4 compared to CG and EG3. In the course of time, higher infection rates of burn wounds were recorded in CG, SG, and EG3 compared to EG1, EG2, and EG4 groups.

Planimetric evaluation. The mean TBSA of the five-month Wistar rats used in this study was 356 cm², whereas the burn wound area of 4 cm² represented in average 1.12% of TBSA of the animals used.

The results of planimetric evaluation of burn wound area, as well as the differences among the groups on days 0, 3, 6, 12, 21, and 31 after induction of burn wounds are represented in Figure 3, and listed in detail in Tables I and II.

On day 3, all groups presented a significant increase of burn surface area ($p<0.05$). The burn surface area of CG, SG, EG3 and EG4 remained almost stable on days 6 and 12, whereas in EG1 and EG2, it significantly decreased ($p<0.05$). On day 21 compared to day 0, the burn surface area was significantly lower in all groups, except for EG3 in which it was slightly increased. Faster re-epithelialization was noticed in EG1 and EG2 groups.

On day 31 compared to day 0, the burn surface area was significantly lower in all groups ($p<0.05$). Furthermore, the burn wound was almost completely re-epithelialized in EG1 (Figure 4), EG2 and EG4 (data not shown).

Histological evaluation. Day 3: The findings in the sections of rats of CG were identical to those of SG, including a central area of blood clot formation, accumulation of polymorphonuclear cells underneath the eschar, tissue oedema, as well as denatured skin and subcutaneous fat tissue. The findings in groups EG1, EG2, EG3, and EG4 were similar, except for the presence of more pronounced oedema. No remarkable granulation tissue development (stage 1) was observed in any group.

Day 6: In the sections of rats in CG and SG, characteristics of acute inflammation were still apparent.
Although a few fibroblasts appeared in the area, there was still no development of granulation tissue (stage 1). In EG1, EG2, EG3, and EG4, despite the absence of well-developed granulation tissue, abundant oedema was accompanied by the presence of more fibroblasts than in rats of CG and SG, and few neo-capillaries (stage 2).

**Day 12:** In the sections from rats of CG and SG, the presence of immature granulation tissue was noted (stage 2). In EG1, EG2, EG3, and EG4 rats, a decreased number of acute inflammatory cells was accompanied by the presence of denser connective tissue with a clearly developed capillary network and several fibroblasts (stage 3).

**Day 21:** In the sections from rats of CG and SG, mature granulation tissue with flattened fibroblasts and dense mature capillary network was noted (stage 3). There was only a small central area with acute inflammatory cells. This area was larger in the rat from EG3. In all other groups, inflammatory cells were no longer observed, and the granulation tissue was more mature. Re-epithelialization appeared (stage 4). In addition, the granulation tissue and re-epithelialization were more mature and extensive in groups EG1 and EG2, respectively, as compared to EG4 (Figure 5).

**Day 31:** In all groups, scar tissue with almost complete re-epithelialization was observed. In the CG, SG, and EG3 groups, the squamous epithelial cell layer consisted of only a few layers of immature keratinocytes (stage 4). In EG1, EG2, EG4 groups, there was almost complete coverage of the wound by keratinocytes forming a mature epidermis. Underneath, dense fibrous connective tissue was present (stage 5).

**Discussion**

Burns are one of the most common and devastating forms of trauma, and account for over 300,000 deaths each year throughout the world. However, the number of cases of mortality after burn injury has declined in the past decades, mainly due to better respiratory, fluid, and sepsis management (20, 21). It has also been estimated that approximately 75% of deaths from thermal injuries are related to infection leading to sepsis (22-24). Destruction of the skin barrier in combination with concomitant depression of local and systemic immune defence in patients with burns are the main factors contributing to infectious complications. Therefore, the burned skin is extremely vulnerable to invasive infections of any kind until complete epithelial repair has occurred (25).

Topical antimicrobial therapy is a particularly important method of burn wound care. The rationale for its use is to reduce the chances for infection, thus optimizing re-epithelialization of superficial burns and skin graft take in deep burns. Consequently, a great variety of topically acting agents have been developed (5). Among them, silver has a long history as an antimicrobial agent, and many articles refer to the use of topical agents containing several forms of silver in burn wound healing.

Figure 2. Macroscopic findings on post-burn days 0, 3, 6, 12, 21 and 31. TI: Total infections. CG, Control group; SG, solvent group; EG1-EG4, animals treated with S1-S4, respectively.
There are two suggested mechanisms for the biological activities of silver: reaction of silver with bacterial cell membranes; preferential binding of silver atoms with DNA, preventing DNA from unwinding. As a consequence, silver can cause fatal structural changes in bacterial wall and membranes by altering DNA and RNA. These mechanisms may also have a synergic action (25, 26). This is why microbes rarely develop resistance to silver, unlike that to antibiotics which usually target one only cell function (6). Silver causes disruption of bacterial cell wall structure, degradation of key bacterial enzymes, such as cytochrome b and a3, and interaction with nucleic acids, due to its preferential binding to nitrogenous groups of guanine and other nucleotides (27). Finally, monovalent silver complexes have been reported to possess antifungal, antivirus, and antitreponemal activities (5, 28).

