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Wash-In Silver Nanoparticle Laundry Additive Was Not Effective in Reducing Bacterial Load on Wrestling Apparel

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Context: The best practice for cleaning wrestling mats is using a residual disinfectant with continued antibacterial action. Recently available wash-in silver additives claim to confer a residual effect to fabric.

Objective: To test the efficacy of laundering with a wash-in silver additive in reducing athletes' exposure to potentially infectious microbes on apparel.

Design: A 4-part controlled laboratory study/parallel group comparison study. (1) To test whether fabrics in athletic clothing would be affected differently, we applied bacteria to control fabrics washed in detergent alone and test counterparts washed in detergent plus wash-in silver additive. Bacteria were applied to fabrics, extracted, plated, incubated, and counted. (2) To see if wash-in silver affected various bacteria differently, we washed cotton t-shirts with detergent alone or with detergent plus wash-in silver. We applied 4 bacterial species commonly found in the wrestling environment. Bacteria were extracted, plated, incubated, and counted. (3) To see if wash-in silver was effective in reducing bacterial contamination during practice, 32 collegiate wrestlers paired off with one wearing a test silver-treated t-shirt and their partner wearing a control shirt. Wrestler rotations exposed shirts to 2, 4, or 8 wrestlers. Identical swatches of fabric were cut from the t-shirts. Bacteria were extracted, plated, incubated, and counted. (4) We simulated prolonged/repeated bacterial exposure as occurs during tournaments by applying bacteria directly to silver-treated and untreated singlet material repeatedly over time. Test samples were taken at regular intervals to see if bacterial growth was inhibited by the presence of the silver nanoparticles. Bacteria were extracted, plated, incubated, and counted.

Setting: Laboratory and practice.

Participants: Collegiate Division III wrestling team.

Main Outcome Measure(s): Wash-in silver would be considered effective if a statistically significant reduction in bacterial count was observed at 95% confidence.

Results: Wash-in silver reduced bacterial growth at low levels of contamination but did not significantly reduce bacterial growth at levels seen during contact sport competitions. This was true for all bacterial species and all fabrics tested.

Conclusions: The environmental and potential health risks in using a wash-in silver nanoparticle laundry additive in the wash cycle for clothing worn by wrestlers outweigh any potential infection control benefits to these athletes. We do not currently recommend adopting wash-in silver treatment as part of the laundering regimen for wrestling programs until further testing of alternate methods of silver impregnation into sports fabrics has been investigated.

Key Words: bacterial contamination, residual antibacterial, wash-in silver nanoparticle treatment

Key Points

- Silver antibacterial efficacy varied with species, showing significant activity against *Streptococcus pneumoniae* but not against *Staphylococcus* species.
- In multiple exposure situations, adding silver to the wash cycle had residual activity and reduced bacterial counts with low bacterial loads but failed to combat heavy contamination.
- In long-term repeated exposures, adding silver nanoparticles to the wash was ineffective in controlling bacterial load.

The spread of infection in athletes participating in contact sports, such as mixed martial arts, judo, Taekwondo, and wrestling, is of major concern. Skin infections are credited with approximately 10% of time-loss injuries in contact sports.[1] Collegiate and high school injury reports indicate that approximately 25% of wrestling practice injuries are a result of infection, with roughly 14% of infections being impetigo from *Staphylococcus aureus* or *Streptococcus pyogenes* and another 10% being ascribed to other bacteria.[2] Up to 30% of high school wrestlers and 40% of college wrestlers contract *Herpes gladiatorum*, a herpes simplex type 1 viral infection, and an average 31% of wrestlers contract *Tinea corporis gladiatorum*, fungal ringworm infections.[3] Bacterial, viral, and fungal infections can all lead to more serious illnesses.[1] In order to prevent microbial transfer between wrestlers and their

surroundings, best practices recommend frequently washing their hands and cleaning mats with residual antimicrobial cleaners.[4,5]

Washing scrubs and other personal protective equipment with wash-in laundry additives to impregnate fabrics with silver nanoparticles has recently been suggested as an effective method for conferring residual antibacterial activity to fabrics. This would result in preventing the spread of infection in the health care environment.[6] Silver has been used as an antimicrobial agent for thousands of years. Recently, expanded use of silver nanoparticles has blossomed across many medical applications,[7] as is its widespread use in clothing, toys, and cosmetics.[8] However, although these products are widely available and are being marketed to the wrestling and combat sport community to reduce bacterial load in athletic apparel, we found no studies of silver nanoparticle efficacy in athletics.

