Antimicrobial and Cytotoxicity Evaluations of Hydrogen peroxide- Towards Clinical Application of Antimicrobial Biomaterials for Wound Dressings

A Idrees[1,2,3], S Pacharra[3], L Marlinghaus[4], V Chiono[1], G Ciardelli[1], JM Vasquez[1,5], U Greiser[5], W Wang[5], R Viebahn[2], SG Gatermann[4], J Salber[2,3]

[1]Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Corso Duca Degli Abruzzi 24, 10129 Turin, Italy.
[2]Clinic of Surgery, Knappschaftskrankenhaus Bochum, Hospital of the RUHR-University, In der Schornau 23-25, 44892 Bochum, Germany.
[3]Clinical Research Center, RUHR University Bochum, Universitätsstraße 150, 44801 Bochum
[4]Department of Medical Microbiology, Institute for Hygiene and Microbiology, Ruhr-University Bochum, Universitätsstraße 150, 44801, Germany.
[5]Vornia Biomaterials Ltd, Dublin.

Introduction: Chronic wound infections and emerging drug resistance are serious problems in the present world causing a considerable morbidity and a high healthcare costs. For this purpose, investigation on novel antimicrobial strategies is of great interest. Use of honey from ancient times is reputed for its wound-healing and antibacterial properties. It has been reported that the major antibacterial factor in honey is the release of hydrogen peroxide (H_2O_2) produced by glucose oxidase. This approach can be exploited to prepare novel polymer-based antimicrobial biomaterials for wound healing purposes. In this study, the inhibitory effect of H_2O_2 on the growth of numerous bacteria of clinical significance was investigated. To determine the "safe" antimicrobial concentration of H_2O_2, cytocompatibility analysis was also performed for H_2O_2 induced cellular cytotoxicity.

Methods: The effect of externally added H_2O_2 was performed by exposing L929 fibroblasts to various H_2O_2 concentrations. At different time points after exposure with H2O2, cell viability was assessed by measuring cell metabolic activity, cell membrane integrity and cell morphology. Antimicrobial efficacy was evaluated against a wide range of gram-positive and gram-negative bacteria that are involved in chronic wounds namely *S. aureus, S. epidermidis, S. lugdunensis, E. faecalis, E. coli, P. aeruginosa, K. pneumoniae,* and *A. baumanii*. Antimicrobial tests were performed using broth microdilution method for the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). MIC is the lowest concentration of antimicrobial agent that inhibits bacterial growth, while the MBC is the lowest level of antimicrobial agent that kills the bacteria (by reducing the viability of the initial bacterial inoculum by ≥99.9%).

