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

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20

## 1 **Abstract**

2 Novel biocides, which avoid the induction of cross-resistance to antibiotics, are an urgent  
3 societal requirement. Here, we compared the cytotoxic and bactericidal effects of a new  
4 antimicrobial agent, the iodo-thiocyanate complex (ITC), with those of the common antiseptics,  
5 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), povidone iodine (PVP-I) and Lugol's iodine (Lugol). The  
6 antimicrobials were co-incubated for 10 min with HeLa and *Escherichia coli* cells in the  
7 presence and absence of organic matter (Dulbecco's modified Eagle's medium, supplemented  
8 with 10% fetal bovine serum). The cytotoxic concentrations of ITC were equivalent to its  
9 bactericidal concentrations (<7.8 µg ml<sup>-1</sup>). By contrast, cytotoxic effects of H<sub>2</sub>O<sub>2</sub>, PVP-I and  
10 Lugol were apparent at concentrations lower than their bactericidal concentrations (250, 125  
11 and 31.3 µg ml<sup>-1</sup>, respectively). The cellular effects of ITC were not quenched by organic  
12 matter, unlike the other antiseptics. ITC, PVP-I and Lugol had dose-dependent effects on the  
13 viability of horse erythrocytes, while H<sub>2</sub>O<sub>2</sub> showed no hemolysis. ITC, at 30 or 300 µg ml<sup>-1</sup>,  
14 did not cause DNA breakage in HeLa [cells](#); as assessed by an *in vitro* comet assay [in the absence](#)  
15 [of S9 metabolic activation](#), whereas H<sub>2</sub>O<sub>2</sub> caused extensive single-strand DNA breaks. The  
16 pronounced antimicrobial potency of ITC and its favorable cytotoxicity profile suggests that  
17 ITC should be considered for antiseptic applications.

## 18 **Keywords**

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

## 21 **1. Introduction**

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

1 challenge. In the context of growing resistance to antibiotics, wound infection control and  
2 management, along with the control of potentially pathogenic bacteria in healthcare  
3 environments, is becoming challenging and represents a major healthcare burden (WUWHS  
4 2008).

5 ~~Naturally occurring peroxidase-catalyzed antimicrobial systems suggest~~ provide a possible  
6 future direction, ~~in the form of peroxidase-catalyzed antimicrobial systems~~. A peroxidase  
7 enzyme together with hydrogen peroxide, inorganic ion substrate and generated oxidized  
8 products is known as a peroxidase system (Kussendrager and van Hooijdonk 2000). These  
9 ~~naturally occurring~~ systems are part of the host defense network and provide an immediate  
10 defense against invading microorganisms (Davies et al. 2008). A large number of studies have  
11 investigated and demonstrated the antimicrobial potential of peroxidase-catalyzed systems, as  
12 extensively reviewed elsewhere (Bafort et al. 2014; Davies et al. 2008; Kussendrager and van  
13 Hooijdonk 2000; Naidu 2000; Seifu et al. 2005). Peroxidase/hydrogen peroxide/halide systems  
14 have a dual role, however, acting as both a friend and a foe (Davies et al. 2008; Klebanoff  
15 2005). As they possess a non-specific, broad-spectrum target mechanism, aside from mediating  
16 bacterial killing, destroying invading parasites, combating fungal infections and inactivating  
17 viruses, they can also attack a variety of mammalian cells (Clark and Klebanoff 1977; Edelson  
18 and Cohn 1973), including tumor cells (Clark and Klebanoff 1975; Henderson et al. 1981; Jong  
19 and Klebanoff 1980).

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

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

13 A variety of approaches can be used to evaluate and screen the *in vitro* toxicity of antimicrobial  
14 substances. Testing the effects of compounds on the viability of mammalian cells grown in  
15 culture (*in vitro* cytotoxicity tests) is widely used as an indicator of potential toxic effects in  
16 animals (Riss et al. 2011). Among the different cytotoxicity tests, the methyl tetrazolium (MTT)  
17 viability assay is the most popular, low-cost and convenient method (Fotakis and Timbrell  
18 2006). Considering that antimicrobials may potentially come into direct contact with blood, an  
19 evaluation of hemotoxicity is also worthwhile. The standard measure of blood compatibility is  
20 hemolytic activity or the lysis of red blood cells (Li et al. 2012). Genotoxicity testing is also an  
21 important aspect of the safety assessment of broad-spectrum substances, including  
22 pharmaceuticals and biocides (Corvi and Madia 2016). Our previous research (Tonoyan et al.  
23 2017) indicated that the ITC contains reactive species, which may have the potential to induce  
24 bacterial DNA breakage; the detection of DNA breaks by single cell gel electrophoresis (comet  
25 assay), would thus be informative.

1 The aim of the present study was to investigate the *in vitro* cytotoxicity, hemolytic activity and  
2 DNA damaging effect of the novel antimicrobial ITC—in relation to its antimicrobial activity.  
3 The potential suitability of ITC as an antiseptic was then considered and compared with H<sub>2</sub>O<sub>2</sub>,  
4 PVP-I and Lugol's iodine.

## 5 **2. Materials and methods**

### 6 2. 1. Antimicrobial agents and preparation

7 All the materials were purchased from Sigma-Aldrich, unless otherwise stated. The  
8 antimicrobial agents evaluated in this study were H<sub>2</sub>O<sub>2</sub>/KI, H<sub>2</sub>O<sub>2</sub>/KSCN and H<sub>2</sub>O<sub>2</sub>/KI/KSCN  
9 [ITC; (Tonoyan et al. 2017)]. The stock solutions were prepared by combining concentrated  
10 solutions of H<sub>2</sub>O<sub>2</sub> (v/v) with KI (w/v) and/or KSCN (w/v) to obtain 1% final concentrations for  
11 each agent. These solutions were considered as 1% according to the concentration of H<sub>2</sub>O<sub>2</sub>  
12 present in the mixtures, as KI and KSCN do not possess antimicrobial activity alone (data not  
13 shown). The antiseptics H<sub>2</sub>O<sub>2</sub> (30% v/v stock solution), PVP-I (10% w/v available iodine stock)  
14 and Lugol's iodine (1.25% w/v available iodine stock) were also used in this study. The stock  
15 solutions were diluted to the desired working antimicrobial concentration range using sterile  
16 deionized water (dH<sub>2</sub>O).