Silver's effectiveness as an infection control measure varies by microbial species and by the size of the nanoparticles.[9] Silver nanoparticles bind to the plasma membrane of the bacteria and change the charge of the membrane, causing microbial death.[10] Additionally, silver ions can penetrate the plasma membrane and act in a variety of ways internally to inactivate and kill the bacteria.[11] Silver was also found to kill 4 fungi associated with skin infections (*Trichophyton rubrum*, *Candida albicans*, *Microsporum canis*, and *Aspergillus flavus*), although no mechanism of action was suggested.[12] We hypothesized that a wash-in silver product, added when laundering athletic clothing, might act as a residual cleaner (one with a persistent antibacterial effect) to decrease the number of microbes on the clothing. If effective in reducing bacterial load on clothing, it could reduce microbial transmission between athletes and (although not part of this study), hopefully, ultimately reduce the number of skin infections.

Study Aims

The overarching goal of this study was to assess wash-in silver treatment and its efficacy in acting as a residual antimicrobial to reduce bacterial load in the wrestling environment. Our hypothesis for all experiments was that the wash-in silver would be an effective residual antimicrobial. Because athletic clothing is made from a variety of materials, experiment 1 asked if there was a residual effect of laundering with (test) and without (control) wash-in silver additive and if it differed between 3 fabrics commonly used in athletic clothing. Because athletes are exposed to multiple bacterial types, experiment 2 tested residual antimicrobial activity of the 4 bacteria most commonly found on mats (*S. epidermidis*, *S. pneumoniae*, *Bacillus subtilis*, and *S. aureus*). In practice, wrestlers are exposed to a bacterial load from multiple individuals. To assess efficacy of wash-in silver at varying loads, in experiment 3, team members wrestled in control and test t-shirts, and the shirts were subsequently assessed for microbial contamination after having been exposed to high, medium, or low levels of bacteria. Practices usually last 1 or 2 hours, but wrestling tournaments last all day. Experiment 4 examined the efficacy of wash-in silver to reduce bacterial load during longer events with repeated exposure to bacteria.

METHODS

Overview

To determine if a wash-in silver nanoparticle laundry additive used to wash practice t-shirts and competition singlets could minimize the exposure of wrestlers to microbial contaminants on these fabrics and potentially reduce their risk of infections, we conducted 4 experiments. All experiments followed a pattern:

STEP 1: We washed the fabric to be tested with either detergent alone (EcoLab), henceforth referred to as the control group, or with detergent and 2 sheets of a wash-in silver laundry additive (SilverWorks!), henceforth referred to as the test group. We followed the manufacturer's directions precisely: "Usage: Add to wash cycle along with your favorite detergent . . . Two (2) sheets for heavily soiled loads." Sheets were not transferred to the dryer.

STEP 2: We added bacteria either in a controlled laboratory environment where a known number of bacteria could be applied or in a practice environment where bacteria were added by having wrestlers wear the clothing during practice. This method is more realistic but harder to measure.

STEP 3: We extracted bacteria from the fabric by placing 5-cm fabric squares into test tubes containing 2 mL of Mueller-Hinton broth and agitating for 10 minutes in a thermoagitator (set at 37°C and 1000 rpm).[13]

STEP 4: We removed the cloth from the tube, squeezing it against the side. The contaminated fabric was disposed of in a biohazardous waste container.

STEP 5: We used a micropipetter to put 50 μL of each extract onto a Petri dish containing blood agar and used a spreader to distribute the bacteria evenly around the plate.

