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Original Article

Inhibition of malignant cells by silver anodes in vitro

Ilke Evrim Secinti

Department of Pathology, Faculty of Medicine, Hatay Mustafa Kemal University, Hatay, Turkey

Abstract

Aim: The aim of this study was to investigate the antitumoral effect of silver anodes.

Materials and Methods: Pure silver electrodes and sham electrodes were placed in Petri dishes. Hep-II cells in culture media were added. The electrodes were connected with a direct current source. After incubation, the cells were examined and counted under an inverted microscope.

Results: With an applied current of 4.0 μA, a clear inhibition zone was seen around the anode but not around the cathode. There was no inhibition zone around the anode or cathode with a 1.0 μA applied current or around the sham electrode.

Conclusion: Metallic silver alone is not capable of inhibiting malignant cell growth, but our findings demonstrated an antitumoral effect of silver anodes in vitro. This effect was both current- and silver ion-dependent. Throughout the experiment, the electrical charges were acceptable in that they do not have harmful effects on normal living and undifferentiated human cells. Further studies are needed to evaluate clinical applications.

Keywords: Electricity; silver; tumor cells

INTRODUCTION

Pure silver metal is antibacterial (1-3) and antifungal (4, 5) due to the spontaneous release of silver ions from the metal surface. This is the oligodynamic effect. When pure silver metal is polarized with a low electric current, it is called a silver anode/cathode, and its antibacterial effect is enhanced approximately 100 fold (6). As expected, an electric current amplifies silver emission from the electrode surface, but the antibacterial effect of the anodic silver is not enhanced parallel to the electric current. Interestingly, greater antibacterial effects cannot be obtained with more silver when an electric current is lacking. In addition, once the effects of a silver anode appear, they do not cease even after the electric current ceases. For example, when pure silver is anodized with 1 μA for 1h, the antibacterial effect lasts at least 10 weeks. These extreme specificities are unique for pure silver electrodes (7).

Electrical parameters that can be used on human skin have been defined as 0.1–2.0 coulomb/day (7). There was no indication of deleterious effects on mammalian cells when/if the charge was held below 2.0 coulomb/day (7). Since the 1980s, silver anode applications have been widely used in clinics. Positively-polarized pure silver electrodes have been used in the treatment of deep bone infections in orthopedics (7, 8) and in the treatment of dental abscesses (9) and vertebral infections in neurosurgery (10). However, the use of electrical parameters has prevented many clinicians from employing such procedures and the application of silver electrodes has not found a broad-based use.

In parallel with developments in nano technology in the 2000s, the use of nano-silver has become popular and attracted great attention. Some studies have indicated that nano-silver has antitumoral effects (11-13). It was reported that healthy cells tolerate silver better than tumor cells do and that tumor cells die at lower silver concentrations than healthy cells do (11-14). However, these studies, which did not use electric currents, are far from examining the possible increase in the effect of nano silver combined with electric current. Given the possible cytotoxic potential of nano-silver, systemic administration is likely to be controversial. The idea of local nano silver application into tumor tissue, which can be theorized to avoid the cytotoxicity, seems quite challenging because of the impossibility of keeping the concentration of silver in the tumor tissue constant. Silver has a very wide tissue distribution. It is known to disperse rapidly throughout the body from where it is applied, and its concentration decreases rapidly in the application area (15). In view of these data, the possibility of sustained and controllable silver release by temporary silver electrodes that can be

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Corresponding Author: Ilke Evrim Secinti, Department of Pathology, Faculty of Medicine, Hatay Mustafa Kemal University, Hatay, Turkey, **E-mail:** ilkevrim@gmail.com

inserted into the tumor may be valuable.

To establish a base for clinical usage of anodic silver on laryngeal squamous carcinoma cases, we examined the antitumoral efficacy of silver anodes on Hep-II cells.

MATERIALS and METHODS

Electrodes were prepared with 99.9% pure silver metal (2.2 mm x 0.8 mm x 30 mm), and the surface area of each electrode was calculated as 5.28 cm^2. Electrodes were bent into a rectangular shape and placed into two standard glass Petri dishes (radius: 9 cm) as previously described (2,-4, 9, 16). The distance between electrodes was adjusted to 7.3 cm for each Petri dish. Cables were removed. A pure silver plate (thickness: 0.08 cm; radius: 3.6 cm; surface area: 1.13 cm^2) was placed as a sham electrode, and no electrode was placed in the control Petri dish. They all were autoclaved.

