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In vitro comparative cytotoxicity study of a novel biocidal iodo-thiocyanate complex

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Abstract

Novel biocides, which avoid the induction of cross-resistance to antibiotics, are an urgent societal requirement. Here, we compared the cytotoxic and bactericidal effects of a new antimicrobial agent, the iodo-thiocyanate complex (ITC), with those of the common antiseptics, hydrogen peroxide (H_2O_2), povidone iodine (PVP-I) and Lugol's iodine (Lugol). The antimicrobials were co-incubated for 10 min with HeLa and *Escherichia coli* cells in the presence and absence of organic matter (Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum). The cytotoxic concentrations of ITC were equivalent to its bactericidal concentrations (<7.8 µg ml^{-1}). By contrast, cytotoxic effects of H_2O_2, PVP-I and Lugol were apparent at concentrations lower than their bactericidal concentrations (250, 125 and 31.3 µg ml^{-1}, respectively). The cellular effects of ITC were not quenched by organic matter, unlike the other antiseptics. ITC, PVP-I and Lugol had dose-dependent effects on the viability of horse erythrocytes, while H_2O_2 showed no hemolysis. ITC, at 30 or 300 µg ml^{-1}, did not cause DNA breakage in HeLa cells, as assessed by an *in vitro* comet assay in the absence of S9 metabolic activation, whereas H_2O_2 caused extensive single-strand DNA breaks. The pronounced antimicrobial potency of ITC and its favorable cytotoxicity profile suggests that ITC should be considered for antiseptic applications.

Keywords

Iodo-thiocyanate complex (ITC); hydrogen peroxide; povidone iodine; Lugol's iodine; HeLa cytotoxicity; *in vitro* comet assay.

1. Introduction

The increasing occurrence of antibiotic resistance in pathogenic bacteria, coupled with a dramatic decline in the number of newly approved antibiotics, represents a major societal

challenge. In the context of growing resistance to antibiotics, wound infection control and management, along with the control of potentially pathogenic bacteria in healthcare environments, is becoming challenging and represents a major healthcare burden (WUWHS 2008).

Naturallye occurring peroxidase-catalyzed antimicrobial systems suggests provide a possible future direction, in the form of peroxidase-catalyzed antimicrobial systems. A peroxidase enzyme together with hydrogen peroxide, inorganic ion substrate and generated oxidized products is known as a peroxidase system (Kussendrager and van Hooijdonk 2000). These naturally occurring systems are part of the host defense network and provide an immediate defense against invading microorganisms (Davies et al. 2008). A large number of studies have investigated and demonstrated the antimicrobial potential of peroxidase-catalyzed systems, as extensively reviewed elsewhere (Bafort et al. 2014; Davies et al. 2008; Kussendrager and van Hooijdonk 2000; Naidu 2000; Seifu et al. 2005). Peroxidase/hydrogen peroxide/halide systems have a dual role, however, acting as both a friend and a foe (Davies et al. 2008; Klebanoff 2005). As they possess a non-specific, broad-spectrum target mechanism, aside from mediating bacterial killing, destroying invading parasites, combating fungal infections and inactivating viruses, they can also attack a variety of mammalian cells (Clark and Klebanoff 1977; Edelson and Cohn 1973), including tumor cells (Clark and Klebanoff 1975; Henderson et al. 1981; Jong and Klebanoff 1980).

Peroxidase-catalyzed systems may also inspire the development of a new generation of antimicrobial agents. Our previous research evaluated three such antimicrobial agents (Tonoyan et al. 2017). The agents, all produced without a peroxidase enzyme, were formed by the reactions of H_2O_2 with iodide (I^-) salt (H_2O_2/KI); or H_2O_2 with thiocyanate (SCN^-) salt (H_2O_2/KSCN); or H_2O_2 with both ion substrates (I^- and SCN^-). The latter reaction forms the iodo-thiocyanate complex (ITC). In the ITC, H_2O_2 serves as a source of oxidation for two

substrates, generating reactive oxygen and iodine species within the reaction mixture, which possess powerful antimicrobial properties. The mixture was shown to contain low concentrations of antimicrobial species such as hypoiodite, hypothiocyanite and hydroxyl radical; with molecular iodine being the major cidal component. The biocidal ITC formulation effectively and rapidly killed Gram-negative and Gram-positive bacteria, including a multidrug-resistant *Staphylococcus aureus*. It was shown that ITC was capable of eradicating mono- and dual-species bacterial biofilms within short exposure times (10 min and 30 s, respectively). In addition, we were unable to induce resistance in bacteria to the ITC, likely due to the presence of multiple cellular targets (Okano et al. 2017). These characteristics suggested the potential for use of ITC as a new antimicrobial to prevent, and possibly treat, bacterial infections. In addition to antimicrobial activity, however, the cytotoxic potential of a new agent must also be evaluated.

A variety of approaches can be used to evaluate and screen the *in vitro* toxicity of antimicrobial substances. Testing the effects of compounds on the viability of mammalian cells grown in culture (*in vitro* cytotoxicity tests) is widely used as an indicator of potential toxic effects in animals (Riss et al. 2011). Among the different cytotoxicity tests, the methyl tetrazolium (MTT) viability assay is the most popular, low-cost and convenient method (Fotakis and Timbrell 2006). Considering that antimicrobials may potentially come into direct contact with blood, an evaluation of hemotoxicity is also worthwhile. The standard measure of blood compatibility is hemolytic activity or the lysis of red blood cells (Li et al. 2012). Genotoxicity testing is also an important aspect of the safety assessment of broad-spectrum substances, including pharmaceuticals and biocides (Corvi and Madia 2016). Our previous research (Tonoyan et al. 2017) indicated that the ITC contains reactive species, which may have the potential to induce bacterial DNA breakage; the detection of DNA breaks by single cell gel electrophoresis (comet assay), would thus be informative.

The aim of the present study was to investigate the *in vitro* cytotoxicity, hemolytic activity and DNA damaging effect of the novel antimicrobial ITC —in relation to its antimicrobial activity. The potential suitability of ITC as an antiseptic was then considered and compared with H_2O_2, PVP-I and Lugol's iodine.