Figure 1: Evaluation of cytotoxic effects of H_2O_2 on L929 fibroblasts

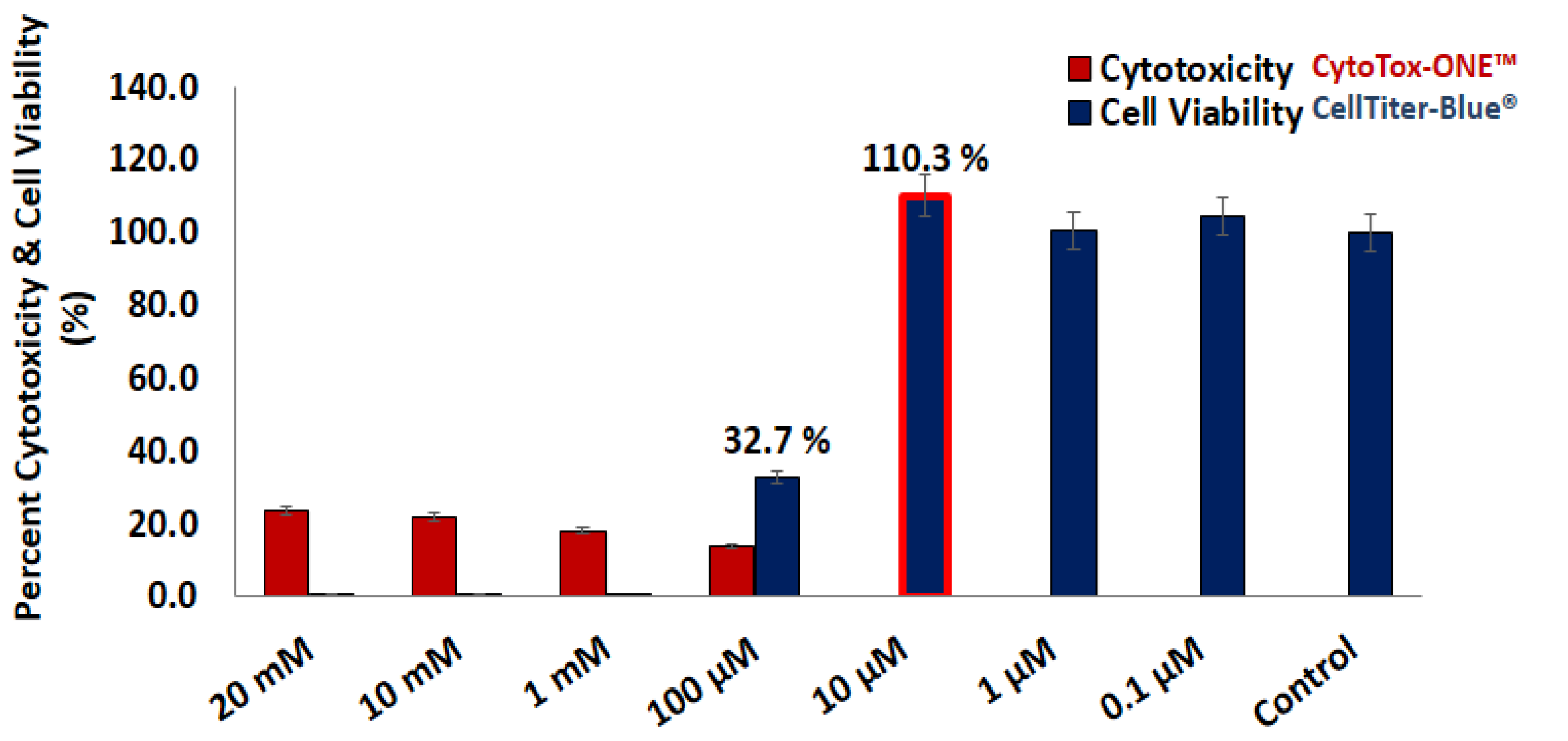


Figure 1: Cell viability data shows that:

1. H_2O_2 did not favor cell viability at 20 mM, 10 mM, and 1 mM concentrations in our experimental set-up (L929 cell culture).
2. H_2O_2 concentration of 10 µM did not alter cell viability with respect to control conditions (cells only) and further that H_2O_2 might be stimulating cell growth at the concentration of 10 µM.[1,2]
3. Cytotoxic effect started decreasing below 100 µM H_2O_2 concentration and was no more relevant at further lower concentrations (10, 1, 0.1 µM). H_2O_2 concentration of 10 µM did not affect cell behavior.

Figure 2: Evaluation of cytotoxic effects of H_2O_2 on L929 fibroblasts over time

