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Antibacterial Effects of Hydrogen Peroxide and Silver Composition on Selected Pathogenic Enterobacteria

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Abstract: The efficacy of 30 ppb silver in 0.3% Hydrogen peroxide solution for disinfection of selected enterobacteria including *Escherichia coli*, Proteus mirabilis and Klebsiella pneumoniae was assessed in suspension and on surface. Mentioned bacteria exposed to the treatment solution for 72 hours in nutrient suspension and for 15 minutes on a steel surface. The bactericidal capability was determined by means of conventional colony counting and optical density (OD) at 450 nm. There were significant differences in OD of K. pneumoniae and P. mirabilis suspension between treatment and control groups during three 24 hours intervals (CI=0.95, P=0.000, for both), along with no growth on solid media at 24 and 48 hours of exposure. Results for *E. coli* were different; during initial exposure times, OD of *E. coli* decreased slightly in treatment group but unexpectedly increased after 24 hours. Colonies grown on plate confirmed this OD increment was because of viable *E. coli* in the suspension. It is possible that decomposition of H_2O_2 and reduction of its concentration after 24 hours allowed undamaged *E. coli* to grow. For surface decontamination test, there were significant differences between pre and post disinfection steps (For all bacteria CI=0.95, P<0.05). In conclusion, results of the current study proposed strong disinfection effect of the treatment solution against three important human pathogens; *E. coli*, *P. mirabilis* and *K. pneumoniae*, both in suspension and on surface.

Key words: Disinfection · Hydrogen peroxide · Silver ion · *E. coli* · K. pneumoniae · P. mirabilis

INTRODUCTION

Surface sterilization in health care settings and drinking water disinfection are two major applications of disinfectants. Currently, chlorine is the most popular disinfectant for water treatment [1-4] and glutaraldehyde as well as peracetic acid are extensively used for sterilization of medical equipments and environmental surfaces [5]. To be an ideal disinfectant, an antimicrobial agent should have no residual toxicity, be safe for human and animal and be stable in applied environments [1, 6, 7]. However, some disadvantages such as formation of toxic disinfection by-products (DBPs) associated with chlorine [6], mutagenic and carcinogenic effects of glutaraldehyde and high instability of peracetic acid [8] have made doubts about their usage.

Two of those best disinfectants known until now are Hydrogen peroxide (H_2O_2) and silver that their strong bactericidal activities have been studied on different bacteria [5, 9, 10]. It has been reported that 30-100 ppm of H_2O_2 killed *E. coli* via DNA damage [11]. Hydrogen Peroxide Vapor (HPV) also has bactericidal activity like its aqueous form. HPV inactivated mycobacterium tuberculosis, an important human pathogen [12, 13] and bacillus spores and its vegetative forms [1, 14]. Dry-mist of Hydrogen Peroxide was found to be more

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efficient than sodium hypochlorite solution for eradication of Clostridium difficile spores [1]. The efficacy of silver, in ionic and nanostructure forms, as antibacterial agent has been established in previous studies [15-19].

While disinfection potency of several concentrations of Ag^+ and H_2O_2 has been investigated separately on different bacteria [1, 11-15, 17, 19-21], just a few studies used a combination of these disinfectants [3, 5, 22-24]. Pedahzur *et al*. reported that combined Silver: Hydrogen Peroxide (1:1000) has higher inhibiting potency on *E. coli* growth than each individual agent [3, 5]. It is suggested that the interference of H_2O_2 in Ag^+ efflux from cell wall as well as interference of Ag^+ with H_2O_2 in cellular detoxification are possible modes of action for H_2O_2 and Ag^+ in a combination [24]. It also has been shown by Nabizadeh *et al.,* (2008) that 2% (20000 mg/L) concentration of a Nanocil (0.05% Ag^+: 50% H_2O_2) kill all target bacteria including Klebsiella pneumoniae in 15 minutes [23].

Although some countries currently use various concentrations of H_2O_2: Ag^+ for disinfecting of drinking water, applicability and efficacy of this agent are questionable [3, 5, 10]. The aim of this study was to evaluate antibacterial effects of a 30 ppb Ag^+ in 0.3% Hydrogen peroxide solution on *Escherichia coli*, Klebsiella pneumoniae and Proteus mirabilis. These Gram-negative, straight rods, facultative anaerobic, oxidase-negative and catalase-positive bacteria comprise 80 to 95% of the clinical isolates and also found in water supplies and bio-films formed on surfaces.