2. Materials and methods

2. 1. Antimicrobial agents and preparation

All the materials were purchased from Sigma-Aldrich, unless otherwise stated. The antimicrobial agents evaluated in this study were H_2O_2/KI, H_2O_2/KSCN and H_2O_2/KI/KSCN [ITC; (Tonoyan et al. 2017)]. The stock solutions were prepared by combining concentrated solutions of H_2O_2 (v/v) with KI (w/v) and/or KSCN (w/v) to obtain 1% final concentrations for each agent. These solutions were considered as 1% according to the concentration of H_2O_2 present in the mixtures, as KI and KSCN do not possess antimicrobial activity alone (data not shown). The antiseptics H_2O_2 (30% v/v stock solution), PVP-I (10% w/v available iodine stock) and Lugol's iodine (1.25% w/v available iodine stock) were also used in this study. The stock solutions were diluted to the desired working antimicrobial concentration range using sterile deionized water (dH_2O).

2. 2. Test bacteria, cell cultures, nutrient solutions and growth conditions

The bacterial strain used in this study was *Escherichia coli* ATCC 25922 (from the American Type Culture Collection), which was cultured aerobically on Lennox agar and lysogeny broth at 37°C. HeLa human cervical epithelial cell lines were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) and were grown as a monolayer in complete DMEM (Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) at 37°C, 5% CO_2.

2. 3. Cytotoxicity studies

2. 3. 1. Determination of simultaneous cellular and bacterial toxicities of antimicrobials towards human and bacterial cells

The cellular toxicity of the test antimicrobials and antiseptics towards human epithelial HeLa cells was assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, whereas, their antimicrobial potential towards bacterial *E. coli* cells was assessed using viable bacterial cell counts.

The simultaneous cytotoxic and bactericidal effects of antimicrobials were determined by co-incubation of HeLa and *E. coli* cells, for 10 min, with the antimicrobials, at a range of concentrations, in two different media [phosphate-buffered saline (PBS) and DMEM supplemented with 10% FBS without addition of antibiotics (DMEM + FBS)], at room temperature. HeLa cells were seeded at a concentration of 8×10^3 cells $well^{-1}$ in 96-well plates in complete DMEM media. After 72 h incubation, the confluent cell monolayer was washed with PBS and was covered with 200 µl PBS, or DMEM + FBS, containing 10^6 cfu ml^{-1} *E. coli* cells and the antimicrobial agent. Eight two-fold dilutions of the selected antimicrobials were tested, and the concentrations in the wells ranged from 7.8 to 1000 µg ml^{-1}. As it was shown previously (Tonoyan et al. 2017)~~,~~ that ITC at this specified concentration range could eradicate *E. coli* biofilms within 10 min, this was thus considered an appropriate duration of exposure to demonstrate antimicrobial activity. Following the 10 min incubations, 10 µl aliquots were taken from the supernatant and ten-fold serially diluted in PBS. Aliquots (5 µl) of the dilutions were then replica (three per sample dilution) spot-plated on agar plates to enumerate viable *E. coli*. This method can be used to enumerate bacteria on a high-throughput scale in a fast, easy-to-use, labor-efficient and cost-efficient manner (Sieuwerts et al. 2008). Bactericidal activity (expressed as a minimum bactericidal concentration; MBC) was defined as a >3 $\log_{10}$ reduction

(99.9% kill) in cfu ml^{-1} from the starting *E. coli* inoculum concentration (Rose and Poppens 2009). The HeLa monolayers were washed twice with PBS and incubated with complete DMEM, supplemented with 0.6 mg ml^{-1} MTT, for 4 h at 37°C. The medium containing MTT was aspirated, formazan crystals were dissolved in 150 µl dimethyl sulfoxide and the absorbance was measured at 595 nm (Tecan GENios, Salzburg, Austria). Cellular viability was expressed as a percentage of the untreated controls. ~~All the treatments and their concentration range were tested in duplicate, and all experiments were repeated at least twice. Statistical analysis of treated versus untreated HeLa cells was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (SigmaPlot Version 11, Systat Software, Inc., San Jose, USA).~~

2. 3. 2. Visualization of morphological changes in Hela cells

The impact of the antimicrobial treatments on HeLa cells was also evaluated microscopically. Untreated cells, cells lysed with 10% Triton X-100, and cells exposed to the highest concentrations of the antimicrobials (1000 µg ml^{-1}) in either PBS, or DMEM + FBS, were visualized in 96-well plates, after 10 min antimicrobial treatments, using bright-field optic microscopy on an inverted microscope (Leica DM IL LED, Leica Microsystems Ltd.) fitted with a DFC420C digital camera using LAS at 20× objective magnification.

2. 3. 3. Recovery of HeLa cells after withdrawal of antimicrobials

To assess whether the cytotoxic effects of the antimicrobials on human cells were reversible or irreversible, cytotoxicity was measured either immediately after 10 min exposure of HeLa cells to antimicrobials, or after recovery based on a previously published protocol (Müller and Kramer 2007). Briefly, HeLa cells were exposed to antimicrobials in PBS, or DMEM + FBS media, for 10 min, and the MTT assay was performed. Subsequently, another set of HeLa cells were exposed to antimicrobials in PBS, or DMEM + FBS, for 10 min, carefully washed twice

to remove the antimicrobials, and then cultured for a further 24 h. Following incubation, the MTT assay was performed and cell viability was determined. ~~To estimate the significant recovery of cells exposed to different concentrations of antimicrobials, the data from HeLA cells exposed for 10 min and then immediately tested was compared to data from the revived HeLA cells by two-way ANOVA followed by Tukey's pairwise multiple comparison test (SigmaPlot Version 11).~~

2. 4. Hemolytic activity studies

2. 4. 1. Determination of hemolytic activity of antimicrobials towards horse red blood cells (hRBC)

The hemolytic activity of the antimicrobials was tested by determining the release of hemoglobin in horse erythrocyte suspension as a measure of red blood cell lysis (Evans et al. 2013).