### 17 2. 2. Test bacteria, cell cultures, nutrient solutions and growth conditions

18 The bacterial strain used in this study was *Escherichia coli* ATCC 25922 (from the American  
19 Type Culture Collection), which was cultured aerobically on Lennox agar and lysogeny broth  
20 at 37°C. HeLa human cervical epithelial cell lines were obtained from the DSMZ (German  
21 Collection of Microorganisms and Cell Cultures) and were grown as a monolayer in complete  
22 DMEM (Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine  
23 serum (FBS), 100 units ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin) at 37°C, 5% CO<sub>2</sub>.

## 1 2. 3. Cytotoxicity studies

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

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

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

1 (99.9% kill) in cfu ml<sup>-1</sup> from the starting *E. coli* inoculum concentration (Rose and Poppens  
2 2009). The HeLa monolayers were washed twice with PBS and incubated with complete  
3 DMEM, supplemented with 0.6 mg ml<sup>-1</sup> MTT, for 4 h at 37°C. The medium containing MTT  
4 was aspirated, formazan crystals were dissolved in 150 µl dimethyl sulfoxide and the  
5 absorbance was measured at 595 nm (Tecan GENios, Salzburg, Austria). Cellular viability was  
6 expressed as a percentage of the untreated controls. ~~All the treatments and their concentration  
7 range were tested in duplicate, and all experiments were repeated at least twice. Statistical  
8 analysis of treated versus untreated HeLa cells was performed using one way analysis of  
9 variance (ANOVA) followed by Tukey's multiple comparison test (SigmaPlot Version 11,  
10 Systat Software, Inc., San Jose, USA).~~

### 11 2. 3. 2. Visualization of morphological changes in HeLa cells

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

### 18 2. 3. 3. Recovery of HeLa cells after withdrawal of antimicrobials

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



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

## 7 2. 4. Hemolytic activity studies

### 8 2. 4. 1. Determination of hemolytic activity of antimicrobials towards horse red blood cells 9 (hRBC)

10 The hemolytic activity of the antimicrobials was tested by determining the release of  
11 hemoglobin in horse erythrocyte suspension as a measure of red blood cell lysis (Evans et al.  
12 2013).

13 The horse RBCs were prepared by centrifugation of defibrinated horse blood (TCS  
14 Biosciences; packed cell volume 40%) at  $3000 \times g$  for 5 min. Plasma, white blood cells and  
15 platelets were removed as supernatant by washing the blood with PBS. After obtaining a clear  
16 supernatant, the hRBC pellet was resuspended in PBS to 4% hRBC suspension. Aliquots (900  
17  $\mu\text{l}$ ) of hRBC suspensions were then added to micro-centrifuge tubes containing 100  $\mu\text{l}$  of  
18 antimicrobial solutions to give final concentrations of antimicrobials at a range of 7.8 – 1000  
19  $\mu\text{g ml}^{-1}$ . PBS and 10% Triton X-100 were used as 0% and 100% hemolysis controls,  
20 respectively. The samples were incubated for 1 h at  $37^\circ\text{C}$ . Subsequently, the tubes were  
21 centrifuged at  $3000 \times g$  for 5 min and 20  $\mu\text{l}$  aliquots from supernatant were transferred into 96-  
22 well plates containing 180  $\mu\text{l}$  PBS. Hemoglobin release was monitored by measuring the  
23 absorbance at 540 nm (Tecan GENios). Hemolysis was calculated as a percentage of the  
24 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  $\mu\text{g ml}^{-1}$ ) for 1 h, were visualized microscopically. Aliquots (10  $\mu\text{l}$ ) from the antimicrobial-treated hRBC suspensions were transferred to a hemocytometer chamber and viewed by inverted microscope (Leica DM IL LED) at 40 $\times$  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  $5.75 \times 10^5$  cells  $\text{ml}^{-1}$ . ITC was added to cell suspensions, at final concentrations of either 30 or 300  $\mu\text{g ml}^{-1}$ , and incubated for 30 min. Exposure of cells to either  $\text{H}_2\text{O}_2$  at 300  $\mu\text{g ml}^{-1}$  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  $\mu\text{l}$  aliquot of each cell suspension~~ at final density of  $1 \times 10^4$  cells  $\text{ml}^{-1}$  was ~~mixed with added to 450  $\mu\text{l}$  1% low melting point agarose in PBS (LMPA) and 150  $\mu\text{l}$  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  $\mu\text{l}$  slide $^{-1}$ ) and dried at 50°C. Thus prepared,

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

## 20 2. 6. Statistical analysis

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

1 significance of differences between the treatment groups and controls, one-way analysis of  
2 variance (ANOVA) followed by Tukey's multiple comparison test was applied. To estimate  
3 the significant recovery of HeLa cells exposed to different concentrations of antimicrobials, the  
4 data from HeLa cells exposed for 10 min and then immediately tested was compared to data  
5 from the revived HeLa cells by two-way ANOVA followed by Tukey's test. A value of  $p <$   
6 0.05 was considered statistically significant and  $p < 0.01$  statistically highly significant.

### 7 **3. Results**

#### 8 3. 1. Cytotoxic and antibacterial activities of antimicrobials

9 The cytotoxic and antibacterial effects of the antimicrobials were determined simultaneously  
10 by co-incubation of human HeLa, bacterial *E. coli* cells in different concentrations of  
11 antimicrobials, for 10 minutes, in either PBS, or in DMEM + FBS, and then applying the MTT  
12 assay and viable cell counting. When incubated in PBS (Fig. 1A), H<sub>2</sub>O<sub>2</sub>/KI and H<sub>2</sub>O<sub>2</sub>/KSCN  
13 were cytotoxic at all concentrations tested (7.8 – 1000 µg ml<sup>-1</sup>) with very slight antimicrobial  
14 activity, with the exception of the highest dose (1000 µg ml<sup>-1</sup>) of H<sub>2</sub>O<sub>2</sub>/KSCN, which caused a  
15 5 log<sub>10</sub> reduction in *E. coli* (Fig. 1A). ITC, when incubated in PBS, was highly toxic towards  
16 both cell types after 10 min exposure; even at the lowest test concentration of ITC (7.8 µg ml<sup>-1</sup>)  
17 no viable *E. coli* and HeLa cells were detected (Fig. 1A). Amongst the antiseptics, the  
18 antimicrobial potential of H<sub>2</sub>O<sub>2</sub> increased in a concentration-dependent manner, with a  
19 bactericidal effect noted at a concentration of 250 µg ml<sup>-1</sup> (the MBC value). It was, however,  
20 highly cytotoxic at all concentrations tested (Fig. 1A). The cellular and bacterial toxicities of  
21 PVP-I also increased in a manner depending on its dose. PVP-I exhibited the least cytotoxic  
22 performance among the tested antimicrobials – around 20% cytotoxicity at 31.3 µg ml<sup>-1</sup>.  
23 However, the bactericidal concentration of PVP-I (125 µg ml<sup>-1</sup>) was accompanied by toxicity