MATERIALS AND METHODS

Reagents and Media: Stock solutions were 30% hydrogen peroxide (Merck) and 800 ppm silver prepared from $AgNO_3$ (Merck). The treatment solution containing 0.3% H_2O_2 plus 30 ppb Ag^+ was freshly made in deionized water. Peptone Broth (Gibco) plus pure glucose powder (Sigma) in a concentration of 100 mg/dl was used as base medium. Bacterial culture was done on Eosin Methylen Blue (EMB) agar (Gibco) and Nutrient Agar (NA) (Gibco). All glasswares were soaked in 10% nitric acid (Merck) overnight, rinsed with deionized water and sterilized by autoclave before use.

Bacteria Preparation: *E. coli*, K. pneumoniae and P. mirabilis, taken from hospital samples and confirmed by specific diagnostic and differential tests, were sub-cultured on EMB and transferred to NA.

Experiment 1: the Disinfectant and Bacterial Suspension: A suspension of each bacterium was prepared in the base medium and optical density (OD) of suspensions was adjusted on 0.1-0.2 at 450nm. Each bacteria suspension was divided into 15 tubes followed by both OD assay and bacterial culture on EMB agar. In the next step, the treatment solution (30 ppb silver in 0.3% H_2O_2) was added to 10 tubes as test group and no additives to 5 tubes as control group.

Bacterial growth was assayed during three 24 hours intervals at 450 nm spectrophotometrically as well as colony counting on EMB agar; all bacterial suspensions were kept at room temperature in dark conditions during experimental period.

Experiment 2: the Disinfectant and Steel Surface: To determine the efficacy of the disinfectant on contaminated surface, a steel bench was divided into thirty 20×20 cm areas and sterilized by a reliable method, i.e. alcohol and fire. Bacterial culture was performed to confirm this sterilization procedure. Divided surface was contaminated with a heavy suspension of *E. coli*, K. pneumoniae and P. mirabilis, ten areas for each one, followed by culturing. At the end, Ag^+: H_2O_2 solution was applied and bacterial culture was repeated after 15 minutes. Culture media were incubated on 37°C for 24 hours in a microbiological incubator (Memert). In all steps, contact with surfaces was accomplished using sterile swabs.

Statistical Analysis: Data obtained from cultures and absorbance were analyzed using SPSS software Ver.14. We used paired samples t-test, independent samples t-test, Chi-square/Fisher exact methods to compare bacterial growth (mean colony forming units (CFU) and OD) and to determine disinfection efficacy.

RESULTS

Efficacy of the Disinfectant on Suspension: The efficacy of treatment solution against K. pneumoniae is shown in Figure 1. OD in the test group had a reduction within 24 hours of exposure, while in the control group it increased over time rapidly (CI=0.95, $P<0.05$). For longer exposure times (>24 hours) OD was virtually unchanged in the treatment group (CI=0.95, $P>0.05$); but not in control tubes (CI=0.95, $P<0.05$). At the end of experiment, data analysis showed a significant difference in OD between treatment and control groups (CI=0.95, P=0.000). For K. pneumoniae, bacterial culture of treatment group

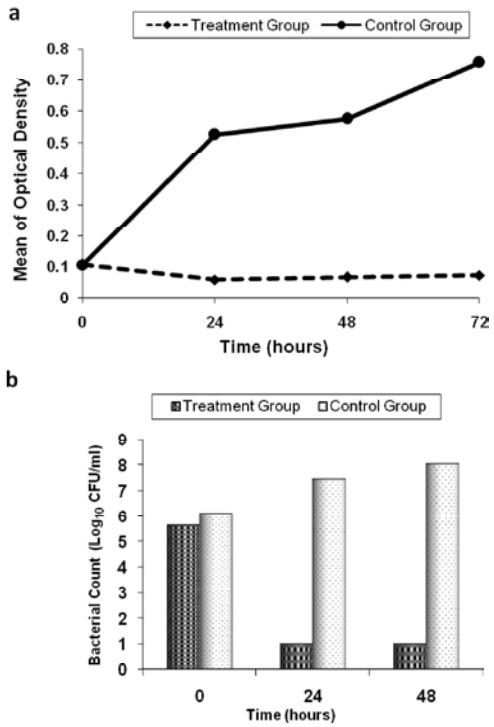

Fig. 1: Effects of hydrogen peroxide and silver combined solution on K. pneumoniae suspension in terms of: a) Optical Density and b) Colony Forming Units