The horse RBCs were prepared by centrifugation of defibrinated horse blood (TCS Biosciences; packed cell volume 40%) at 3000 × *g* for 5 min. Plasma, white blood cells and platelets were removed as supernatant by washing the blood with PBS. After obtaining a clear supernatant, the hRBC pellet was resuspended in PBS to 4% hRBC suspension. Aliquots (900 µl) of hRBC suspensions were then added to micro-centrifuge tubes containing 100 µl of antimicrobial solutions to give final concentrations of antimicrobials at a range of 7.8 – 1000 µg ml^{-1}. PBS and 10% Triton X-100 were used as 0% and 100% hemolysis controls, respectively. The samples were incubated for 1 h at 37°C. Subsequently, the tubes were centrifuged at 3000 × *g* for 5 min and 20 µl aliquots from supernatant were transferred into 96-well plates containing 180 µl PBS. Hemoglobin release was monitored by measuring the absorbance at 540 nm (Tecan GENios). Hemolysis was calculated as a percentage of the maximum hemolysis achieved by Triton X-100. ~~All the treatments were carried out in duplicate~~

~~and all experiments were repeated at least twice. The statistical significance between different concentrations, by comparison to untreated cells, was assessed using one-way ANOVA with follow-up Tukey's post hoc test (SigmaPlot Version 11).~~

2. 4. 2. Visualization of morphological changes in horse erythrocytes

To assess erythrocyte lysis hRBCs, exposed to the highest concentration of the antimicrobials (1000 µg ml^{-1}) for 1 h, were visualized microscopically. Aliquots (10 µl) from the antimicrobial-treated hRBC suspensions were transferred to a hemocytometer chamber and viewed by inverted microscope (Leica DM IL LED) at 40× objective magnification.

2. 5. Genotoxicity studies

2. 5. 1. Assessment of ITC-induced DNA breaks in HeLa cells

The comet assay was used to analyze and quantify any DNA strand breaks caused by ITC at the individual cell level. The assay was performed as described elsewhere~~, but with some modifications~~ (Olive and Banath 2006). ~~Specifically~~Briefly, 80% confluent HeLa cells were detached and resuspended in PBS at a density of 5.75×10^5 cells ml^{-1}. ITC was added to cell suspensions, at final concentrations of either 30 or 300 µg ml^{-1}, and incubated for 30 min. Exposure of cells to either H_2O_2 at 300 µg ml^{-1} or UV-C irradiation (performed under the Philips TUV 64T5/SP UV-C disinfection lamp of laminar SterilGard Hood, producing UV radiation of 253.7 nm) for 30 min were used as two positive controls, while untreated cell suspensions in PBS represented the negative controls. After treatment, ~~a 50 µl aliquot of~~ each cell suspension at final density of 1×10^4 cells ml^{-1} was mixed with ~~added to 450 µl~~ 1% low melting point agarose in PBS ~~(LMPA)~~ and ~~150 µl cell suspension/LMPA mix were subsequently~~ placed onto a microscope slide precoated ~~, which had been previously coated~~ with a film of 1% normal melting point agarose. ~~(200 µl $slide^{-1}$) and dried at 50°C. Thus prepared,~~

each slide contained approximately 10000 cells. Coverslips were added to the slides and the preparations were allowed to gelAfter solidification at 4°C, the . Once gelled, the coverslips were removed and slides were submerged in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% sodium lauroyl sarcosine, 1% Triton X-100 and 10% DMSO). Lysis was performed for 2 h at 4°C. Afterwards, the microgels were rinsed with, and then placed in, cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) to equilibrate for 30 min. Electrophoresis was conducted in the same buffer at 20 V (1 V cm^{-1}) and about /200 mA (adjusted by raising or lowering the buffer level) for 30 min. Immediately after electrophoresis, the The slides were neutralized in cold 0.4 M Tris pH 7.4 buffer (3 × 5 min washes), and then washed with cold dH_2O. Microgels were furtherand stained with 200 µl of 1 µg ml^{-1} 4',6-diamidino-2-phenylindole (DAPI) for 10 min and then washed with dH_2O. All steps were performed under dimmed light to reduce additional light-induced DNA damage. The slides were then observed by inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems Ltd.) at 20× objective magnification and comets were scored using Comet Assay IV image analyzing software (Perceptive Instruments Ltd.). To quantify DNA damage, the results were expressed in the average extent tail moment ± SD of 50 randomly selected comets per treatment. The significance of difference between control and treated groups was statistically analyzed by one-way ANOVA, followed by Tukey's post-hoc test (IBM SPSS Statistics 21 Inc., Chicago, USA).

2. 6. Statistical analysis

The presented data are displayed as a mean ± standard deviation (SD) of at least two independent experiments conducted in duplicates. To quantify DNA damage, the results were expressed in the average extent tail moment ± SD of 50 randomly selected comets per treatment. The statistical analyses were performed using the SigmaPlot Version 11 (Systat Software, Inc., San Jose, USA) or IBM SPSS Statistics 21 (Chicago, USA). To assess the

significance of differences between the treatment groups and controls, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied. To estimate the significant recovery of HeLa cells exposed to different concentrations of antimicrobials, the data from HeLA cells exposed for 10 min and then immediately tested was compared to data from the revived HeLA cells by two-way ANOVA followed by Tukey's test. A value of $p < 0.05$ was considered statistically significant and $p < 0.01$ statistically highly significant.

3. Results

3. 1. Cytotoxic and antibacterial activities of antimicrobials

The cytotoxic and antibacterial effects of the antimicrobials were determined simultaneously by co-incubation of human HeLa, bacterial *E. coli* cells in different concentrations of antimicrobials, for 10 minutes, in either PBS, or in DMEM + FBS, and then applying the MTT assay and viable cell counting. When incubated in PBS (Fig. 1A), H_2O_2/KI and H_2O_2/KSCN were cytotoxic at all concentrations tested (7.8 – 1000 µg ml^{-1}) with very slight antimicrobial activity, with the exception of the highest dose (1000 µg ml^{-1}) of H_2O_2/KSCN, which caused a 5 $\log_{10}$ reduction in *E. coli* (Fig. 1A). ITC, when incubated in PBS, was highly toxic towards both cell types after 10 min exposure~~,~~; even at the lowest test concentration of ITC (7.8 µg ml^{-1}) no viable *E. coli* and HeLa cells were detected (Fig. 1A). Amongst the antiseptics, the antimicrobial potential of H_2O_2 increased in a concentration-dependent manner, with a bactericidal effect noted at a concentration of 250 µg ml^{-1} (the MBC value). It was, however, highly cytotoxic at all concentrations tested (Fig. 1A). The cellular and bacterial toxicities of PVP-I also increased in a manner depending on its dose. PVP-I exhibited the least cytotoxic performance among the tested antimicrobials – around 20% cytotoxicity at 31.3 µg ml^{-1}. However, the bactericidal concentration of PVP-I (125 µg ml^{-1}) was accompanied by toxicity

towards HeLa. Lugol was cytotoxic even at concentrations below the MBC (31.3 µg ml^{-1}; Fig. 1A).