1 towards HeLa. Lugol was cytotoxic even at concentrations below the MBC ( $31.3 \mu\text{g ml}^{-1}$ ; Fig.  
2 1A).

3 Microscopic observations supported the results from the MTT cytotoxicity assay. With respect  
4 to the PBS incubations (Fig. 2A),  $\text{H}_2\text{O}_2/\text{KI}$ ,  $\text{H}_2\text{O}_2/\text{KSCN}$  and  $\text{H}_2\text{O}_2$ , at  $1000 \mu\text{g ml}^{-1}$ , completely  
5 lysed and “dissolved” HeLa cells after a 10 min contact time. However, upon exposure to ITC,  
6 PVP-I and Lugol, the cells seemed to not be fully lysed, but looked unhealthier in comparison  
7 to untreated control (Fig. 2A).

8 The antimicrobial and cytotoxic effects of the antimicrobials were notably different in the  
9 presence of the cell culture media supplemented with 10% fetal bovine serum (DMEM + FBS;  
10 Fig. 1B), by comparison to the PBS incubations.  $\text{H}_2\text{O}_2/\text{KI}$  had a similar modest antimicrobial  
11 activity profile, but its cytotoxic impact decreased (Fig. 1B).  $\text{H}_2\text{O}_2/\text{KSCN}$  had no antimicrobial  
12 activity at all test concentrations and was also less cytotoxic towards HeLa cells (Fig. 1B). ITC  
13 was still toxic towards both cell types in a similar manner to that observed in PBS, but the  
14 toxicity concentration thresholds increased two-fold (from  $7.8$  to  $15.6 \mu\text{g ml}^{-1}$ ; Fig. 1B).  $\text{H}_2\text{O}_2$   
15 displayed almost no antimicrobial activity and reduced cytotoxicity in the presence of organic  
16 matter. PVP-I displayed decreased antimicrobial and cytotoxic potential. Lugol had very small  
17 antimicrobial activity, except at the highest concentration tested, while cytotoxicity was  
18 considerable even at the concentrations without any antimicrobial effect (Fig. 1B).

19 In agreement with the trends recorded from the MTT assay, microscopic observations revealed  
20 that, when incubated in DMEM + FBS (Fig. 2B), the HeLa cells exposed to  $\text{H}_2\text{O}_2/\text{KI}$  looked  
21 intact, healthier but with a reduced population when exposed to  $\text{H}_2\text{O}_2/\text{KSCN}$  and  $\text{H}_2\text{O}_2$ ,  
22 whereas, the impact of ITC, PVP-I and Lugol on HeLa morphology was comparable to that  
23 observed with cells incubated in PBS (Fig. 2A).

24 3. 2. Recovery of HeLa cells from cytotoxic effect of antimicrobials

1 The potential for recovery of HeLa cells from the cytotoxic effects of the test antimicrobials  
2 was assessed using MTT assays either immediately after 10 min exposure of HeLa cells to  
3 antimicrobials, or after withdrawal of antimicrobials and 24 h incubation of HeLa cells in drug-  
4 free culture medium. The recovery study in PBS (Fig. 3A) with H<sub>2</sub>O<sub>2</sub>/KI showed that the dose-  
5 response curve was shifted to a less cytotoxic response range, indicating that H<sub>2</sub>O<sub>2</sub>/KI  
6 cytotoxicity to HeLa cells was, at least partially, reversible. There was no recovery of cells after  
7 exposure to H<sub>2</sub>O<sub>2</sub>/KSCN and H<sub>2</sub>O<sub>2</sub>, however, indicating an irreversible detrimental effect  
8 against HeLa cells. ITC, PVP-I and Lugol showed significant recovery when cells were  
9 exposed to the lowest test concentrations. Interestingly, concentrations of PVP-I and Lugol  
10 between 15.6 and 62.5 µg ml<sup>-1</sup> were significantly more cytotoxic after the removal of these  
11 antimicrobials and further culturing of the cells than after the initial 10 min exposure.

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

### 17 3. 3. Hemolytic effect of antimicrobials

18 The hemolytic effect of all the test antimicrobials on mammalian red blood cells was  
19 investigated by assessing their ability to lyse horse red blood cells following 1 h of co-  
20 incubation (Fig. 4). H<sub>2</sub>O<sub>2</sub>/KI and H<sub>2</sub>O<sub>2</sub> did not show any hemolytic effect within the measured  
21 concentrations. The microscopic images of hRBC exposed to the highest test concentration  
22 (1000 µg ml<sup>-1</sup>) of H<sub>2</sub>O<sub>2</sub>/KI and H<sub>2</sub>O<sub>2</sub> supported the results from spectrometric evaluation, with  
23 no morphological changes in the cells evident (Fig. 5). The exposure of hRBC to H<sub>2</sub>O<sub>2</sub>/KSCN,  
24 ITC and PVP-I at dosage levels of 7.8 to 1000 µg ml<sup>-1</sup> resulted in dose-dependent hemolysis

1 (Fig. 4). Micrographs of the hRBCs, exposed to the highest concentration of these  
2 antimicrobials, confirmed that at 1000  $\mu\text{g ml}^{-1}$  ITC ruptured the cells, whereas  $\text{H}_2\text{O}_2/\text{KSCN}$   
3 and PVP-I reduced the number of unimpaired cells (Fig. 5). Lugol exhibited the highest  
4 hemolytic activity at lower concentrations of all the tested antimicrobials, with 30% hemolysis  
5 at a 62.5  $\mu\text{g ml}^{-1}$  concentration (Fig. 4). At concentrations of Lugol above 250  $\mu\text{g ml}^{-1}$  an  
6 apparent drop of activity was observed, but visualization of the actual color of the supernatants  
7 (Fig. 4, inset in Lugol) explained that this decrease was due to the color interference of Lugol  
8 and hemoglobin. These observations were additionally confirmed by microscopic images of  
9 extensive cell debris and complete lysis caused by Lugol at the highest tested concentration  
10 (Fig. 5).