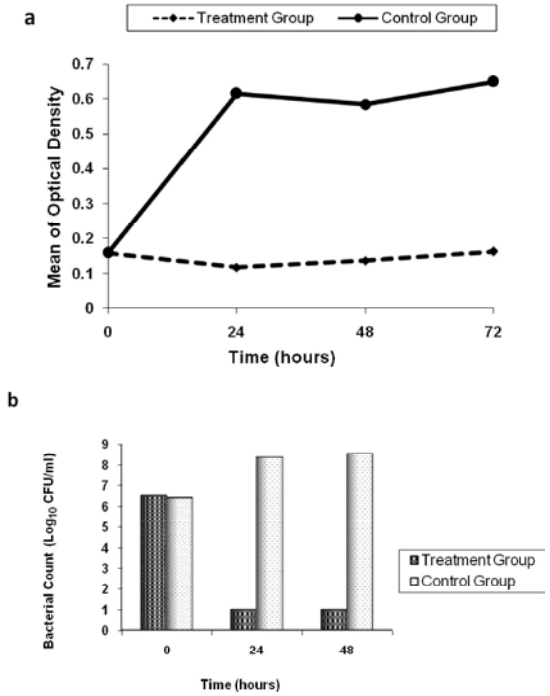

Fig. 2: Effects of hydrogen peroxide and silver combined solution on P. mirabilis suspension in terms of: a) Optical Density and b) Colony Forming Units

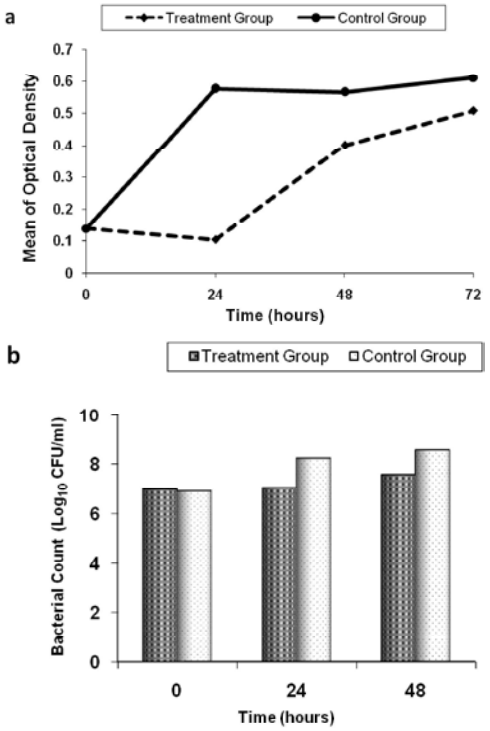

Fig. 3: Effects of hydrogen peroxide and silver combined solution on *E. coli* suspension in terms of: a) Optical Density and b) Colony Forming Units

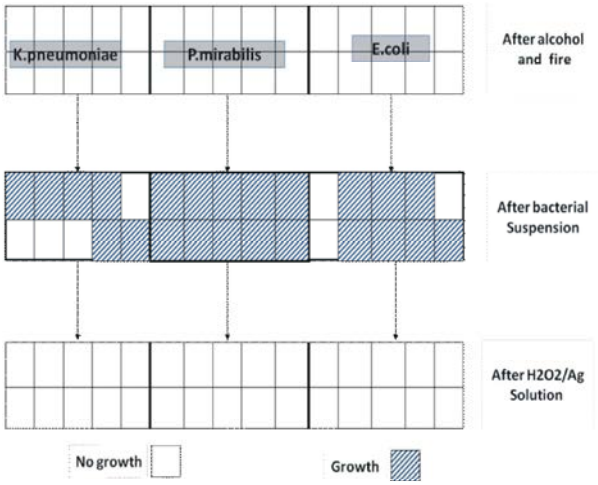

Fig. 4: Schematic of surface disinfection procedure and results of H_2O_2+Ag^+ solution effects on *E. coli*, K. pneumoniae and P. mirabilis

was negative at 24 and 48 hours of exposure. A similar trend was observed for P. mirabilis in terms of both OD and colony counting (Figure 2).

Results for *E. coli* were different; during initial exposure times; OD of *E. coli* suspension decreased slightly in treatment group but increased significantly

after 24 hours. In treatment group, number of *E. coli* colony grown on plate increased during 48 hours (Figure 3).