Hep-II cells (obtained from the Refik Saydam Institute of Virology Laboratory, Ankara, Turkey) in Eagle's Minimum Essential Medium (EMEM; 9.2 g/L Basal Medium Eagle (BME; Gibco), 2.2 g/L $NaHCO_3$, 25 mM/L HEPES, and deionized water) were added to the plates. Cells were incubated for 96 h under 10% CO_2 at 37o C. Cells were washed with 5 ml phosphate buffered saline (PBS; 8.0 g/L NaCl, 0.2 g/L HCl, 2.37 g/L $NaHPO_3$•12 H_2O, 0.2 g/L HPO) with 1 g/L dextrose and 0.02 g/L phenol red in deionized water. Versene trypsin (8.0 g/L NaCl, 0.2 g/L HCl, 2.37 g/L $NaHPO_3$•12 H_2O, 0.2 g/L HPO_3, 1 g/L Versene, 1.25 g/L trypsin, and deionized water) was added. Then 5 ml of EMEM was added and centrifuged. The initial cell count was adjusted to 100,000 per ml. 15 ml of medium (EMEM) with sterile calf serum (10%) was added to each of four Petri dishes. The electrical impedance of the medium was measured with the two-terminal method (17) using a separate Petri dish. The medium's impedance was measured as 8+/-1.04 K ohm. Measurements of reactance that originated from proteins in the medium were ignored because a direct current was being used. The electrodes were connected with a battery-driven constant direct-current source. The first group received 1 μA of direct current and the second group received 4 μA of direct current. No electrical current was applied to the sham group.

After 96 h incubation, the medium was collected for harvesting the dead cells and replaced with fresh medium, and cells that were attached to the bottom of the Petri dishes and the inhibition zones were investigated and photographed under an inverted microscope (Figure 1A). A Thoma Lam was placed under the Petri dishes, and cells that were attached to the bottom of the Petri dishes near or away from the electrodes were counted by one investigator. Counting of cells was repeated 10 times, and mean cell counts were calculated (Table 1).

Table 1. Cell counts which were attached to the bottom of dishes

PRIVATE	Cell Counts (n=10)						
	Behind anode			Inter electrode area			
Distance from anode (mm)	>4	<4	ANODE	<9	9	>9	Most nearer to cathode	CATHODE
		Female:12 (33.3%)					
		Male: 24 (66.6%)					
1 μA	1248 ± 192	93.5 ± 11		0	392 ± 109	1392 ± 103	384 ± 119	
4 μA	848 ± 110	0		0	120 ± 27	976 ± 207.2	856 ±140	
Sham				1368 ± 221.76				
Control				1488 ± 369.9				

Table 1. Behind the anode, no cell was detected until 4 mm away from electrode in 4 uA current applied group, while cell count was decreased in 1 uA current applied group at the same area. At inter-electrode area; no cell was detected until 9 mm away from electrode in both 1 uA and 4 uA current applied groups. There was no difference between control group and sham group.

Table 2. The total dead cell counts among the groups

Groups	Total Dead Cell Count
Control	28 (± 4)
Sham	32 (± 3)
1 μA	61 (± 7)
4 μA	239 (± 24)

Table 2. Note that the dead cell counts are similar in control and sham groups but so distinct from 4 mA current group. This is indicating that the inhibition zone is not the result of electrophoresis.

For all Petri dishes, the collected media was centrifuged, supernatant was removed, and the cell pellet was diluted in 1 ml of fresh medium. The medium was divided into 10 equal volumes (100 μL each), which were distributed into the wells of a 96-well plate to facilitate the counting of whole dead cells. Dead cell counts, morphological changes of cells, and intensity of dead cells are shown in Table 2, Figure 1B, and Figure 1C–D, respectively.

RESULTS

No corrosion on the surface of the electrodes or discoloration of the media was seen after 96 h of