STEP 6: We put plates in an incubator overnight at 37°C with 5% CO_2.

STEP 7: After 24 hours, we counted the number and type of bacterial colonies on the plates by either visual inspection or, for plates with a lot of colonies, using a Flash 'N Go Colony Counter. Counts were recorded.

STEP 8: All experiments were done in triplicate, at a minimum (assume triplicates unless specified). Means were calculated, and an analysis of variance was used to determine the significance of differences when more than 2 groups were compared, whereas Student's *t* tests were used to compare means when only 2 groups were compared. Significance was set at a 95% CI.

Experiment 1: Does the Type of Fabric Used Affect Efficacy of Silver in Preventing Bacterial Growth?

Although most of the studies on silver efficacy use cotton,[14,15] a variety of fabrics are used to make singlets, t-shirts, and other clothing worn in the wrestling environment. Therefore, we performed a test to determine if the fabric type affected efficacy of a wash-in silver additive in preventing bacterial growth. Because we needed to have enough bacteria to provide reasonable colony counts but not so many that the plate was overloaded, we conducted pilot studies that determined that if we applied 100 μL of bacteria at a concentration of a 0.5 McFarland standard, that would give us exactly 1.5×10^7 bacteria applied to the fabric. This allowed us to accurately calculate the percentage of surviving bacteria retrieved. We therefore applied 100 μL of a 0.5 McFarland standard of

S. pneumoniae to 5-cm squares of cotton, polyester, or Lycra test and control fabrics. After 10 minutes, the bacteria were extracted, plated, incubated, and counted as described above.

Experiment 2: Does the Type of Bacteria Used Affect Efficacy of Silver in Preventing Bacterial Growth?

Because experiment 1 found no difference in the efficacy of the wash-in silver treatment on the fabrics that we tested, and most of the published work on silver-treating fabrics was done on cotton or cotton blends, we used cotton in this simulation to allow for better comparison of our work with that of others.[14,15] Previously, we determined the 4 bacterial species most commonly found on wrestling mats during a typical practice and their relative concentrations (*S. epidermidis* [75%], *S. pneumoniae* [16%], *B. subtilis* [6%], and *S. aureus* [3%]).[4] To examine whether fabric treated with wash-in silver laundry additive affected these species differently, we prepared a proportional mixture of the 4 species and added 100 μL of a 0.5 McFarland standard of the mixture to 5-cm squares of cloth from 100% cotton test and control t-shirts. After 10 minutes, the bacteria were extracted, plated, incubated, and counted as described above.

Experiment 3: Does the Wash-In Silver Laundry Additive Function in a Real-Life Situation Like Wrestling Practice to Reduce Bacterial Contamination on Clothing?

In experiment 3, our goal was to look at a real-life situation (wrestling practice) and test bacterial load from wrestlers as opposed to applying a highly specific standard number of bacteria.

We assigned cotton t-shirts to test or control groups and laundered accordingly. As soon as the shirts were removed from the dryer, they were individually packaged (wearing nitrile gloves while handling to prevent accidental contamination). Because the source of bacteria (STEP 2 above) for experiment 3 was the wrestlers, we were very careful when handling the shirts to avoid any other source of bacteria. Whenever shirts were not in use, they were maintained in new Ziplock plastic bags. In the lab, before practice, a 5-cm diameter circle was drawn on the forearm region of each sleeve with an indelible marker just below the elbow where the coaches indicated wrestlers were likely to grab during sparring, and shirts were resterilized using ultraviolet radiation.