Microscopic observations supported the results from the MTT cytotoxicity assay. With respect to the PBS incubations (Fig. 2A), H_2O_2/KI, H_2O_2/KSCN and H_2O_2, at 1000 µg ml^{-1}, completely lysed and "dissolved" HeLa cells after a 10 min contact time. However, upon exposure to ITC, PVP-I and Lugol, the cells seemed to not be fully lysed, but looked unhealthier in comparison to untreated control (Fig. 2A).

The antimicrobial and cytotoxic effects of the antimicrobials were notably different in the presence of the cell culture media supplemented with 10% fetal bovine serum (DMEM + FBS; Fig. 1B), by comparison to the PBS incubations. H_2O_2/KI had a similar modest antimicrobial activity profile, but its cytotoxic impact decreased (Fig. 1B). H_2O_2/KSCN had no antimicrobial activity at all test concentrations and was also less cytotoxic towards HeLa cells (Fig. 1B). ITC was still toxic towards both cell types in a similar manner to that observed in PBS, but the toxicity concentration thresholds increased two-fold (from 7.8 to 15.6 µg ml^{-1}; Fig. 1B). H_2O_2 displayed almost no antimicrobial activity and reduced cytotoxicity in the presence of organic matter. PVP-I displayed decreased antimicrobial and cytotoxic potential. Lugol had very small antimicrobial activity, except at the highest concentration tested, while cytotoxicity was considerable even at the concentrations without any antimicrobial effect (Fig. 1B).

In agreement with the trends recorded from the MTT assay, microscopic observations revealed that, when incubated in DMEM + FBS (Fig. 2B), the HeLa cells exposed to H_2O_2/KI looked intact, healthier but with a reduced population when exposed to H_2O_2/KSCN and H_2O_2, whereas, the impact of ITC, PVP-I and Lugol on HeLa morphology was comparable to that observed with cells incubated in PBS (Fig. 2A).

3. 2. Recovery of HeLa cells from cytotoxic effect of antimicrobials

The potential for recovery of HeLa cells from the cytotoxic effects of the test antimicrobials was assessed using MTT assays either immediately after 10 min exposure of HeLa cells to antimicrobials, or after withdrawal of antimicrobials and 24 h incubation of HeLa cells in drug-free culture medium. The recovery study in PBS (Fig. 3A) with H_2O_2/KI showed that the dose-response curve was shifted to a less cytotoxic response range, indicating that H_2O_2/KI cytotoxicity to HeLa cells was, at least partially, reversible. There was no recovery of cells after exposure to H_2O_2/KSCN and H_2O_2, however, indicating an irreversible detrimental effect against HeLa cells. ITC, PVP-I and Lugol showed significant recovery when cells were exposed to the lowest test concentrations. Interestingly, concentrations of PVP-I and Lugol between 15.6 and 62.5 µg ml^{-1} were significantly more cytotoxic after the removal of these antimicrobials and further culturing of the cells than after the initial 10 min exposure.

In contrast to the observations in PBS, in the presence of the cell culture media and additional serum (Fig. 3B), there was generally no significant recovery of HeLa cells after the exposure and withdrawal of any antimicrobial at any concentration, with the exception of the lowest test concentration of ITC. This could be explained by the generally lower cytotoxicity of antimicrobials in the presence of DMEM + FBS.

3. 3. Hemolytic effect of antimicrobials

The hemolytic effect of all the test antimicrobials on mammalian red blood cells was investigated by assessing their ability to lyse horse red blood cells following 1 h of co-incubation (Fig. 4). H_2O_2/KI and H_2O_2 did not show any hemolytic effect within the measured concentrations. The microscopic images of hRBC exposed to the highest test concentration (1000 µg ml^{-1}) of H_2O_2/KI and H_2O_2 supported the results from spectrometric evaluation, with no morphological changes in the cells evident (Fig. 5). The exposure of hRBC to H_2O_2/KSCN, ITC and PVP-I at dosage levels of 7.8 to 1000 µg ml^{-1} resulted in dose-dependent hemolysis

(Fig. 4). Micrographs of the hRBCs, exposed to the highest concentration of these antimicrobials, confirmed that at 1000 µg ml^{-1} ITC ruptured the cells, whereas H_2O_2/KSCN and PVP-I reduced the number of unimpaired cells (Fig. 5). Lugol exhibited the highest hemolytic activity at lower concentrations of all the tested antimicrobials, with 30% hemolysis at a 62.5 µg ml^{-1} concentration (Fig. 4). At concentrations of Lugol above 250 µg ml^{-1} an apparent drop of activity was observed, but visualization of the actual color of the supernatants (Fig. 4, inset in Lugol) explained that this decrease was due to the color interference of Lugol and hemoglobin. These observations were additionally confirmed by microscopic images of extensive cell debris and complete lysis caused by Lugol at the highest tested concentration (Fig. 5).

3. 4. Genotoxic effect of ITC

The presence of DNA breakage, induced by ITC, was evaluated using comet assay, by comparison with the known DNA damaging agents active in the *in vitro* comet assay in the absence of S9 metabolic activation – UV-C irradiation and H_2O_2 oxidative stress. HeLa cells were exposed to ITC, at either 30 or 300 µg ml^{-1} concentrations, for 30 min and then examined for DNA breakage. The concentrations and the duration of ITC exposure were chosen based on our previous study (Tonoyan et al. 2017), which revealed that 30 min treatment of *E. coli* cells with ITC at 300 µg ml^{-1}, but not 30 µg ml^{-1}, produced a bacterial DNA smear, as assessed by gel electrophoresis. In the same study, we also observed a bacterial DNA smear when cells were challenged for 30 min with 300 µg ml^{-1} H_2O_2. Here, upon inspection of comet assay micrographs it was clear that ITC was apparently not causing DNA breaks in HeLa cells at either 30 µg ml^{-1} (Fig. 6B) or 300 µg ml^{-1} (Fig. 6C) concentrations, by comparison to the untreated controls (Fig. 6A), exhibiting round-shaped images of intact DNA. By contrast, UV-C induced elongated-shaped double strand breaks of DNA (Fig. 6D), while H_2O_2 generated droplet-shaped extensive single-strand breaks (Fig. 6E). The assessment of the extent tail

moments showed that there was no difference between the control and the ITC 30 and 300 µg ml^{-1} treatments, while H_2O_2 300 µg ml^{-1} and UV-C treatments were significantly different from the control and also from each other (Fig. 6F).