A series of studies verify the broad spectrum of the antimicrobial effect of local application of silver. The antimicrobial activity of silver iontophoresis against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolated from fatal burn wound infections has been confirmed in vitro as well as in animal models (25, 29). Silver complexes with nicotinate-type ligands have shown considerable activity against *P. aeruginosa*, *S. aureus*, *S. pyogenes*, and *P. mirabilis*, all clinically isolated in foot ulcers in diabetics, whereas the same bacteria were found to be resistant to at least 10 antibiotics commonly used for the treatment of such ulcers (30). Silver *N*-heterolytic carbene complexes have shown antimicrobial activity against *Bacillus subtilis* (31), and shell crosslinked nanoparticles carrying silver cations against *Escherichia coli* (32). Silver complex Ag(DEPE)2NO was found to have activity comparable to that of fungizone (Amphotericin B) against strains of *Candida albicans* (29). Moreover, monovalent silver (I) complexes of thiocarboxylates such as thiomalic acid and 2-mercaptonicotinic acid have also shown remarkable antibacterial activity against bacteria, yeast and mould (33, 34).

Nevertheless, there are certain side-effects associated with the use of silver. Absorption of silver from the use of silver sulfadiazine in extensive burns is associated with systemic toxicities and transient leucopenia. Systemic silver absorption from wound management products is higher through wounded skin than through intact skin and is related to silver content, formulation, mode and frequency of application of the product (6). Topical treatment also offers the advantage of immediate effect by increasing local tissue bioavailability and thus lowering systemic levels (35). The final concern with silver products is the potential adverse effect of silver on wound healing, since certain studies reported that silver is toxic to keratinocytes and fibroblasts (7-13). In addition, as Gram-negative bacteria are destroyed, and exotoxins are released that could potentially exert a negative effect on wound healing (5).
In the present study, the healing effect of four different silver complexes was investigated in experimentally created full-thickness burn wounds. According to our findings, the significantly lower rate of wound infection, shown by absence of purulent discharge, is evident in groups EG1, EG2, and EG4 compared to both the CG and SG groups. It was also evident that the solvent (glycerine trioctanoate) facilitated burn eschar detachment, since it occurred significantly faster in SG, EG1, EG2, EG3, and EG4 groups as compared with CG.

Regarding the burn surface area, there was an initial (post-burn days 3 and 6) statistically significant increase of the burn surface area in EG1, EG2, EG3, and EG4 groups compared with the CG and SG groups. This increase might be due to the toxic effect of silver on keratinocytes and fibroblasts already affected by the burn injury (zones of stasis or hyperemia) (7-13). This might suggest that the selected

![Figure 3](image1.png)

**Figure 3.** Planimetric measurement of open burn wound size (cm²) and comparison between the control group (CG) and the other groups on post-burn days 0, 3, 6, 12, 21 and 31. SG, Solvent group; EG1-EG4: animals treated with S1-S4, respectively. Data are presented as the means±SD. *Statistically significant difference at p<0.05.

![Figure 4](image2.png)

**Figure 4.** Appearance of burn wound on post-burn day 0 (a) and 31 (b) in a rat of the group treated with S1 (EG1).

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<th>Comparison between groups</th>
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**Table II. Comparison of wound surface area, and statistical analysis from post-burn day (PBD) 6 to 31 between groups treated with S1-S4 (EG1-EG4).** p-Values <0.05 were considered to be statistically significant.
dose of silver solutions applied daily failed to lead to rapid healing of full-thickness burns which require skin graft as soon as possible. However, in the course of time (post-burn days 21 and 31), the burn surface area became significantly smaller in groups EG1, EG2, and EG4 compared to the CG and SG groups, probably due to the significantly lower infection rates. Interestingly this did not occur in rats of the EG3 group, in which development of infection was recorded on post-burn day 21. Overall, re-epithelialization was faster in groups EG1 and EG2 compared to the other groups.

Figure 5. Histological evaluation of representative sections of all groups on post-burn day 21. In the sections of control group (CG) (a) and solvent group (SG) (b), a central area of ulceration with polymorphonuclear cells is noted and is surrounded by mature granulation tissue. This central area is larger in the group treated with S3 (EG3) and absent from rats of groups treated with S1 (EG1) (c), S2 (EG2) (d) and S4 (EG4) (f). In the EG1 (c) and EG2 (d) rats, more mature granulation tissue and more extensive re-epithelization are apparent as compared to EG4 (f). Haematoxylin-eosin stain, ×200).
However, on post-burn day 31, the final outcome was similar in groups EG1, EG2, and EG4, since no statistically significant difference of the burn surface area was recorded between these groups. On the contrary in EG3, a statistically significant delay of wound re-epithelization was noted. A striking difference that may explain this delay is the presence of a different halide in the structure of S1 and S2 as compared to S3, namely chloride rather than iodide. However, further research is needed to support this hypothesis.

Histological findings were also in support of accelerated wound healing and faster re-epithelization in groups EG1, EG2, and EG4, since they were consistently better than those of groups CG, SG, and EG3. The main histological findings indicating increased healing rate in groups EG1, EG2, and EG4 were the early appearance of loose connective tissue and recently formed capillaries, as well as at later stages, the better quality and structure of both epithelium and collagen fibres.

**Conclusion**

According to our findings, substances S1, S2, and S4 seem to accelerate re-epithelization and improve the quality of burn wound healing in our rat model. Nevertheless, they failed to deliver rapid healing of the full-thickness burn in order to prevent surgery. Further studies are needed to define the exact biological activities and characteristics of these compounds, as well as the optimal dose of the substances used, in order to ensure a faster and more effective burn wound healing process.

**Conflicts of Interest**

The Authors report no conflicts of interest in regard to this study.

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