Thirty-two wrestlers signed Institutional Review Board informed consent forms (protocol LY-AS-030223-1). Each time they wrestled, they were assigned a shirt, and individual wrestlers were always in either the test or control group with no crossover. Shirts were passed from wrestler to wrestler so that we could get more users per shirt, as if each student had wrestled with several others. We had to do this for the safety of our wrestlers because there were a limited number of wrestlers in each weight class. We tested 3 sets of shirts with the aim of representing light contamination, medium contamination, and high contamination. Four control and 4 test shirts ($N = 4$) were worn by 1 wrestler who grappled with a second wrestler, thus exposing the shirt to 2 wrestlers (light contamination); an additional 4 shirts were worn by 2 wrestlers, so the shirt was exposed to 4 wrestlers (doubling the bacterial load [medium contamination]); and a final 4 shirts from each washing regimen were worn by 4 wrestlers, and the process was repeated, so the shirt was exposed to 8 wrestlers (quadrupling the initial bacterial load [high contamination]). Although this is standard practice, wrestlers were reminded to shower with an antibacterial soap immediately following this practice because they had shared shirts "for science."

Bacterial load on the shirts was assessed by cutting out the marked fabric circles and pressing them onto Petri plates containing Mueller-Hinton agar with 5% sheep red blood cells and extracting them as previously described and plating a 50-μL aliquot. This is half of what we used in controlled experiments because the process of wrestling leads to exceedingly heavy contamination. All plates were incubated overnight at 37°C with 5% CO_2, and colonies were counted.

Experiment 4: Does the Wash-In Silver Laundry Additive Function as a Residual Antibacterial Disinfectant Over Prolonged Exposure to Bacterial Load as Experienced During a Wrestling Tournament?

To simulate wrestling tournament conditions, Lycra singlet fabric was laundered as before, and 24 5-cm squares of each wash regimen were inoculated with 50 μL of a 0.5 MacFarland standard of *S. aureus* as a representative bacterium (simulating bacterial exposure of a bout). After 15 minutes, 3 squares from each treatment were pressed directly onto blood agar plates and stroked 10 times with an inoculating spreader vertically and then horizontally. The fabric square was disposed of in a biohazardous waste container. After 1 hour, this process was repeated with 3 additional pieces of singlet from each treatment to determine if extended exposure to silver nanoparticles reduced bacterial load compared with control fabric. Simulation of a second bout was performed by applying an additional 50 μL of *S. aureus* to each of the remaining squares, and the sampling process was repeated at 15 and 60 minutes. As wrestling tournaments take hours and wrestlers can face 6 or more different opponents, this process was repeated twice more for a total of 4 times (simulating 4 bouts total). Plates were incubated overnight, and colonies were counted.

RESULTS

Experiment 1: Does the Type of Fabric Used Affect Efficacy of Silver in Preventing Bacterial Growth?

There was no significant difference ($P = .22$) between total bacterial count on silver-treated cotton, polyester, or Lycra and the same fabrics washed with detergent alone (Figure 1A). In all fabrics, at lower bacterial contamination, there was a reduction of bacterial counts in the silver-treated fabrics. With that said, the deviations in the silver-treated fabrics exceeded those of the untreated fabric.

Experiment 2: Does the Type of Bacteria Used Affect Efficacy of Silver in Preventing Bacterial Growth?

Although *S. pneumoniae* demonstrated poor retrieval, there was a 60% reduction in bacterial survival ($P = .03$ by a Student's *t* test, significant at a 95% CI) on the silver-treated fabric (Figure 1B). There was no significant difference between the percentage of surviving *Staphylococcus* species tested on silver-treated and control fabric (*S. epidermidis* $P = .28$; *S. aureus* $P = .27$), nor were there significant differences