4. Discussion

Antiseptics are widely used in hospitals and other healthcare settings to inactivate, reduce or eliminate pathogenic microorganisms in, or on, living tissue (Drosou et al. 2003). An ideal antiseptic should have a broad, potent and rapid antimicrobial spectrum with no risk of developing antimicrobial resistance (McDonnell and Russell 1999). In a previous study (Tonoyan et al. 2017), we characterized three antimicrobial formulations (H_2O_2/KI, H_2O_2/KSCN and the novel ITC) that exhibited evident potential as wound antiseptics. From the viewpoint of antimicrobial activity, ITC, out of the three, best met the requirements of an optimal antiseptic. However, an ideal antiseptic needs also to be biocompatible (Müller and Kramer 2008). The term "biocompatibility" refers to different properties of the antimicrobial and a few of the most important aspects are *in vitro* cytotoxicity, hemolytic activity and genotoxicity (Fischer et al. 2003).

Unfortunately, many frequently used antiseptics are cytotoxic. Therefore, the biological balance between microbial and cellular toxicities should be taken into consideration. Here, the three newly described antimicrobial compositions were tested for their bacterial and cellular toxicities and compared to commonly used antiseptics. H_2O_2, PVP-I and Lugol are some of the most widely used and oldest antiseptics, despite controversy about their cytotoxicity (Block 2001; Cooper 2007; Khan and Naqvi 2006; Sibbald et al. 2011). Additionally, H_2O_2, being one of the components of ITC mixture, and iodine, being the prominent product of ITC, explain the choice of these antiseptics.

For a more realistic portrayal of "antiseptic at work" our study employed a simultaneous assessment of the potential cytotoxic effects and bactericidal efficacy of the test antimicrobials, upon 10 min exposure to human and bacterial cells, in two different media. In this work, HeLa cervical epithelial cell lines and *E. coli* cells were used as representatives of human and bacterial cells, respectively. ~~Whereas~~ On the other hand, phosphate-buffered saline and cell culture media supplemented with 10% fetal bovine serum were selected as two media for performance of antimicrobial and cytotoxic activities in the absence and presence of bioburden, respectively. The HeLa cell line serves as a model system not only in cancer research, but also to gain knowledge of every fundamental process that occurs in human cells (Masters 2002). Besides, HeLa cells are human epithelial cells and so cytotoxicity demonstrated towards them may potentially translate to toxicity towards other epithelial cells. On the other hand, *E. coli* is another conventional model organism, which is extensively used to study bacterial processes, is a recommended reference strain for antimicrobial susceptibility testing and is one of the common pathogens found in wound infections (Bowler et al. 2001). PBS is widely used in cell and bacterial culturing, as well as in wound rinsing (Atiyeh et al. 2009), ~~whereas~~while, nutrient-rich cell culture medium DMEM supplemented with 10% FBS represents conditions simulating wound bioburden (Kramer et al. 2016; Müller and Kramer 2008; Schedler et al. 2017). Despite the differences in experimental design, conditions and test organisms, several studies examined both the cytotoxic effect and the microbicidal activity of antimicrobial agents (Alaçam et al. 1993; Hidalgo et al. 2002; Lineaweaver et al. 1985a; Lineaweaver et al. 1985b; Müller and Kramer 2008). However, these studies evaluated cytotoxic and antimicrobial aspects of the chosen antiseptics in separate assays, as opposed to the present work.

In this study, the co-incubation of *E. coli*, HeLa cells and ~~H_2O_2/KI~~ITC upon 10 min in PBS (Fig. 1A) revealed that ~~H_2O_2/KI had a weak antibacterial effect during the short exposure time, while being cytotoxic at all doses tested. H_2O_2/KSCN also damaged HeLa cells to a greater~~

extent than *E. coli*. The ITC was shown to be similarly toxic towards both human HeLa and bacterial *E. coli* cells. In our study, the cellular toxicity of H_2O_2 remarkably exceeded its antibacterial potency in PBS. Lineaweaver and co-workers also reported a higher cellular toxicity of H_2O_2 compared to its antimicrobial potency when tested at full strength (3%), and when serially diluted in saline (Lineaweaver et al. 1985a; Lineaweaver et al. 1985b). Lugol was also highly cytotoxic at microbicidal concentrations. In our experiments, PVP-I was the only antiseptic that had concentrations non-lethal to human cells, coupled with concentrations causing moderate reduction of bacterial cell numbers. Similarly, Lineaweaver and co-workers identified bactericidal and non-cytotoxic dilutions of PVP-I in saline (Lineaweaver et al. 1985a; Lineaweaver et al. 1985b). WhereasBy contrast, Sanchez et al. (1988) reported that non-lethal concentrations of PVP-I in PBS allowed significant bacterial survival.

Generally, the antimicrobial activity of biocides is dependent on number of factors, such as biocide concentration, exposure time, presence of organic matter, etc. (Maillard 2005). It is commonly accepted that H_2O_2 (WFBSC) and iodine solutions (Cooper 2007; Khan and Naqvi 2006) lose their germicidal activity once they come into contact with organic load. So the questions arise whether publicallypublicly accepted antiseptics are adequate as wound antiseptics and if the new antimicrobials described here hasve more suitable antimicrobial properties. Thus, in order to more realistically picture the wound environment, the simultaneous assessment of antimicrobial and cytotoxic activities of the antimicrobials under consideration was also conducted in cell culture medium supplemented with fetal bovine serum as an indicator of organic challenge. In the presence of organic load, H_2O_2/KI, H_2O_2/KSCN and H_2O_2 waserе minimally cytotoxic (Fig. 1B), by contrast with our observations during culture in PBS (Fig. 1A). However, it also lost its antimicrobial activity Only modest antimicrobial activity was also noted with H_2O_2/KI and H_2O_2 (Fig. 1B), while H_2O_2/KSCN displayed no antimicrobial activity following 10 min exposure, at the concentrations tested. The cytotoxicity

of PVP-I was reduced to some extent in DMEM + FBS, but its antimicrobial capacity was also negatively impacted. Similarly, Müller and Kramer (2008) reported that PVP-I was well-tolerated by murine fibroblasts, but had low bactericidal activity against *E. coli* and *S. aureus* when tested in FBS supplemented culture media. ~~ITC remained cytotoxic and bactericidal, matching the performance observed in PBS.~~ Lugol again exhibited a greater cytotoxic influence than an antimicrobial effect, though the two effects were milder in the presence of organic matter. ITC remained cytotoxic and bactericidal, matching the performance observed in PBS.

