Investigation and review on the efficacy of super-oxidized solution (HYDROCYN aqua®) against biofilm





Super-oxidized solution (SOS) is widely known for its effectiveness in aiding chronic wounds to heal compared with other traditional antiseptic solutions. There is a growing body of literature that recognizes the role of SOS in wound management, but studies on their efficacy toward biofilms are scarce. In this paper, we provide a short review on the effectiveness of SOS in biofilm removal. In addition, SOS (HYDROCYN aqua®) was tested against *Pseudomonas aeruginosa* biofilm and achieved more than 99.999% reduction of biofilm within 15 minutes of exposure. Within the confines of this review, SOS was found to be effective in eradicating biofilms.

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For centuries, infections have been one of the critical challenges faced by millions of people globally and are categorized by the types of microbes (biological agents) involved. Biofilm development within chronic wounds and medical device-related infections are a leading cause of patient morbidity and mortality and pose a huge threat to healthcare services (Tenke et al, 2017; Zimmerli and Sendi, 2017). Römling and Balsalobre (2012) have mentioned that up to 80% of chronic bacterial infections are involving the formation of biofilms by the associated microorganisms.

The most prevalent cause of biofilm-associated infections in hospitals are *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridians*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (Donlan, 2001). Generally, biofilm forms following expression of adhesins by planktonic (free-floating) microorganisms and then develop on the surface of the wound or medical device. The construction of an extracellular polymeric substances (EPS) by the microbes provides a three-dimensional structure (Bjarnsholt, 2013) that acts as a protective barrier to the embedded bacterial cells from cellular or chemical attack. Therefore, biofilms are known to be resistant to antibiotic as well as immune response, thus challenging the clinical treatment of chronic infections (Wu et al, 2014).

Currently, anti-biofilm strategies mainly focus on the disruption of the biofilm forming process by active anti-biofilm agents (Kiedrowski and Horswill, 2011; Ma et al, 2012) or repelling the biofilm formation through bioengineering approaches, such as the anti-biofilm coatings (Otto, 2008). Despite the success of anti-biofilm technologies, patients remain at risk of developing infections in the wound. Hence, there is a need for an antiseptic solution that is effective against biofilms.

Role of super-oxidized solution (SOS) in biofilm eradication

Recently, SOS was introduced as a wound rinse owing to its broad spectrum of activity. Most studies have demonstrated that SOS is effective in killing planktonic cells (Huang et al, 2008; Gunaydin et al, 2014), but very little attention has been paid to the role of SOS in destroying biofilms. Conventionally, SOS is produced by applying an electric current to the salt water through the electrolysis process, yielding hypochlorous acid (HOCl), hypochlorite ions (ClO^-), dissolved oxygen, ozone, and superoxide radicals (Gunaydin et al, 2014). SOS is stable, harmless to mammalian cells, inexpensive, and primarily it is excellent in killing microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and yeast (Wang et al, 2007; Sauer et al, 2009; Eftekharizadeh et al, 2016). Examples of SOS-susceptible bacteria are *Staphylococcus aureus*, *Kiebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii*, and *Enterococcus faecalis*, whilst fungi and yeast are *Candida albicans*, *Candida tropicalis*, *Candida parapsolosis*, *Trichosporon* spp. and *Aspergillus* spp (Gunaydin et al, 2014; Ünal et al, 2014; Gupta et al, 2017).

Previous research findings have highlighted that the main antimicrobial components of SOS is the HOCl (Selkon et al, 1999; Wang et al, 2007; Sakarya et al, 2014) and sodium chlorite ($NaClO_2$) (Chen et al, 2013). In the mammalian immune system, HOCl is produced naturally by neutrophils responsible for attacking and fighting against invading pathogens (Wyatt et al, 2014). Based on existing literature investigations, HOCl exerts its antimicrobial and anti-biofilm activities through different modes of action, including penetrating bacterial cell wall and reacting with enzymes to prevent normal respiration (Banwart, 1989); inhibition of RNA and DNA synthesis that will result in disruption of bacterial cell's function (Armstrong et al, 2015), disaggregation of biofilms (Sauer et al, 2009); and electrostatic attraction between neutrally charged HOCl and negatively charged surface of bacterial barrier (Pintaric et al, 2015).