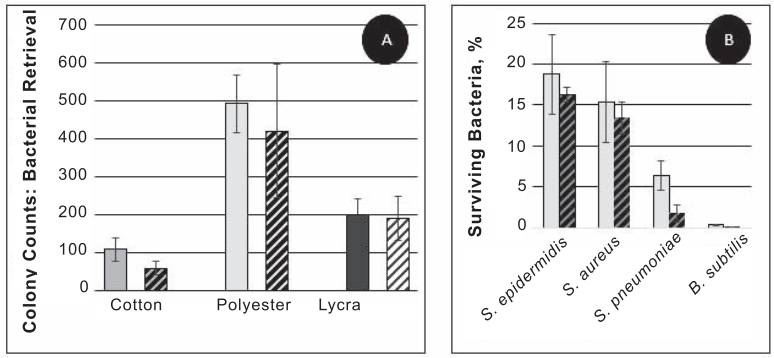

Figure 1. Comparison of silver efficacy on different fabrics and against different bacterial species. (A) Bacterial adhesion differed from fabric to fabric, but adding silver to the wash cycle did not significantly increase the fabric's resistance to bacterial contamination ($P = .22$). (B) The percentage of surviving bacteria shows a trend that fewer bacteria survive on silver-washed shirts than on shirts washed with detergent alone. This trend was significant for *Streptococcus pneumoniae* ($P = .03$) but not for *Staphylococcus epidermidis* (the most common nonpathogenic species found on skin; $P = .28$) nor for *Staphylococcus aureus* (the most common pathogenic species found on skin; $P = .27$) or *Bacillus subtilis* ($P = .08$). Solid bars indicate detergent alone, whereas striped bars indicate detergent + wash-in silver additive.

between the control and test fabrics for survival of *B. subtilis* ($P = .08$), which had low retrieval on all fabrics.

Experiment 3: Does the Wash-In Silver Laundry Additive Function in a Real-Life Situation Such as Wrestling Practice to Reduce Bacterial Contamination on Clothing?

In the practice simulation experiment, silver treatment reduced the number of bacteria on shirts with low exposure to contamination (2 and 4 wrestlers only, $P = .12$ and $P = .20$, respectively). However, at higher levels of exposure, as seen with contact by more than 4 wrestlers, silver treatment was ineffective ($P = .49$; Figure 2).

Experiment 4: Does the Wash-In Silver Laundry Additive Function as a Residual Antibacterial Over Prolonged Exposure to Bacterial Load as Experienced During a Wrestling Tournament?

In our wrestling tournament simulation, periodic reapplication of bacteria to silver-treated and control singlet fabric followed by intermittent incubation times mimicked the bacterial contamination process of extended competition. Unfortunately, there was no reduction in bacterial survival for silver-treated cloth. All samples showed an increase in bacterial load with each application (Figure 3).

DISCUSSION

Interestingly, and in agreement with previous research on silver-treated fabric,[14] the effectiveness in killing different bacterial species varied. Bacterial adhesion to fabrics and other materials varies based on the surface energy and texture of the material to which it attaches as well as the surface charge.[13] In direct negation of our hypothesis, using a wash-in silver additive in the washing cycle did not make the cotton, polyester, or Lycra fabrics significantly more resistant to the high levels of bacterial contamination that might be encountered in a contact sport regardless of the species tested. Bacterial cell membranes contain many sulfur-containing proteins. Because silver nanoparticles react with sulfur-containing proteins, this interferes with the function of the bacterial cell membrane.[16] Consequently, we had expected an across-the-

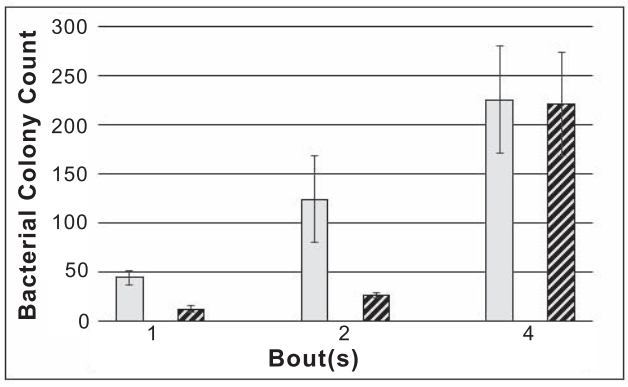

Figure 2. Practice simulation experiment. Mean bacterial count for cotton t-shirts worn during 1, 2, or 4 bouts at a regular practice show that wash-in silver was only effective at very low exposures to bacterial contamination. Solid bars indicate detergent alone, whereas striped bars indicate detergent + wash-in silver additive.