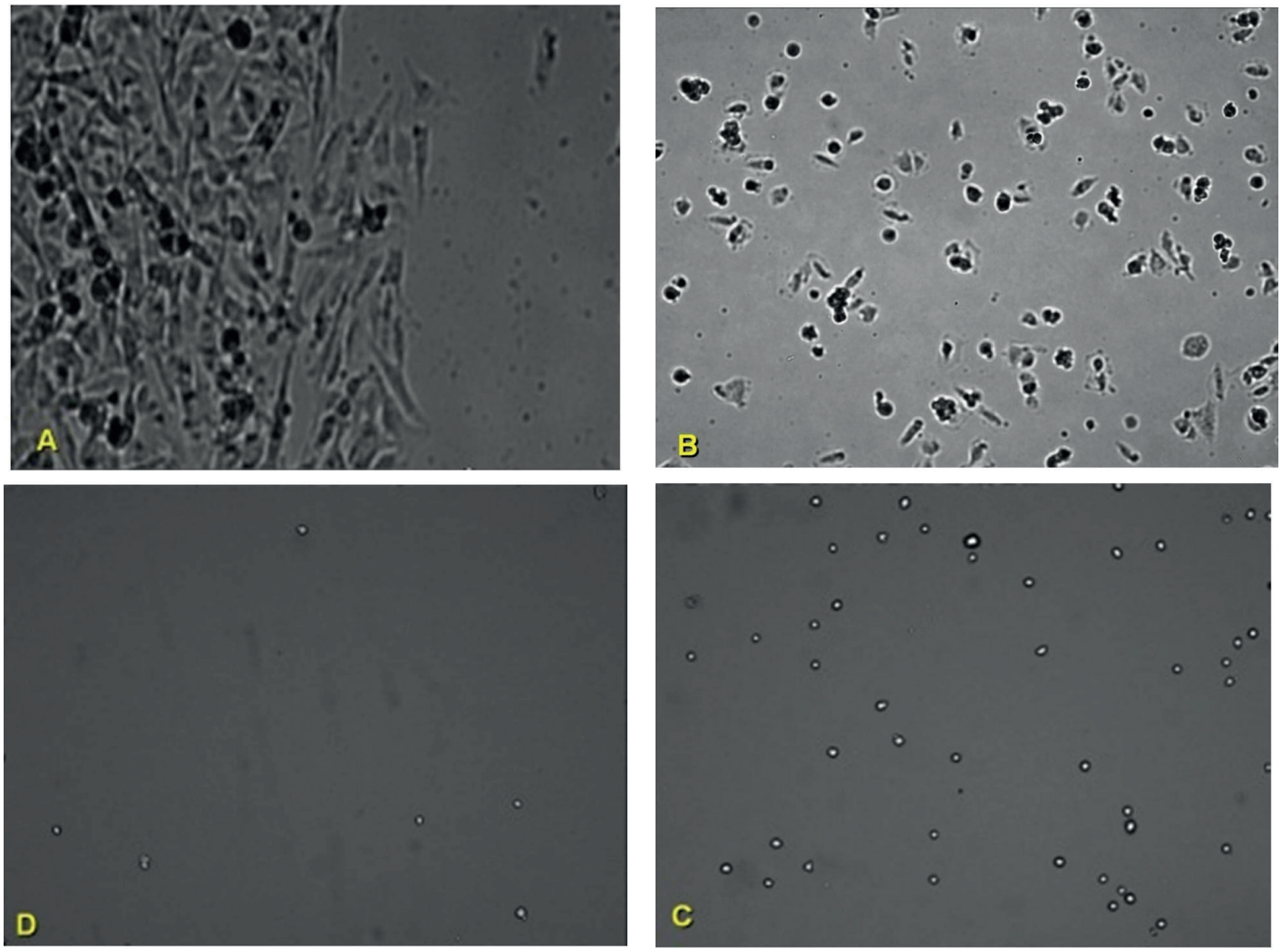

Figure 1. A clear inhibition zone can be seen in 4μA current applied group (Fusiform cells are indicating the viable cells while spherical ones are dead cells) (A). Morphological changes of dead cells can be seen in B. The dead cell intensity can be compared among 4μA current group and the control group (C and D)

incubation. There were no detectable pH changes or breakdown of media. At the end of the experiment, the electric current level was found to be the same as the initial level.

With an applied current of 4 μA, a clear zone of growth inhibition was seen around the anode. There were no cells in the inhibition zone. In other words, the cells near the anode were not attached to the glass surface of Petri dish. The border of the zone was not sharp. Under magnification (40X), vacuole formation was detected in the cells that were located at the border of the inhibition zone (data not shown). Neither an inhibition zone nor vacuole formation was detected around the cathode. Cellular morphology was normal on the cathode side.

There was no inhibition zone around the anode or cathode in the Petri dish with an applied current of 1 μA. However, the cell count around the anode was decreased.

There was no inhibition zone around the sham electrode. Cell attachment to the dish was homogeneous and cell morphology was normal. However, when the sham electrode was removed, there were no cells attached to the plate under the electrode. The cell counts around the electrodes are given in Table 1.

DISCUSSION

The cell count at the sham electrode was not significantly different than that in the control culture plate ($p = 0.73$). Thus, metallic silver alone is not capable of inhibiting malignant cell growth in vitro. The combination of silver ions and a weak direct current significantly inhibited malignant cell growth around the anode ($p < 0.001$). When a 1 μA current used was used, poor cell attachment was seen at the anode, but when a 4 μA current was used, no cells were seen at the anode or within 9 mm of the anode. Thus, anodic silver ions were capable of inhibiting malignant cell growth in vitro. This effect was both current- and silver ion-dependent.

When an electrical current is applied to electrodes, the current will follow the shortest pathway between the negative and positive poles. Thus, the electrical current will dominate any effect due to silver ions emitted from the anode surface, notably in the inter-electrode area. This area will be under the effect of the electrical current rather than the effect of silver ions emitted from electrode surface. When the electrical current is oriented to the cathode as a "silver anode treatment," the effect of the electrical current behind the anode will be minor. In this area, silver ions emitted from the anode surface will be the effecting parameter. Thus, sub-cellular changes in the inter-electrode area will be mostly due to the electrical current, but behind the electrodes, the effects will be mostly due to silver ions liberated from the anode surface.