~~The antibacterial profiles of H_2O_2/KI, H_2O_2/KSCN and H_2O_2 (Fig. 1A, B), against *E. coli*, were somewhat surprising, given the minimum inhibitory and bactericidal concentrations (MIC/MBC) for these agents (31.3/31.3 µg ml^{-1}) reported in our previous study (Tonoyan et al. 2017). In the present study, however, the antimicrobial susceptibility testing was based on viable cell counts after only a short contact time (10 min) between the antimicrobial and *E. coli* in either PBS or DMEM + FBS; and with an MBC definition of a concentration causing >3 log_{10} reduction (99.9% kill). By contrast, in our previous study we used broth microdilution MIC determinations, over 24 h of continuous exposure to the antimicrobials in nutrient broth, with follow-up MBC determination as a concentration causing 100% kill (Tonoyan et al. 2017). Despite this, the antibacterial profile of ITC was comparable in both studies – 100% kill of *E. coli* was detected at 7.8 and 31.3 µg ml^{-1} concentrations after 10 min exposure in PBS and DMEM + FBS, respectively (Fig. 1A, B), and 100% kill was observed at 15. 6 µg ml^{-1} concentration after 24 h exposure in nutrient broth (the MBC value; (Tonoyan et al. 2017). These observations indicate that, regardless of the test conditions and methods used, ITC demonstrated a fast-acting and effective antibacterial profile.~~

Mammalian cells and tissues contain defense systems to detoxify reactive intermediates, thus preventing or limiting cell damage. Toxic processes have reversible, or irreversible, features as a consequence of the interplay with cellular defense and repair mechanisms (Wallace 1997).

Thus, it is not unreasonable to assume that the cytotoxicity of an antimicrobial, based on the action of reactive oxygen species, could be reversible and temporary. To test this hypothesis, we performed drug washout experiments, followed by analyzes of cell viability utilizing MTT assay (Fig. 3). The MTT test depends on dehydrogenase activity in intact mitochondria, and tests of mitochondrial function are sensitive indicators of oxidant injury (Watson et al. 1994). The experiments showed that the incubation of HeLa cells with ~~H_2O_2/KI~~ITC, PVP-I and Lugol for 10 min in PBS with the subsequent removal of the antimicrobial, allowed for recovery of some cells at a treatment concentration~~s of between~~ 7.8 ~~and 125~~ µg ml^{-1} ~~and thus indicated, to some extent, a reduced cytotoxic effect~~ (Fig. 3A). However, th~~ei~~s~~e~~ w~~eas~~re the only recovery case~~s~~ for these antimicrobials amongst all the tested ~~antimicrobials~~concentrations. Interestingly, a similar study by Müller and Kramer (2007) showed that cytotoxic effects of PVP-I containing products (in a defined concentration range ensuring > 20% cell viability) on epithelial cells and fibroblasts, after 30 min exposure, were completely reversible after washout of the compound and follow-up culturing. ~~Whereas~~By contrast, Watson et al. (1994) concluded that even a mild injury induced by H_2O_2 (0.1 mM H_2O_2 upon 1 h) in human colonic carcinoma epithelial cells was not fully reversible. Seemingly, 10 min exposure of HeLa cells to the ~~other~~ test antimicrobials was enough to cause irreversible and permanent cellular destruction, however, the actual machinery involved in oxidative damage and repair cannot be extracted from these observations.

Another conventionally used measure of biocompatibility is the hemolytic activity of the antimicrobial candidate, in particular those nominated for open wound treatment. When ranked according to their hemolytic activities, the antimicrobials ~~tested in this study~~ had the following order: Lugol > ITC > PVP-I > ~~H_2O_2/KSCN >~~ H_2O_2 ~~≥ H_2O_2/KI~~ (Figs. 4, 5). It was shown that H_2O_2/KI and H_2O_2 were the only two antimicrobials, which did not cause hemolysis of horse erythrocytes. This was somewhat surprising, considering that these two agents lysed HeLa

epithelial cells in PBS (Fig. 2A). However, in the presence of organic matter, and under more representative physiological conditions, H_2O_2/KI and H_2O_2 had considerably lesser impact on HeLa membrane integrity (Fig. 2B). By contrast to the results with H_2O_2/KI and H_2O_2, the ITC and Lugol ruptured erythrocytes (Fig. 5), but not epithelial cells (Fig. 2A, B). Our previous work (Tonoyan et al. 2017) also showed that ITC did not compromise the membrane integrity of bacterial cells. Possible explanations may be the extremely fragile nature of erythrocytes, as well as the variable composition of different cellular membranes and, hence, distinct susceptibility to lysis by the different agents. The exact mechanism by which the modes of toxicity differ needs further study, but it was notable that the concentration of ITC needed to cause hemolysis was considerably higher compared to concentration required to exert an antimicrobial effect.