Efficacy of the Disinfectant on Steel Surface: A summary of results is shown in Figure 4. Applying alcohol and fire, all surfaces were sterilized completely confirmed by culture on EMB agar; after contamination of surfaces by bacterial suspensions, respectively 6, 10 and 7 squares were positive for K. pneumoniae, P. mirabilis and *E. coli*. In the ultimate step treatment solution was applied and cultures were repeated. There was not any positive result on EMB. Bacterial growth differences on EMB agar between above described steps were significant (P= 0.008 for *E. coli*, P=0.014 for K. pneumoniae and P=0.002 for P. mirabilis).

DISCUSSION

Here we showed that 30 ppb of silver in 0.3% hydrogen peroxide had bactericidal activity against *E. coli*, K. pneumoniae and P. mirabilis. K. pneumoniae and P. mirabilis responded to the treatment solution in a similar pattern; they both were inactivated completely in suspension which confirmed by no growth on the plates and virtually unchanged OD during 72 hours follow-up. While *E. coli* acted different; CFU of *E. coli* reduced up to 24 hours of exposure but increased within further exposure times. It is possible that the combination of silver and hydrogen peroxide damaged only a few percent of *E. coli* cells and those survived begun to grow after 24 hours, when probably H_2O_2 decomposed and its concentration reduced. These results imply that low concentrations of silver, here 30 ppb, did not inhibit bacterial growth, even if we consider the concentrations of H_2O_2 in the suspension reduced significantly within 24 hours. Unlike our results, Pedahzur *et al*. reported 60 minutes exposure to combination of silver and hydrogen peroxide (30 ppb: 30 ppm) resulted in 5 log reduction of *E. coli* [3]. However, their experimental method and time intervals of disinfection were somewhat different from those of we used. Also in our study we observed that addition of H_2O_2 to all bacterial suspensions resulted in formation of air bubbles which interfere with OD assessment in turbidimeteric assay that was one of our techniques for bacterial growth evaluation. Air bubble formation is due to the reaction of bacterial Catalase enzyme with its main substrate, H_2O_2. However, in our investigation, as same as Pedahzur's study, all cultures for three test bacteria were negative within 6 hours of exposure time in intervention, but positive in control groups (data not shown). Our results for *E. coli* are somewhat in agreement with those studies investigated effect of H_2O_2 or silver on *E. coli* in different status [3, 5, 11, 14, 18, 24-26]. Rincón and co-workers showed that H_2O_2 had positive effect on photocatalytic inactivation rate of *E. coli* [25]. Furthermore, other studies designed on the basis of decontamination with H_2O_2 in solar disinfection process and in the presence of iron and H_2O_2 [27], or photo-Fenton reaction [26], photolysis and photocatalysis inactivation of both vegetative and spore forms of Clostridium perfringens, in the presence of TiO_2 and H_2O_2 [28] confirm our results for *E. coli*. Gangadharan and collages also designed a novel silver nanoparticle that was able to eradicate both gram positive and gram negative bacteria; they proposed it as a beneficial material for water disinfection [16, 18].

Action mechanism of H_2O_2+Ag^+ was explored by Pedahzur and collages. They showed that silver ion act mildly on the promoters of *E. coli* genes *grpE*, *lon* and *dnaK*. They proposed that these effects may be related to the cellular protein damage [24].

Surface disinfection is critical for preventing pathogens distribution especially nosocomial infection agents that continuously are in contact with hospital surfaces. Here, we also determined surface disinfection potency of the treatment solution on the steel surface, inactivating three selected species of enterobacteria family. No colony forming units was seen on EMB agar after surface decontamination using H_2O_2+Ag^+ disinfectant. Brady and team workers have described a silver based technology for surface disinfection. They tested their desired technology on eradication of *Escherichia coli*, Staphylococcus aureus, Klebsiella pneumoniae, Enterobacter aerogenes, Enterococcus faecium and Salmonella choleraesuis; they proposed it as a good disinfectant within home and healthcare settings [29]. Finally all studies regarding antibacterial properties of silver only, hydrogen peroxide only, or their combination confirm results of the current study.

CONCLUSION

This study demonstrated strong disinfection effect of H_2O_2+Ag^+ solution against three important human pathogens including Proteus mirabilis, Klebsiella pneumoniae and *Escherichia coli*, both in suspension and on surface. However, the treatment solution presented better activity against K. pneumoniae and P. mirabilis on suspension. It is recommended that

morphological and ultra structural change of mentioned bacteria be analyzed following disinfection under similar situations. Considering our promising results, it is suggested that further researches be designed to investigate efficacy of this combined disinfectant on other pathogen bacteria and its application in disinfecting of medical settings.

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