Table 1 lists studies conducted on biofilms using SOS and HOCl. As shown in the table, the studies found that HOCl in SOS has a pivotal role in biofilm eradication. Sauer et al (2009) have conducted a study to test the effectiveness of pH neutral-SOSs to combat both planktonic and biofilm cells of *Pseudomonas aeruginosa*. In this study, three different types of SOSs were tested (OIS-80, OIS-125, and OIS-200) at different oxychlorine concentrations (80, 125, 200 ppm). The efficacy of these three SOSs in eliminating *Pseudomonas aeruginosa* biofilms were analyzed quantitatively through a colony-forming unit (CFU) count and qualitatively by confocal laser scanning microscopy (CLSM) visualization. Matured biofilms (6 days old) were established on the interior surfaces of Masterflex silicone tubing and subsequently treated with the three SOSs for 60 mins. Results showed that treatment with OIS-80 and OIS-200 successfully reduced the biofilm viability by 50% within 10 min and 2.5 min, respectively. As the treatment time increased from 5 min to 30 min to 60 min, the value of log reduction of biofilm increased from 0.5 to ~2.5 to 3.3. This indicates that reduction in the number of biofilm viable cells increased from <90% reduction to ~99% to ~99.9% as the treatment time prolonged.

Under the CLSM observations, biofilms treated with OIS-200 showed an increase in the number of red stained cells (dead cells),

Table 1. Studies carried out in treating biofilm using super-oxidized solution (SOS) and hypochlorous acid solution (HOCl)

Title	Objective	Type of Microorganism	Result/Performance
Solutions of 125 and 200 ppm concentration enhanced the disaggregation of biofilms	*Pseudomonas aeruginosa*	Determine the efficacy of three SOSs varying in oxychlorine concentration	Neutral super-oxidized solutions are effective in killing *Pseudomonas aeruginosa* biofilms (Sauer et al, 2009)
Stable HOCl solution decreased the amount of biofilm, and the microorganisms within the biofilm with the dose-dependent manner depending on species	*Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*	Investigate the effect of stabilized HOCl on biofilm formation	Hypochlorous acid: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency (Sakarya et al, 2014)
Super-oxidized water demonstrated a remarkable and similar bactericidal effect as compared to the traditional NaOCl solution against *Enterococcus faecalis* biofilms	*Enterococcus faecalis*	Investigate the antibacterial effects of super-oxidized water in root canals infected	Antibacterial efficacy of super-oxidized water on *Enterococcus faecalis* biofilms in root canal (Zan et al, 2016)
HOCl significantly lowered the LPS concentration of *Porphyromonas gingivalis* when compared with NaOCl and CHX	*Escherichia coli, Porphyromonas gingivalis, Enterococcus faecalis, Streptococcus sanguinis*	Examine anti-biofilm efficiency of HOCl, sodium hypochlorite (NaOCl) and chlorhexidine (CHX)	Effectiveness of HOCl to reduce the biofilms on titanium alloy surfaces *in vitro* (Chen et al, 2016)
Stabilized hypochlorous acid was the only solution tested capable of eradicating *Ralstonia pickettii* biofilm on all implant surfaces tested within the first five-minute soak time	*Ralstonia pickettii*	Evaluate the effectiveness of four antimicrobial solutions on biofilm	Preliminary results of the use of a stabilized HOCl solution in the management of *Ralstonia pickettii* biofilm on silicone breast implants (Brindle et al, 2017)
Electrolyzed water effectively removes bacterial biofilms within short exposure time	*Staphylococcus aureus, Pseudomonas aeruginosa*	Assess new formulated electrolysed water on biofilm removal	The efficacy of an electrolysed water formulation on biofilms (Brindle et al, 2017)

destruction of the three-dimensional structure of the biofilms and reduction in overall biomass attached to the substrate. The disaggregated biofilm structure has allowed the microbial colonies to detach and disperse from the EPS matrices, thus increasing their susceptibility towards the SOS. The authors proposed that the SOS might exert its anti-biofilm action through several modes of action, including the impairment of cell membrane, inactivation of cytoplasmic enzymes, and presence of chlorine-based free radicals that tend to attack the DNA, RNA, and proteins. However, the true mechanism of anti-biofilm action of OIS-200 against the tested *Pseudomonas aeruginosa* is unknown.