The *in vitro* comet assay is a sensitive, low cost and rapid genotoxicity test. It can be the test of choice for preliminary genotoxicity screening of drug candidates early in the development of new pharmaceuticals (Giannotti et al. 2002; Žegura and Filipič 2004). In principle, any type of eukaryotic cell may be used for comet assay genotoxicity testing. For the *in vitro* comet assay, well-characterized cell lines or primary cells are preferred, which are generally exposed to test compounds in the presence and absence of metabolic activation system, such as S9 (Tice et al. 2000; Žegura and Filipič 2004). In this study, we used the alkaline comet assay tool to assess the genotoxicity of ITC on human HeLa cell line only in the absence of S9 metabolic activation. The alkaline comet assay detects both single and double-stranded DNA breaks (Karbaschi and Cooke 2014) producing special comet-like appearances. H_2O_2 is known to primarily produce single-stranded breaks (Cortés-Gutiérrez et al. 2014), ~~whereas~~on the other hand, double-strand breaks are the major feature of the UV-radiation (Bogdanov et al. 1997). In the present study we have revealed that, indeed, H_2O_2 induced single-strand breaks in the shape of droplet-like comets (Fig. 6E), and UV-C irradiation produced double-stranded breaks in the shape of

elongated comets (Fig. 6D). However, the exposure to ITC resulted in round-shaped images (Fig. 6B, C), suggesting that the new test antiseptic ITC was not causing DNA breaks in HeLa cells at low (30 µg ml^{-1}) or at high (300 µg ml^{-1}) concentrations as evaluated by an *in vitro* comet assay.

5. Conclusion

The *in vitro* cytotoxicity of a novel, biocidal, iodo-thyocyanate complex, ITC, was investigated and compared to commonly used antiseptics. The antimicrobial potency of ITC, together with a comparable cytotoxicity profile to existing antiseptics, suggests that it could be considered for future antiseptic and therapeutic applications. Indeed, the concentrations of ITC resulting in cytotoxicity were equivalent to those resulting in potent bactericidal activity. By contrast, the cellular toxicity of the commonly used antiseptics H_2O_2, PVP-I and Lugol towards HeLa cells was apparent at levels too low to cause significant bactericidal effects. The activity of ITC was not quenched by organic matter, ~~whereas~~while, the activities of commonly used non-selective antiseptics were suppressed by organic load. In addition, ITC did not produce DNA breaks at 30 and 300 µg ml^{-1} concentrations, as detected by an *in vitro* comet assay in the absence of S9 metabolic activation~~, at 30 and 300 µg ml^{-1} concentrations~~.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Abbreviations

ITC, iodo-thiocyanate complex; PVP-I, povidone iodine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cfu, colony-forming units; hRBD, horse red blood cells; ~~LMPA, low melting point agarose;~~ DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation.

References

Alaçam T, Ömürlü H, Özkul A, Görgül G, Misirligil A (1993) Cytotoxicity versus antibacterial activity of some antiseptics *in vitro*. J Nihon Univ Sch Dent 35(1):22-27 doi:10.2334/josnusd1959.35.22

Atiyeh BS, Dibo SA, Hayek SN (2009) Wound cleansing, topical antiseptics and wound healing. Int Wound J 6(6):420-430 doi:10.1111/j.1742-481X.2009.00639.x

Bafort F, Parisi O, Perraudin J-P, Jijakli MH (2014) Mode of action of lactoperoxidase as related to its antimicrobial activity: A review. Enzyme Res 2014:13 doi:10.1155/2014/517164

Block SS (2001) Disinfection, sterilization, and preservation, 5th edn. Lippincott Williams & Wilkins, Philadelphia, USA

Bogdanov KV, Chukhlovin AB, Zaritskey AY, Frolova OI, Afanasiev BV (1997) Ultraviolet irradiation induces multiple DNA double-strand breaks and apoptosis in normal granulocytes and chronic myeloid leukaemia blasts. Br J Haematol 98(4):869-72

Bowler PG, Duerden BI, Armstrong DG (2001) Wound microbiology and associated approaches to wound management. Clin Microbiol Rev 14(2):244-269 doi:10.1128/CMR.14.2.244-269.2001

Clark RA, Klebanoff SJ (1975) Neutrophil-mediated tumor cell cytotoxicity: Role of the peroxidase system. J Exp Med 141(6):1442-1447 doi:10.1084/jem.141.6.1442

Clark RA, Klebanoff SJ (1977) Myeloperoxidase - H_2O_2 - halide system: Cytotoxic effect on human blood leukocytes. Blood 50(1):65-70

Cooper RA (2007) Iodine revisited. Int Wound J 4(2):124-137 doi:10.1111/j.1742-481X.2007.00314.x

Cortés-Gutiérrez EI, López-Fernández C, Fernández JL, Dávila-Rodríguez MI, Johnston SD, Gosálvez J (2014) Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. Front Genet 5:404

Corvi R, Madia F (2016) *In vitro* genotoxicity testing–Can the performance be enhanced? Food Chem Toxicol doi:http://doi.org/10.1016/j.fct.2016.08.024

Davies MJ, Hawkins CL, Pattison DI, Rees MD (2008) Mammalian heme peroxidases: From molecular mechanisms to health implications. Antioxid Redox Signal 10(7):1199-1234 doi:10.1089/ars.2007.1927

Drosou A, Falabella A, Kirsner RS (2003) Antiseptics on wounds: An area of controversy. Wounds 15(5):149-166

Edelson PJ, Cohn ZA (1973) Peroxidase-mediated mammalian cell cytotoxicity. J Exp Med 138(1):318-323 doi:10.1084/jem.138.1.318

Evans BC, Nelson CE, Yu SS, et al. (2013) *Ex vivo* red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J Vis Exp(73):e50166 doi:10.3791/50166

Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T (2003) *In vitro* cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. Biomaterials 24(7):1121-1131 doi:http://dx.doi.org/10.1016/S0142-9612(02)00445-3

Fotakis G, Timbrell JA (2006) *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett 160(2):171-177 doi:http://dx.doi.org/10.1016/j.toxlet.2005.07.001

Giannotti E, Vandin L, Repeto P, Comelli R (2002) A comparison of the *in vitro* Comet assay with the *in vitro* chromosome aberration assay using whole human blood or Chinese