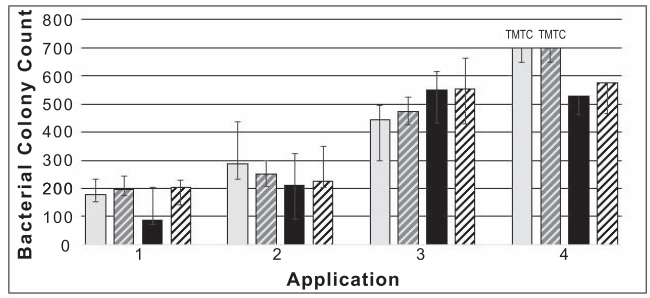

Figure 3. Wrestling tournament simulation experiment. Bacterial colony counts following repeated inoculation of singlet fabric with *Staphylococcus aureus* showed no significant effect of adding silver sheets to the wash. Solid bars indicate detergent alone, whereas striped bars indicate detergent + wash-in silver additive. Light gray bars indicate 15 minutes, whereas dark gray bars indicate 60 minutes. Abbreviation: TMTC, too many to count.

board reduction in bacterial survival. It is likely that when applied appropriately to produce the desirable low-density population of evenly scattered particles,[15] silver nanoparticles would be effective.

The high standard deviations seen in our study may be due to differences between bacterial load of individual wrestlers, which is fairly common, or uneven distribution of silver nanoparticles during washing. This might have occurred if fabrics stuck together in the washer or if an excessively dense distribution of the nanoparticles resulted due to 2 sheets being the recommended protocol rather than a single sheet. Thus, a wash-in approach may not be the most effective way to uniformly impregnate fabrics with silver nanoparticles. Furthermore, high levels of silver are less effective at killing microbes, and there is concern about bacteria developing silver resistance.[17]

During a tournament, a wrestler's singlet is contaminated beginning with their first bout, and the bacterial load increases with each subsequent event. If the singlet fabric is impregnated with an effective residual antimicrobial agent, then contamination can be reduced between bouts, minimizing the risk of infection. Although the manufacturer of the wash-in silver additive claims that its product has residual activity, our data do not support this assertion for the level of bacterial loads seen during either practice or wrestling tournament conditions. If, as we hypothesized, residual activity was present, we would expect bacterial counts to be high immediately after application, to decrease after 15 minutes, and fall precipitously after 1 hour. This pattern was not seen.

Having not observed significant efficacy of this silver nanoparticle wash-in additive, future explorations may consider other brands of wash-in silver nanoparticles or, when available, pretreated sports fabrics using alternate silver impregnation methods. Once the efficacy of silver treatment is demonstrated in control settings, testing in the actual wrestling environment would need to be conducted. Additionally, a study should be performed to see if the silver wash-in additive must be used every wash cycle or if some residual activity remains after the initial washing.

Many studies indicate that as silver use rises, so too do negative effects on human health and the environment. It is important to not only consider the potential benefit to athletes by reducing bacterial contamination but also consider any negative impact of contact with elevated silver levels. Silver nanoparticles can be released from fabric easily by washing and abrasion.[9] Alarmingly, silver is released more readily from fabric in the presence of sweat,[18] and ingestion of silver nanoparticles has been linked to blood diseases and colon cancer.[19] Worse yet, nanoparticles may be small enough to cross the blood-brain barrier.[20] Although it is thought that the deadly effects of silver are more greatly pronounced in bacteria and fungi than in mammals, both the Natural Resources Defense Council and the Friends of the Earth have expressed concern about the rate at which silver use is increasing without sufficient research and oversight.[20] Silver appears to be a more effective antimicrobial agent when it is released from the fabric to act on the bacterial membrane, which makes it more likely to cause collateral environmental damage, potentially leaching into drinking water and into ground water and interfering with microbes beneficial to native flora and agriculture.[17]

Therefore, we conclude that the environmental and potential health risks in using a wash-in silver nanoparticle laundry additive for clothing worn by wrestlers outweigh any potential infection control benefits to these wrestlers. We do not currently recommend adopting wash-in silver treatment as part of the laundering regimen for wrestling programs until further testing of alternate methods of silver impregnation into sports fabrics have been investigated.

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