Neoplastic cell growth is consistent, and the malignancy of such cells is well correlated with the cell number in culture plates at any location at any time. The number of cells at any location reflects growth capability of where the material were isolated from. In this study, cells that were attached to the glass bottom of the Petri dishes were counted at multiple areas of the dishes. The cell numbers that we observed reflected the anti-neoplastic effect of the electrical current and electrically activated silver ions.

The antitumoral effect of anodic silver has been extensively studied by Spadaro (18). He clearly showed the death of Ehrlich ascites tumor cells in Dulbecco's Eagle medium with the use of silver electrodes driven by 0.2 µA, 2.0 µA and 20 µA direct currents after 5 hours of exposure. Then, the silver anode-treated and untreated tumor cells were inoculated into Albino Swiss mice. After four weeks, all control mice, which had received untreated tumor cells, had died. Half of the mice receiving tumor cells from the 0.2 µA silver anode groups survived. All animals in both 2.0 µA and 20 µA anode groups survived and showed neither signs of cancer nor tumor growth. The silver anode inhibited growth of neoplastic cells, judging by the lack of cells in the inhibition zone. This led to the following questions: What component of the silver anode is dominant—silver ions emitted by the anode, the electric current, or both? Is the cell-free zone a simple cellular migration due to electrophoresis?

If inhibition of growth was due to simple electrophoresis, another zone would have been established by cells accumulating around the opposite pole (cathode), but this was not observed (2, 3, 4). The relation between the amount of silver ion emitted and its polarization voltage is not linear (8, 19). Thus, many studies have clearly demonstrated that silver anode treatment is not a simple electrophoresis (2, 3, 5, 16). Aydin et al. (9) previously showed the nature of silver emissions and described a formula for their exponential scattering from the metal surface to agar medium with the use of an atomic absorption spectrophotometer. Electrophoretic forces may have briefly helped cell migration only along the electric current trace between the two electrodes, but it did not cause the complete inhibition of cell growth. We clearly detected a continuous and uninterrupted inhibition zone in every direction (see Table 1), despite electrophoretic forces resisting ion emissions behind the anode. Thus, the inhibition zone did not result from electrophoresis or the potential difference between electrodes (2, 9).

Throughout the experiment, the applied electrical charges remained under 2.0 coulomb/day. These are acceptable parameters for mammalian cells and have no harmful effects on normal living and undifferentiated human cells (6, 7). The electrical charge used in this experiment was 1.7 %– 4.3% of threshold. For this reason, we do not think the electric current completely destroyed malignant cells inside the inhibition zone. On the other hand, scattering non-polar silver did not inhibit malignant cell growth because there was no significant difference between cell counts of the control group and those of the non-polar silver group ($p = 0.73$). We think the growth inhibition of tumor cells were originated from the combination of the silver and the electric current.

It is difficult to know what was happening in the neoplastic cells after they were exposed to anodic silver. However, vacuolization was not a surprise. Aydin et al. previously showed by electron microscopically vacuolization in either bacterial cells or yeast cells with silver anode treatment (4, 16). Additionally, they showed initial cytoplasmic density lost, cytoplasm leakage due to possible osmotic imbalance, lysis of intra-cytoplasmic organelles (including the nucleus and mitochondria), and inhibition of budding in yeast cells (4, 16). Similar sub-cellular changes can expected from these malignant cells. After exposure to a silver anode, yeast cells be examined by electron microscopy showed vacuole formation, clumping in the endoplasmic reticulum, and pervasive cell destruction (4, 16). Vacuolization may be an early sign and key process of silver anode treatment for differentiated cells.

To establish a base for in vivo studies, one more question addressing what is the expected penetration of depth of silver emitted from the anode into the liquid medium. Previous studies demonstrated a silver penetration depth of approximately 10 mm in bone tissue (8), 14 mm in agar medium (2), and 1.34 mm in teeth (9). In this study, it was difficult to detect a radius of the zone of emitted silver because of the liquid medium's nature. However, it can be estimated that was no less than 14 mm.

Another question is how long the antibacterial, antifungal or antitumoral effect will continue after the electric current ceases. Spadaro followed the antibacterial effect of a single 1-hour silver anode exposure for 10 weeks (7). The antitumoral effects of a silver anode exposure are expected to be at least as long-lasting as its antibacterial effects, because irreversible loss of malignant cells occurs at the silver anode (18).

CONCLUSION

Our findings demonstrated the antitumoral effect of

a silver anode on Hep-II cells and encourage the idea of treatment of malignant cells by a silver anode as a possible alternative therapy.

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Conflict of interest : The authors declare that they have no competing interest.

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