hamster lung cells: Validation study using a range of novel pharmaceuticals. Mutagenesis 17(2):163-70 doi:https://doi.org/10.1093/mutage/17.2.163
Henderson WR, Chi EY, Jong EC, Klebanoff SJ (1981) Mast cell-mediated tumor cell cytotoxicity. Role of the peroxidase system. J Exp Med 153(3):520-33 doi:10.1084/jem.153.3.520
Hidalgo E, Bartolome R, Dominguez C (2002) Cytotoxicity mechanisms of sodium hypochlorite in cultured human dermal fibroblasts and its bactericidal effectiveness. Chem Biol Interact 139(3):265-282 doi:http://dx.doi.org/10.1016/S0009-2797(02)00003-0
Jong EC, Klebanoff SJ (1980) Eosinophil-mediated mammalian tumor cell cytotoxicity: Role of the peroxidase system. J Immunol 124(4):1949-53
Karbaschi M, Cooke MS (2014) Novel method for the high-throughput processing of slides for the comet assay. Sci Rep 4:7200 doi:10.1038/srep07200
Khan MN, Naqvi AH (2006) Antiseptics, iodine, povidone iodine and traumatic wound cleansing. J Tissue Viability 16(4):6-10 doi:http://dx.doi.org/10.1016/S0965-206X(06)64002-3
Klebanoff SJ (2005) Myeloperoxidase: Friend and foe. J Leukoc Biol 77(5):598-625 doi:10.1189/jlb.1204697
Kramer A, Assadian O, Koburger-Janssen T (2016) Antimicrobial efficacy of the combination of chlorhexidine digluconate and dexpanthenol. GMS Hyg Infect Control 11:Doc24 doi:10.3205/dgkh000284
Kussendrager KD, van Hooijdonk A (2000) Lactoperoxidase: Physico-chemical properties, occurrence, mechanism of action and applications. Br J Nutr 84(Suppl 1):S19-S25 doi:https://doi.org/10.1017/S0007114500002208
Li X, Wang L, Fan Y, Feng Q, Cui F-z (2012) Biocompatibility and toxicity of nanoparticles and nanotubes. J Nanomater 2012:19 doi:10.1155/2012/548389
Lineaweaver W, Howard R, Soucy D, et al. (1985a) Topical antimicrobial toxicity. Arch Surg 120(3):267-270 doi:10.1001/archsurg.1985.01390270007001
Lineaweaver W, McMorris S, Soucy D, Howard R (1985b) Cellular and bacterial toxicities of topical antimicrobials. Plast Reconstr Surg 75(3):394-6
Maillard J-Y (2005) Antimicrobial biocides in the healthcare environment: Efficacy, usage, policies, and perceived problems. Ther Clin Risk Manag 1(4):307-320
Masters JR (2002) HeLa cells 50 years on: The good, the bad and the ugly. Nat Rev Cancer 2(4):315-319 doi:10.1038/nrc775
McDonnell G, Russell AD (1999) Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev 12(1):147-179
Müller G, Kramer A (2007) Revitalisation of mammalian cells exposed to antiseptics. GMS Hyg Infect Control 2(2):Doc 35
Müller G, Kramer A (2008) Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. J Antimicrob Chemother 61(6):1281-1287 doi:10.1093/jac/dkn125
Naidu AS (2000) Lactoperoxidase. In: Naidu AS (ed) Natural food antimicrobial systems. CRC Press, Boca Raton, USA, p 103-132
Okano A, Isley NA, Boger DL (2017) Peripheral modifications of [Ψ[CH_2NH]Tpg^4]vancomycin with added synergistic mechanisms of action provide durable and potent antibiotics. Proc Natl Acad Sci USA doi:10.1073/pnas.1704125114
Olive PL, Banath JP (2006) The comet assay: A method to measure DNA damage in individual cells. Nat Protoc 1(1):23-29 doi:10.1038/nprot.2006.5

Riss TL, Moravec RA, Niles AL (2011) Cytotoxicity testing: Measuring viable cells, dead cells, and detecting mechanism of cell death. In: Stoddart MJ (ed) Mammalian cell viability: Methods and protocols. Humana Press, Totowa, NJ, p 103-114

Rose WE, Poppens PT (2009) Impact of biofilm on the *in vitro* activity of vancomycin alone and in combination with tigecycline and rifampicin against *Staphylococcus aureus*. J Antimicrob Chemother 63(3):485-488 doi:10.1093/jac/dkn513

Sanchez IR, Nusbaum KE, Swaim SF, Hale AS, Henderson RA, McGuire JA (1988) Chlorhexidine diacetate and povidone-iodine cytotoxicity to canine embryonic fibroblasts and *Staphylococcus aureus*. Vet Surg 17(4):182-185 doi:10.1111/j.1532-950X.1988.tb00995.x

Schedler K, Assadian O, Brautferger U, et al. (2017) Proposed phase 2/ step 2 *in-vitro* test on basis of EN 14561 for standardised testing of the wound antiseptics PVP-iodine, chlorhexidine digluconate, polihexanide and octenidine dihydrochloride. BMC Infect Dis 17:143 doi:10.1186/s12879-017-2220-4

Seifu E, Buys EM, Donkin EF (2005) Significance of the lactoperoxidase system in the dairy industry and its potential applications: A review. Trends Food Sci Tech 16(4):137-154 doi:http://dx.doi.org/10.1016/j.tifs.2004.11.002

Sibbald RG, Leaper DJ, Queen D (2011) Iodine made easy Wounds Int 2(2)

Sieuwerts S, De Bok FAM, Mols E, De Vos WM, Van Hylckama Vlieg JET (2008) A simple and fast method for determining colony forming units. Lett Appl Microbiol 47(4):275-278 doi:10.1111/j.1472-765X.2008.02417.x

Tice RR, Agurell E, Anderson D, et al. (2000) Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environmental and molecular mutagenesis 35(3):206-21 doi:10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J

Tonoyan L, Fleming G, Mc Cay P, Friel R, O'Flaherty V (2017) Antibacterial potential of an antimicrobial agent inspired by peroxidase-catalyzed systems. Frontiers in Microbiology 8(680) doi:10.3389/fmicb.2017.00680

Wallace KB (1997) Free Radical Toxicology. Taylor & Francis, Washington DC, USA

Watson AJ, Askew JN, Sandle GI (1994) Characterisation of oxidative injury to an intestinal cell line (HT-29) by hydrogen peroxide. Gut 35(11):1575-1581 doi:10.1136/gut.35.11.1575

WFBSC Disinfectant chart. In. http://www.cleaning-for-health.org/disinfectant-chart/ Accessed 19.12.2016 2016

WUWHS (2008) Principles of best practice: Wound infection in clinical practice. An international consensus World Union of Wound Healing Societies MEP Ltd, London

Žegura B, Filipič M (2004) Application of *in vitro* Comet assay for gnotoxicity testing. In: Yan Z, Caldwell GW (eds) Optimization in Drug Discovery: *In Vitro* Methods. Humana Press, Totowa, NJ, p 301-313