#### 11 3. 4. Genotoxic effect of ITC

12 The presence of DNA breakage, induced by ITC, was evaluated using comet assay, by  
13 comparison with the known DNA damaging agents active in the *in vitro* comet assay in the  
14 absence of S9 metabolic activation – UV-C irradiation and  $\text{H}_2\text{O}_2$  oxidative stress. HeLa cells  
15 were exposed to ITC, at either 30 or 300  $\mu\text{g ml}^{-1}$  concentrations, for 30 min and then examined  
16 for DNA breakage. The concentrations and the duration of ITC exposure were chosen based on  
17 our previous study (Tonoyan et al. 2017), which revealed that 30 min treatment of *E. coli* cells  
18 with ITC at 300  $\mu\text{g ml}^{-1}$ , but not 30  $\mu\text{g ml}^{-1}$ , produced a bacterial DNA smear, as assessed by  
19 gel electrophoresis. In the same study, we also observed a bacterial DNA smear when cells  
20 were challenged for 30 min with 300  $\mu\text{g ml}^{-1}$   $\text{H}_2\text{O}_2$ . Here, upon inspection of comet assay  
21 micrographs it was clear that ITC was apparently not causing DNA breaks in HeLa cells at  
22 either 30  $\mu\text{g ml}^{-1}$  (Fig. 6B) or 300  $\mu\text{g ml}^{-1}$  (Fig. 6C) concentrations, by comparison to the  
23 untreated controls (Fig. 6A), exhibiting round-shaped images of intact DNA. By contrast, UV-  
24 C induced elongated-shaped double strand breaks of DNA (Fig. 6D), while  $\text{H}_2\text{O}_2$  generated  
25 droplet-shaped extensive single-strand breaks (Fig. 6E). The assessment of the extent tail

1 moments showed that there was no difference between the control and the ITC 30 and 300  $\mu\text{g}$   
2  $\text{ml}^{-1}$  treatments, while  $\text{H}_2\text{O}_2$  300  $\mu\text{g}$   $\text{ml}^{-1}$  and UV-C treatments were significantly different from  
3 the control and also from each other (Fig. 6F).

#### 4 **4. Discussion**

5 Antiseptics are widely used in hospitals and other healthcare settings to inactivate, reduce or  
6 eliminate pathogenic microorganisms in, or on, living tissue (Drosou et al. 2003). An ideal  
7 antiseptic should have a broad, potent and rapid antimicrobial spectrum with no risk of  
8 developing antimicrobial resistance (McDonnell and Russell 1999). In a previous study  
9 (Tonoyan et al. 2017), we characterized three antimicrobial formulations ( $\text{H}_2\text{O}_2/\text{KI}$ ,  
10  $\text{H}_2\text{O}_2/\text{KSCN}$  and the novel ITC) that exhibited evident potential as wound antiseptics. From the  
11 viewpoint of antimicrobial activity, ITC, out of the three, best met the requirements of an  
12 optimal antiseptic. However, an ideal antiseptic needs also to be biocompatible (Müller and  
13 Kramer 2008). The term “biocompatibility” refers to different properties of the antimicrobial  
14 and a few of the most important aspects are *in vitro* cytotoxicity, hemolytic activity and  
15 genotoxicity (Fischer et al. 2003).

16 Unfortunately, many frequently used antiseptics are cytotoxic. Therefore, the biological  
17 balance between microbial and cellular toxicities should be taken into consideration. Here, the  
18 three newly described antimicrobial compositions were tested for their bacterial and cellular  
19 toxicities and compared to commonly used antiseptics.  $\text{H}_2\text{O}_2$ , PVP-I and Lugol are some of the  
20 most widely used and oldest antiseptics, despite controversy about their cytotoxicity (Block  
21 2001; Cooper 2007; Khan and Naqvi 2006; Sibbald et al. 2011). Additionally,  $\text{H}_2\text{O}_2$ , being one  
22 of the components of ITC mixture, and iodine, being the prominent product of ITC, explain the  
23 choice of these antiseptics.



1 For a more realistic portrayal of “antiseptic at work” our study employed a simultaneous  
2 assessment of the potential cytotoxic effects and bactericidal efficacy of the test antimicrobials,  
3 upon 10 min exposure to human and bacterial cells, in two different media. In this work, HeLa  
4 cervical epithelial cell lines and *E. coli* cells were used as representatives of human and  
5 bacterial cells, respectively. ~~Whereas~~ On the other hand, phosphate-buffered saline and cell  
6 culture media supplemented with 10% fetal bovine serum were selected as two media for  
7 performance of antimicrobial and cytotoxic activities in the absence and presence of bioburden,  
8 respectively. The HeLa cell line serves as a model system not only in cancer research, but also  
9 to gain knowledge of every fundamental process that occurs in human cells (Masters 2002).  
10 Besides, HeLa cells are human epithelial cells and so cytotoxicity demonstrated towards them  
11 may potentially translate to toxicity towards other epithelial cells. On the other hand, *E. coli* is  
12 another conventional model organism, which is extensively used to study bacterial processes,  
13 is a recommended reference strain for antimicrobial susceptibility testing and is one of the  
14 common pathogens found in wound infections (Bowler et al. 2001). PBS is widely used in cell  
15 and bacterial culturing, as well as in wound rinsing (Atiyeh et al. 2009), ~~whereas~~ while, nutrient-  
16 rich cell culture medium DMEM supplemented with 10% FBS represents conditions simulating  
17 wound bioburden (Kramer et al. 2016; Müller and Kramer 2008; Schedler et al. 2017). Despite  
18 the differences in experimental design, conditions and test organisms, several studies examined  
19 both the cytotoxic effect and the microbicidal activity of antimicrobial agents (Alaçam et al.  
20 1993; Hidalgo et al. 2002; Lineaweaver et al. 1985a; Lineaweaver et al. 1985b; Müller and  
21 Kramer 2008). However, these studies evaluated cytotoxic and antimicrobial aspects of the  
22 chosen antiseptics in separate assays, as opposed to the present work.