Apart from that, Chen et al (2016) has conducted an *in vitro* anti-biofilm study of HOCl, sodium hypochlorite (NaOCl) and chlorhexidine (CHX); three main components of SOS. These compounds were tested against Gram-positive (*Enterococcus faecalis* and *Streptococcus sanguinis*) and Gram-negative (*Escherichia coli* and *Porphyromonas gingivalis*) bacterial biofilms at different SOS volumes and treatment times. Among the three components, HOCl appeared to be the most effective anti-biofilm compound against all tested bacteria. Moreover, the antimicrobial activity of HOCl was volume-dependent and also time-dependent. The 4:1 volume ratio of HOCl to bacterial solution completely killed the bacteria.

The three compounds were also tested on their efficiency to eliminate biofilm-contaminated implant surfaces (titanium alloy). Indeed, HOCl was the most effective in removing biofilm from contaminated implants as compared to NaOCl and CHX. The concentration of HOCl tested was 0.018%, whilst NaOCl and CHX were 1.3% and 0.2%, respectively. Chen et al (2016) found that HOCl is effective as both an antimicrobial and biofilm remover, even at lower concentration than those NaOCl and CHX. This finding is consistent with that of Sakarya et al (2014) who conducted a study on stabilized HOCl solution against different types of biofilms (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*). Results demonstrated that treatment with HOCl successfully decreased the amount of biofilms and embedded microbial cells within the biofilms, with the dose-dependent manner and depending on the species.

Besides, newly formulated electrolyzed water (EW) was tested *in vitro* against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Salisbury and Percival, 2018). This EW consisted of HOCl, ClO^-, hydroperoxyl (HO_2), hydroxide (OH^-), and hydrogen peroxide (H_2O_2), chlorine (Cl) and singlet oxygen. In the test, 48-hr biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were developed on polycarbonate coupons. The biofilm of each strain was treated with EW at concentrations of 100%, 75%, 50%, 25% and at three different contact times (5, 15, and 60 mins). Post-treatment, the biofilm density was determined through bacterial enumeration of the disaggregated biofilms. At 100, 75, 50, and 25% of EW concentrations, biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were significantly reduced within 5 mins of contact time. It has been suggested that HOCl and ClO^- are the primary components in eradicating biofilms (Sakarya et al, 2014; Chen et al, 2016; Salisbury and Percival, 2018).

In-vitro study of SOS (HYDROCYN aqua®) efficacy against *Pseudomonas aeruginosa* biofilm

HYDROCYN aqua® is an advanced wound wash solution with electrolyzed SOS. The components of this particular SOS are HOCl, sodium hypochlorite (NaOCl), sodium chloride (NaCl) and purified water. HYDROCYN aqua® has been tested and proven to be effective against biofilms of *Pseudomonas aeruginosa* ATCC 15442, according to the method of E2799-12: Standard Test Method for Testing Disinfectant Efficacy using the MBEC Assay. *Pseudomonas aeruginosa* ATCC 15442 is a common strain widely used in anti-biofilm studies due to its capability to produce confluent biofilms (Harrison et al, 2008; Sauer et al, 2009; Lineback et al, 2018). During the test, *Pseudomonas aeruginosa* ATCC 15442 biofilm was established for 24 hrs on pegs in a 96-well plate and exposed to the HYDROCYN aqua® solution at different concentrations (100%, 10%, 1%, and 0.1%). In the post-treatment, the number of surviving cells in the biofilms were quantified by cultivation and optical density measurements. The number of surviving microorganisms were compared with a control sample in which the solution was replaced with buffer solution.