23 In this study, the co-incubation of *E. coli*, HeLa cells and ~~H<sub>2</sub>O<sub>2</sub>/KI~~ H<sub>2</sub>O<sub>2</sub>/KHITC upon 10 min in PBS  
24 (Fig. 1A) revealed that ~~H<sub>2</sub>O<sub>2</sub>/KI had a weak antibacterial effect during the short exposure time,~~  
25 ~~while being cytotoxic at all doses tested. H<sub>2</sub>O<sub>2</sub>/KSCN also damaged HeLa cells to a greater~~

1 ~~extent than *E. coli*. The~~ ITC was ~~shown to be~~ similarly toxic towards both human HeLa and  
2 bacterial *E. coli* cells. In our study, the cellular toxicity of H<sub>2</sub>O<sub>2</sub> remarkably exceeded its  
3 antibacterial potency in PBS. Lineaweaver and co-workers also reported a higher cellular  
4 toxicity of H<sub>2</sub>O<sub>2</sub> compared to its antimicrobial potency when tested at full strength (3%), and  
5 when serially diluted in saline (Lineaweaver et al. 1985a; Lineaweaver et al. 1985b). Lugol was  
6 also highly cytotoxic at microbicidal concentrations. In our experiments, PVP-I was the only  
7 antiseptic that had concentrations non-lethal to human cells, coupled with concentrations  
8 causing moderate reduction of bacterial cell numbers. Similarly, Lineaweaver and co-workers  
9 identified bactericidal and non-cytotoxic dilutions of PVP-I in saline (Lineaweaver et al. 1985a;  
10 Lineaweaver et al. 1985b). ~~Whereas~~ By contrast, Sanchez et al. (1988) reported that non-lethal  
11 concentrations of PVP-I in PBS allowed significant bacterial survival.

12 Generally, the antimicrobial activity of biocides is dependent on number of factors, such as  
13 biocide concentration, exposure time, presence of organic matter, etc. (Maillard 2005). It is  
14 commonly accepted that H<sub>2</sub>O<sub>2</sub> (WFBSC) and iodine solutions (Cooper 2007; Khan and Naqvi  
15 2006) lose their germicidal activity once they come into contact with organic load. So the  
16 questions arise whether ~~publically~~ publicly accepted antiseptics are adequate as wound  
17 antiseptics and if the new antimicrobials described here ~~has~~ ve more suitable antimicrobial  
18 properties. Thus, in order to more realistically picture the wound environment, the simultaneous  
19 assessment of antimicrobial and cytotoxic activities of the antimicrobials under consideration  
20 was also conducted in cell culture medium supplemented with fetal bovine serum as an  
21 indicator of organic challenge. In the presence of organic load, ~~H<sub>2</sub>O<sub>2</sub>/KI, H<sub>2</sub>O<sub>2</sub>/KSCN and H<sub>2</sub>O<sub>2</sub>~~  
22 ~~was~~ ere minimally cytotoxic (Fig. 1B), by contrast with our observations during culture in PBS  
23 (Fig. 1A). ~~However, it also lost its antimicrobial activity~~ Only modest antimicrobial activity  
24 ~~was also noted with H<sub>2</sub>O<sub>2</sub>/KI and H<sub>2</sub>O<sub>2</sub> (Fig. 1B), while H<sub>2</sub>O<sub>2</sub>/KSCN displayed no~~  
25 ~~antimicrobial activity following 10 min exposure, at the concentrations tested.~~ The cytotoxicity

1 of PVP-I was reduced to some extent in DMEM + FBS, but its antimicrobial capacity was also  
2 negatively impacted. Similarly, Müller and Kramer (2008) reported that PVP-I was well-  
3 tolerated by murine fibroblasts, but had low bactericidal activity against *E. coli* and *S. aureus*  
4 when tested in FBS supplemented culture media. ~~ITC remained cytotoxic and bactericidal,~~  
5 ~~matching the performance observed in PBS.~~ Lugol again exhibited a greater cytotoxic influence  
6 than an antimicrobial effect, though the two effects were milder in the presence of organic  
7 matter. ~~ITC remained cytotoxic and bactericidal, matching the performance observed in PBS.~~  
8 ~~The antibacterial profiles of H<sub>2</sub>O<sub>2</sub>/KI, H<sub>2</sub>O<sub>2</sub>/KSCN and H<sub>2</sub>O<sub>2</sub> (Fig. 1A, B), against *E. coli*, were~~  
9 ~~somewhat surprising, given the minimum inhibitory and bactericidal concentrations~~  
10 ~~(MIC/MBC) for these agents (31.3/31.3 µg ml<sup>-1</sup>) reported in our previous study (Tonoyan et al.~~  
11 ~~2017). In the present study, however, the antimicrobial susceptibility testing was based on~~  
12 ~~viable cell counts after only a short contact time (10 min) between the antimicrobial and *E. coli*~~  
13 ~~in either PBS or DMEM + FBS; and with an MBC definition of a concentration causing >3~~  
14 ~~log<sub>10</sub> reduction (99.9% kill). By contrast, in our previous study we used broth microdilution~~  
15 ~~MIC determinations, over 24 h of continuous exposure to the antimicrobials in nutrient broth,~~  
16 ~~with follow up MBC determination as a concentration causing 100% kill (Tonoyan et al. 2017).~~  
17 ~~Despite this, the antibacterial profile of ITC was comparable in both studies—100% kill of *E.*~~  
18 ~~*coli* was detected at 7.8 and 31.3 µg ml<sup>-1</sup> concentrations after 10 min exposure in PBS and~~  
19 ~~DMEM + FBS, respectively (Fig. 1A, B), and 100% kill was observed at 15.6 µg ml<sup>-1</sup>~~  
20 ~~concentration after 24 h exposure in nutrient broth (the MBC value; (Tonoyan et al. 2017).~~  
21 ~~These observations indicate that, regardless of the test conditions and methods used, ITC~~  
22 ~~demonstrated a fast acting and effective antibacterial profile.~~

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

1 Thus, it is not unreasonable to assume that the cytotoxicity of an antimicrobial, based on the  
2 action of reactive oxygen species, could be reversible and temporary. To test this hypothesis,  
3 we performed drug washout experiments, followed by analyzes of cell viability utilizing MTT  
4 assay (Fig. 3). The MTT test depends on dehydrogenase activity in intact mitochondria, and  
5 tests of mitochondrial function are sensitive indicators of oxidant injury (Watson et al. 1994).  
6 The experiments showed that the incubation of HeLa cells with [H<sub>2</sub>O<sub>2</sub>/KHTC](#), [PVP-I](#) and [Lugol](#)  
7 for 10 min in PBS with the subsequent removal of the antimicrobial, allowed for recovery of  
8 some cells at [a treatment concentrations of between 7.8 and 125 μg ml<sup>-1</sup>](#) ~~and thus indicated, to~~  
9 ~~some extent, a reduced cytotoxic effect~~ (Fig. 3A). However, ~~these~~ [weasre](#) the only recovery  
10 cases [for these antimicrobials](#) amongst all the tested [antimicrobials concentrations](#).  
11 Interestingly, a similar study by Müller and Kramer (2007) showed that cytotoxic effects of  
12 PVP-I containing products (in a defined concentration range ensuring > 20% cell viability) on  
13 epithelial cells and fibroblasts, after 30 min exposure, were completely reversible after washout  
14 of the compound and follow-up culturing. ~~Whereas~~ [By contrast](#), Watson et al. (1994) concluded  
15 that even a mild injury induced by H<sub>2</sub>O<sub>2</sub> (0.1 mM H<sub>2</sub>O<sub>2</sub> upon 1 h) in human colonic carcinoma  
16 epithelial cells was not fully reversible. Seemingly, 10 min exposure of HeLa cells to the ~~other~~  
17 [test](#) antimicrobials was enough to cause irreversible and permanent cellular destruction,  
18 however, the actual machinery involved in oxidative damage and repair cannot be extracted  
19 from these observations.

20 Another conventionally used measure of biocompatibility is the hemolytic activity of the  
21 antimicrobial candidate, in particular those nominated for open wound treatment. When ranked  
22 according to their hemolytic activities, the antimicrobials ~~tested in this study~~ had the following  
23 order: Lugol > ITC > PVP-I > [H<sub>2</sub>O<sub>2</sub>/KSCN](#) > H<sub>2</sub>O<sub>2</sub> ≥ [H<sub>2</sub>O<sub>2</sub>/KI](#) (Figs. 4, 5). It was shown that  
24 H<sub>2</sub>O<sub>2</sub>/KI and H<sub>2</sub>O<sub>2</sub> were the only two antimicrobials, which did not cause hemolysis of horse  
25 erythrocytes. This was somewhat surprising, considering that these two agents lysed HeLa

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

12 The *in vitro* comet assay is a sensitive, low cost and rapid genotoxicity test. It can be the test of  
13 choice for preliminary genotoxicity screening of drug candidates early in the development of  
14 new pharmaceuticals (Giannotti et al. 2002; Žegura and Filipič 2004). In principle, any type of  
15 eukaryotic cell may be used for comet assay genotoxicity testing. For the *in vitro* comet assay,  
16 well-characterized cell lines or primary cells are preferred, which are generally exposed to test  
17 compounds in the presence and absence of metabolic activation system, such as S9 (Tice et al.  
18 2000; Žegura and Filipič 2004). In this study, we used the alkaline comet assay tool to assess  
19 the genotoxicity of ITC on human HeLa cell line only in the absence of S9 metabolic activation.  
20 The alkaline comet assay detects both single and double-stranded DNA breaks (Karbachi and  
21 Cooke 2014) producing special comet-like appearances. H<sub>2</sub>O<sub>2</sub> is known to primarily produce  
22 single-stranded breaks (Cortés-Gutiérrez et al. 2014), [whereason the other hand](#), double-strand  
23 breaks are the major feature of the UV-radiation (Bogdanov et al. 1997). In the present study  
24 we have revealed that, indeed, H<sub>2</sub>O<sub>2</sub> induced single-strand breaks in the shape of droplet-like  
25 comets (Fig. 6E), and UV-C irradiation produced double-stranded breaks in the shape of

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

## 5 **5. Conclusion**

6 The *in vitro* cytotoxicity of a novel, biocidal, iodo-thiocyanate complex, ITC, was investigated  
7 and compared to commonly used antiseptics. The antimicrobial potency of ITC, together with  
8 a comparable cytotoxicity profile to existing antiseptics, suggests that it could be considered  
9 for future antiseptic and therapeutic applications. Indeed, the concentrations of ITC resulting  
10 in cytotoxicity were equivalent to those resulting in potent bactericidal activity. By contrast,  
11 the cellular toxicity of the commonly used antiseptics  $\text{H}_2\text{O}_2$ , PVP-I and Lugol towards HeLa  
12 cells was apparent at levels too low to cause significant bactericidal effects. The activity of ITC  
13 was not quenched by organic matter, [whereaswhile](#), the activities of commonly used non-  
14 selective antiseptics were suppressed by organic load. In addition, ITC did not produce DNA  
15 breaks [at 30 and 300  \$\mu\text{g ml}^{-1}\$  concentrations](#), as detected by an *in vitro* comet assay [in the](#)  
16 [absence of S9 metabolic activation, at 30 and 300  \$\mu\text{g ml}^{-1}\$  concentrations](#).

## 17 **Conflict of interest**

18 The authors declare that there are no conflicts of interest.

## 19 **Acknowledgment**

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22 Natural Sciences at National University of Ireland, Galway.