Table 2 shows the test results of HYDROCYN aqua® efficacy at different concentrations against *Pseudomonas aeruginosa* ATCC 15422 biofilm. It is apparent from the table that undiluted and 1:10 dilution HYDROCYN

Table 2. Test results of HYDROCYN aqua® efficacy at different concentrations against biofilms of *Pseudomonas aeruginosa* ATCC 15422

Percent of Reduction (%)	Log Reduction (initial – surviving)	Surviving biofilm population (CFU/ mm^2)	Initial biofilm population (CFU/ mm^2)	Sample concentrations
99.999	≥ *5.95*	< 1	9.01×10^5	Undiluted
99.999	≥ *5.95*	< 1		1:10
3.21	*53.00*	5.6×10^2		1:100
50.00	2.98	9.5×10^2		1:1000

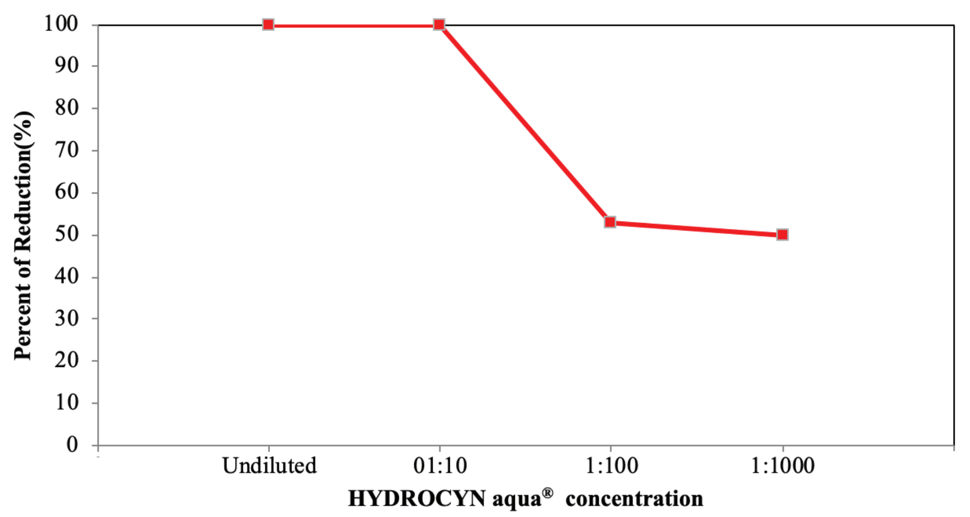

Figure 1: Percentage of Pseudomonas aeruginosa *ATCC 15422 biofilm reduction at different HYDROCYN aqua® concentrations*

aqua® significantly eradicated *Pseudomonas aeruginosa* ATCC 15422 biofilms with 99.999% of reduction from the initial population size. The efficacy of HYDROCYN aqua® in removing biofilm reduces as the concentration decreases *[Figure 1]*. In fact, the presence of HOCl as an active compound in the HYDROCYN aqua® plays the prime role in eradicating the biofilms. These results are in accord with those of previous studies indicating that super-oxidized solution has potential in eradicating biofilm (Sauer et al, 2009; Chen et al, 2016, Salisbury and Percival, 2018).

Conclusion

All the studies included in this review suggest that SOS is effective in eradicating biofilm. Super-oxidized water has been introduced in recent years as one of the effective antimicrobial solutions compared to the conventional solutions like bromine solution that are cytotoxic to cells or tissues. The major component that demonstrated its effectiveness in eradicating biofilms is HOCl, which is produced naturally by white blood cells to combat pathogenic microbes (attacking the microbe cell membrane by dissolving protective membrane's biofilm). HOCl is present and serves as the active component in HYDROCYN aqua®; therefore, affirms the clinical application of HYDROCYN aqua® (super-oxidizing solution) in eradicating bacterial loads and biofilms without cytotoxic effects. Moreover, HYDROCYN aqua® was able to remove 99.999% of *Pseudomonas aeruginosa* ATCC 15422 biofilm based on this *in-vitro* study. The results of this research support the idea that SOS (HYDROCYN aqua®) is an ideal choice for treatment of wound biofilm including chronic and infected surgical or burn wounds. WAS

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