## 23 **Abbreviations**

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

6

## 1   **References**

- 2   Alaçam T, Ömürlü H, Özkul A, Görgül G, Misirligil A (1993) Cytotoxicity versus  
3       antibacterial activity of some antiseptics *in vitro*. J Nihon Univ Sch Dent 35(1):22-27  
4       doi:10.2334/josnugd1959.35.22
- 5   Atiyeh BS, Dibo SA, Hayek SN (2009) Wound cleansing, topical antiseptics and wound  
6       healing. Int Wound J 6(6):420-430 doi:10.1111/j.1742-481X.2009.00639.x
- 7   Bafort F, Parisi O, Perraudin J-P, Jijakli MH (2014) Mode of action of lactoperoxidase as  
8       related to its antimicrobial activity: A review. Enzyme Res 2014:13  
9       doi:10.1155/2014/517164
- 10  Block SS (2001) Disinfection, sterilization, and preservation, 5th edn. Lippincott Williams &  
11       Wilkins, Philadelphia, USA
- 12  Bogdanov KV, Chukhlovin AB, Zaritskey AY, Frolova OI, Afanasiev BV (1997) Ultraviolet  
13       irradiation induces multiple DNA double-strand breaks and apoptosis in normal  
14       granulocytes and chronic myeloid leukaemia blasts. Br J Haematol 98(4):869-72
- 15  Bowler PG, Duerden BI, Armstrong DG (2001) Wound microbiology and associated  
16       approaches to wound management. Clin Microbiol Rev 14(2):244-269  
17       doi:10.1128/CMR.14.2.244-269.2001
- 18  Clark RA, Klebanoff SJ (1975) Neutrophil-mediated tumor cell cytotoxicity: Role of the  
19       peroxidase system. J Exp Med 141(6):1442-1447 doi:10.1084/jem.141.6.1442
- 20  Clark RA, Klebanoff SJ (1977) Myeloperoxidase - H<sub>2</sub>O<sub>2</sub> - halide system: Cytotoxic effect on  
21       human blood leukocytes. Blood 50(1):65-70
- 22  Cooper RA (2007) Iodine revisited. Int Wound J 4(2):124-137 doi:10.1111/j.1742-  
23       481X.2007.00314.x
- 24  Cortés-Gutiérrez EI, López-Fernández C, Fernández JL, Dávila-Rodríguez MI, Johnston SD,  
25       Gosálvez J (2014) Interpreting sperm DNA damage in a diverse range of mammalian  
26       sperm by means of the two-tailed comet assay. Front Genet 5:404
- 27  Corvi R, Madia F (2016) *In vitro* genotoxicity testing—Can the performance be enhanced?  
28       Food Chem Toxicol doi:<http://doi.org/10.1016/j.fct.2016.08.024>
- 29  Davies MJ, Hawkins CL, Pattison DI, Rees MD (2008) Mammalian heme peroxidases: From  
30       molecular mechanisms to health implications. Antioxid Redox Signal 10(7):1199-  
31       1234 doi:10.1089/ars.2007.1927
- 32  Drosou A, Falabella A, Kirsner RS (2003) Antiseptics on wounds: An area of controversy.  
33       Wounds 15(5):149-166
- 34  Edelson PJ, Cohn ZA (1973) Peroxidase-mediated mammalian cell cytotoxicity. J Exp Med  
35       138(1):318-323 doi:10.1084/jem.138.1.318
- 36  Evans BC, Nelson CE, Yu SS, et al. (2013) *Ex vivo* red blood cell hemolysis assay for the  
37       evaluation of pH-responsive endosomolytic agents for cytosolic delivery of  
38       biomacromolecular drugs. J Vis Exp(73):e50166 doi:10.3791/50166
- 39  Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T (2003) *In vitro* cytotoxicity testing of  
40       polycations: Influence of polymer structure on cell viability and hemolysis.  
41       Biomaterials 24(7):1121-1131 doi:[http://dx.doi.org/10.1016/S0142-9612\(02\)00445-3](http://dx.doi.org/10.1016/S0142-9612(02)00445-3)
- 42  Fotakis G, Timbrell JA (2006) *In vitro* cytotoxicity assays: Comparison of LDH, neutral red,  
43       MTT and protein assay in hepatoma cell lines following exposure to cadmium  
44       chloride. Toxicol Lett 160(2):171-177  
45       doi:<http://dx.doi.org/10.1016/j.toxlet.2005.07.001>
- 46  Giannotti E, Vandin L, Repeto P, Comelli R (2002) A comparison of the *in vitro* Comet assay  
47       with the *in vitro* chromosome aberration assay using whole human blood or Chinese



1 hamster lung cells: Validation study using a range of novel pharmaceuticals.  
2 Mutagenesis 17(2):163-70 doi:<https://doi.org/10.1093/mutage/17.2.163>  
3 Henderson WR, Chi EY, Jong EC, Klebanoff SJ (1981) Mast cell-mediated tumor cell  
4 cytotoxicity. Role of the peroxidase system. J Exp Med 153(3):520-33  
5 doi:10.1084/jem.153.3.520  
6 Hidalgo E, Bartolome R, Dominguez C (2002) Cytotoxicity mechanisms of sodium  
7 hypochlorite in cultured human dermal fibroblasts and its bactericidal effectiveness.  
8 Chem Biol Interact 139(3):265-282 doi:[http://dx.doi.org/10.1016/S0009-](http://dx.doi.org/10.1016/S0009-2797(02)00003-0)  
9 [2797\(02\)00003-0](http://dx.doi.org/10.1016/S0009-2797(02)00003-0)  
10 Jong EC, Klebanoff SJ (1980) Eosinophil-mediated mammalian tumor cell cytotoxicity: Role  
11 of the peroxidase system. J Immunol 124(4):1949-53  
12 Karbaschi M, Cooke MS (2014) Novel method for the high-throughput processing of slides  
13 for the comet assay. Sci Rep 4:7200 doi:10.1038/srep07200  
14 Khan MN, Naqvi AH (2006) Antiseptics, iodine, povidone iodine and traumatic wound  
15 cleansing. J Tissue Viability 16(4):6-10 doi:[http://dx.doi.org/10.1016/S0965-](http://dx.doi.org/10.1016/S0965-206X(06)64002-3)  
16 [206X\(06\)64002-3](http://dx.doi.org/10.1016/S0965-206X(06)64002-3)  
17 Klebanoff SJ (2005) Myeloperoxidase: Friend and foe. J Leukoc Biol 77(5):598-625  
18 doi:10.1189/jlb.1204697  
19 Kramer A, Assadian O, Koburger-Janssen T (2016) Antimicrobial efficacy of the  
20 combination of chlorhexidine digluconate and dexpanthenol. GMS Hyg Infect Control  
21 11:Doc24 doi:10.3205/dgkh000284  
22 Kussendrager KD, van Hooijdonk A (2000) Lactoperoxidase: Physico-chemical properties,  
23 occurrence, mechanism of action and applications. Br J Nutr 84(Suppl 1):S19-S25  
24 doi:<https://doi.org/10.1017/S0007114500002208>  
25 Li X, Wang L, Fan Y, Feng Q, Cui F-z (2012) Biocompatibility and toxicity of nanoparticles  
26 and nanotubes. J Nanomater 2012:19 doi:10.1155/2012/548389  
27 Lineaweaver W, Howard R, Soucy D, et al. (1985a) Topical antimicrobial toxicity. Arch Surg  
28 120(3):267-270 doi:10.1001/archsurg.1985.01390270007001  
29 Lineaweaver W, McMorris S, Soucy D, Howard R (1985b) Cellular and bacterial toxicities of  
30 topical antimicrobials. Plast Reconstr Surg 75(3):394-6  
31 Maillard J-Y (2005) Antimicrobial biocides in the healthcare environment: Efficacy, usage,  
32 policies, and perceived problems. Ther Clin Risk Manag 1(4):307-320  
33 Masters JR (2002) HeLa cells 50 years on: The good, the bad and the ugly. Nat Rev Cancer  
34 2(4):315-319 doi:10.1038/nrc775  
35 McDonnell G, Russell AD (1999) Antiseptics and disinfectants: Activity, action, and  
36 resistance. Clin Microbiol Rev 12(1):147-179  
37 Müller G, Kramer A (2007) Revitalisation of mammalian cells exposed to antiseptics. GMS  
38 Hyg Infect Control 2(2):Doc 35  
39 Müller G, Kramer A (2008) Biocompatibility index of antiseptic agents by parallel  
40 assessment of antimicrobial activity and cellular cytotoxicity. J Antimicrob  
41 Chemother 61(6):1281-1287 doi:10.1093/jac/dkn125  
42 Naidu AS (2000) Lactoperoxidase. In: Naidu AS (ed) Natural food antimicrobial systems.  
43 CRC Press, Boca Raton, USA, p 103-132  
44 Okano A, Isley NA, Boger DL (2017) Peripheral modifications of  
45 [ $\Psi$ [CH<sub>2</sub>NH]Tpg<sup>4</sup>]vancomycin with added synergistic mechanisms of action provide  
46 durable and potent antibiotics. Proc Natl Acad Sci USA  
47 doi:10.1073/pnas.1704125114  
48 Olive PL, Banath JP (2006) The comet assay: A method to measure DNA damage in  
49 individual cells. Nat Protoc 1(1):23-29 doi:10.1038/nprot.2006.5

- 1 Riss TL, Moravec RA, Niles AL (2011) Cytotoxicity testing: Measuring viable cells, dead  
2 cells, and detecting mechanism of cell death. In: Stoddart MJ (ed) Mammalian cell  
3 viability: Methods and protocols. Humana Press, Totowa, NJ, p 103-114
- 4 Rose WE, Poppens PT (2009) Impact of biofilm on the *in vitro* activity of vancomycin alone  
5 and in combination with tigecycline and rifampicin against *Staphylococcus aureus*. J  
6 Antimicrob Chemother 63(3):485-488 doi:10.1093/jac/dkn513
- 7 Sanchez IR, Nusbaum KE, Swaim SF, Hale AS, Henderson RA, McGuire JA (1988)  
8 Chlorhexidine diacetate and povidone-iodine cytotoxicity to canine embryonic  
9 fibroblasts and *Staphylococcus aureus*. Vet Surg 17(4):182-185 doi:10.1111/j.1532-  
10 950X.1988.tb00995.x
- 11 Schedler K, Assadian O, Brautferger U, et al. (2017) Proposed phase 2/ step 2 *in-vitro* test on  
12 basis of EN 14561 for standardised testing of the wound antiseptics PVP-iodine,  
13 chlorhexidine digluconate, polihexanide and octenidine dihydrochloride. BMC Infect  
14 Dis 17:143 doi:10.1186/s12879-017-2220-4
- 15 Seifu E, Buys EM, Donkin EF (2005) Significance of the lactoperoxidase system in the dairy  
16 industry and its potential applications: A review. Trends Food Sci Tech 16(4):137-154  
17 doi:<http://dx.doi.org/10.1016/j.tifs.2004.11.002>
- 18 Sibbald RG, Leaper DJ, Queen D (2011) Iodine made easy Wounds Int 2(2)
- 19 Siewerts S, De Bok FAM, Mols E, De Vos WM, Van Hylckama Vlieg JET (2008) A simple  
20 and fast method for determining colony forming units. Lett Appl Microbiol 47(4):275-  
21 278 doi:10.1111/j.1472-765X.2008.02417.x
- 22 Tice RR, Agurell E, Anderson D, et al. (2000) Single cell gel/comet assay: Guidelines for *in*  
23 *vitro* and *in vivo* genetic toxicology testing. Environmental and molecular mutagenesis  
24 35(3):206-21 doi:10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J
- 25 Tonoyan L, Fleming G, Mc Cay P, Friel R, O'Flaherty V (2017) Antibacterial potential of an  
26 antimicrobial agent inspired by peroxidase-catalyzed systems. Frontiers in  
27 Microbiology 8(680) doi:10.3389/fmicb.2017.00680
- 28 Wallace KB (1997) Free Radical Toxicology. Taylor & Francis, Washington DC, USA
- 29 Watson AJ, Askew JN, Sandle GI (1994) Characterisation of oxidative injury to an intestinal  
30 cell line (HT-29) by hydrogen peroxide. Gut 35(11):1575-1581  
31 doi:10.1136/gut.35.11.1575
- 32 WFBSC Disinfectant chart. In. <http://www.cleaning-for-health.org/disinfectant-chart/>  
33 Accessed 19.12.2016 2016
- 34 WUWHS (2008) Principles of best practice: Wound infection in clinical practice. An  
35 international consensus World Union of Wound Healing Societies MEP Ltd, London
- 36 Žegura B, Filipič M (2004) Application of *in vitro* Comet assay for gnototoxicity testing. In:  
37 Yan Z, Caldwell GW (eds) Optimization in Drug Discovery: *In Vitro* Methods.  
38 Humana Press, Totowa, NJ, p 301-313  
39  
40