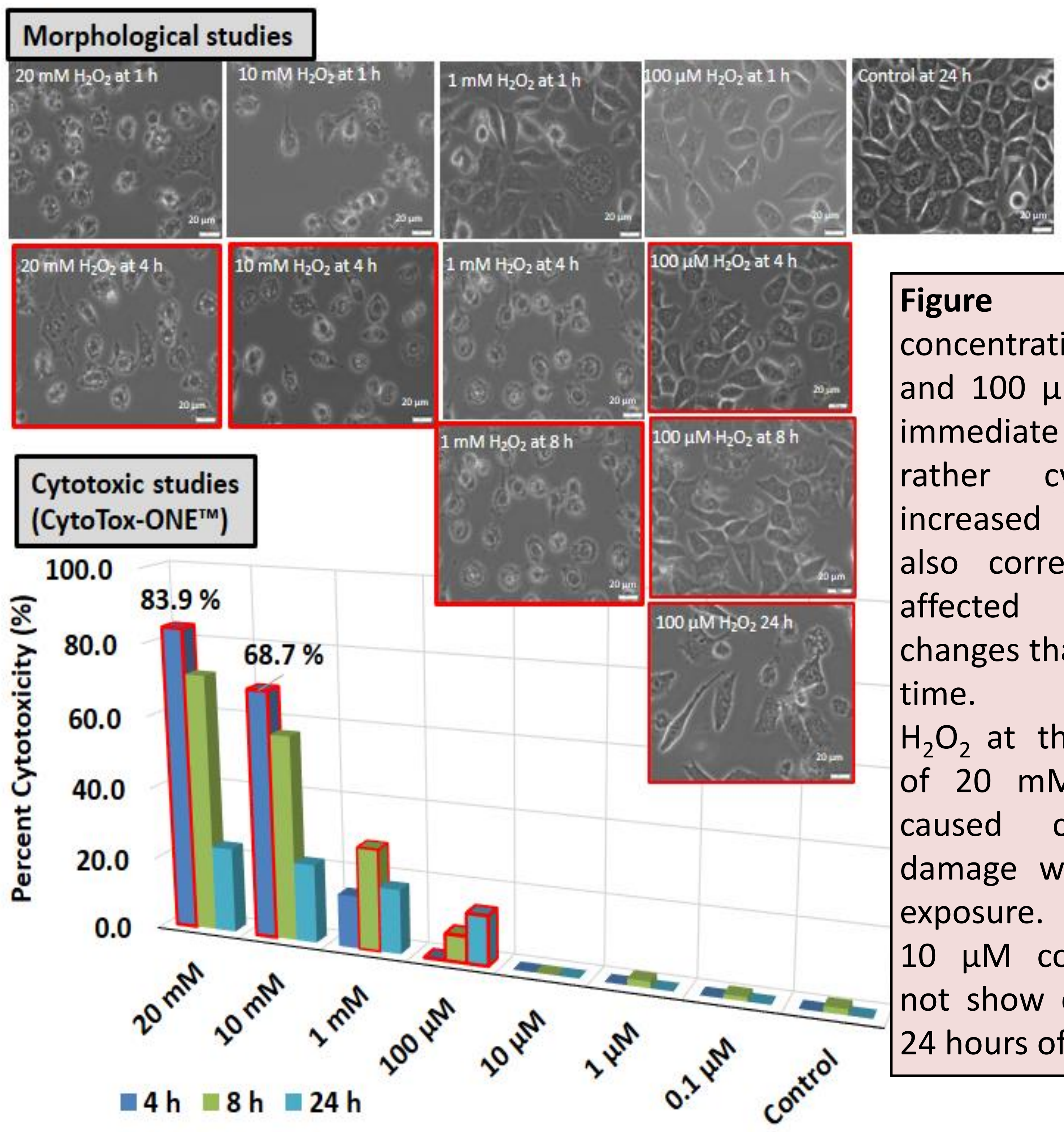
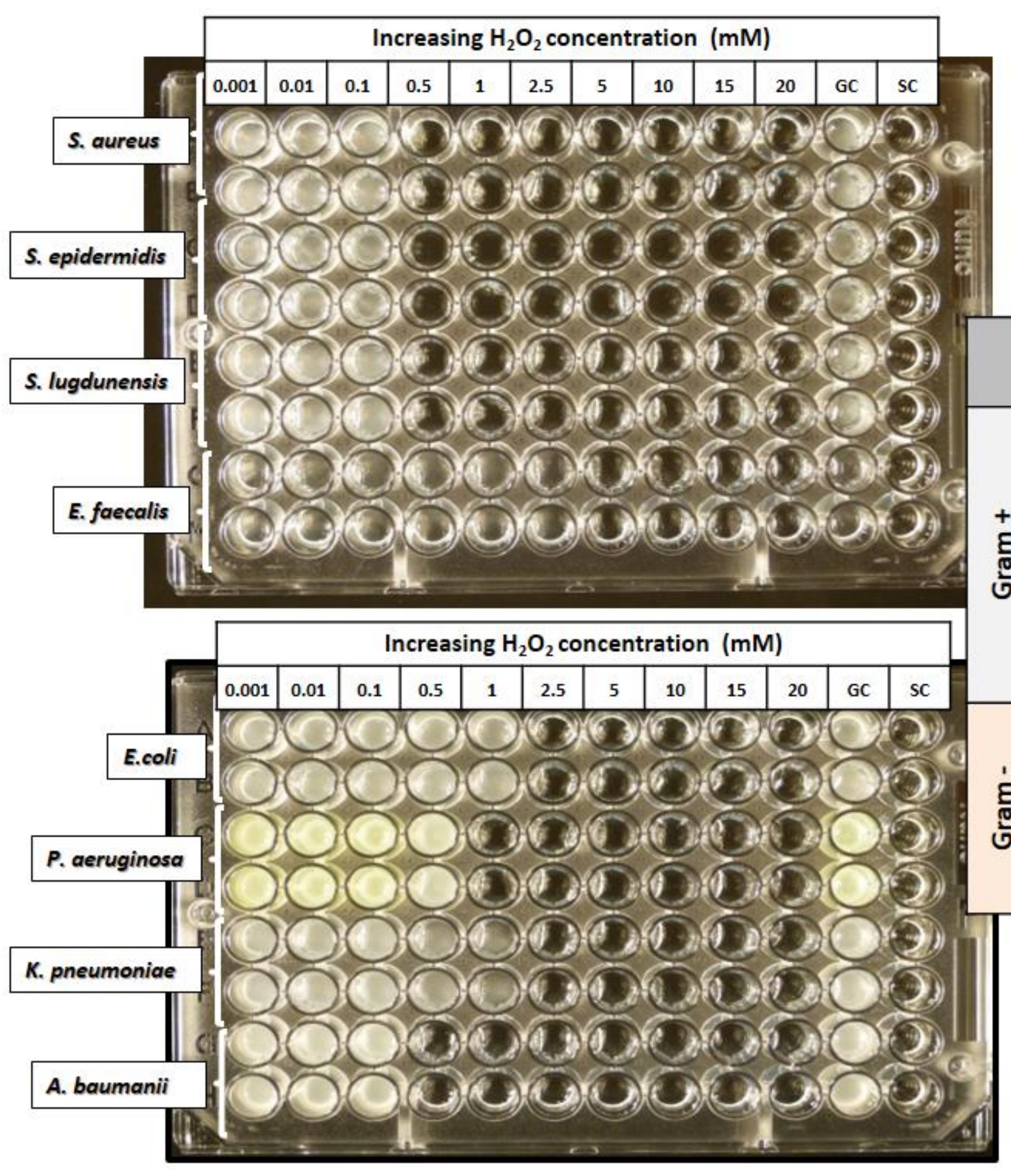


Figure 2: H_2O_2 concentrations of 1 mM and 100 µM did not show immediate cytotoxic effect, rather cytotoxic effect increased with time. This also correlated with the affected morphological changes that appeared over time.

H_2O_2 at the concentration of 20 mM and 10 mM, caused cell membrane damage within 4 hour of exposure.

10 µM concentration did not show cytotoxicity until 24 hours of exposure.

Figure 3: Antimicrobial activity of H_2O_2 against different clinically significant bacterial species



	Bacterial species	MIC mM	MBC mM
Gram +	*S. aureus*	0.5	99.9% reduction at MIC & above
	S. epidermidis	0.5	5
	S. lugdunensis	0.5	2.5
	E. faecalis	5	99.99% reduction at MIC & above
Gram -	*E. coli*	2.5	2.5
	P. aeruginosa	1	1
	K. pneumoniae	2.5	2.5
	A. baumanii	0.5	1

Figure 4: MIC of H_2O_2 against different bacterial species

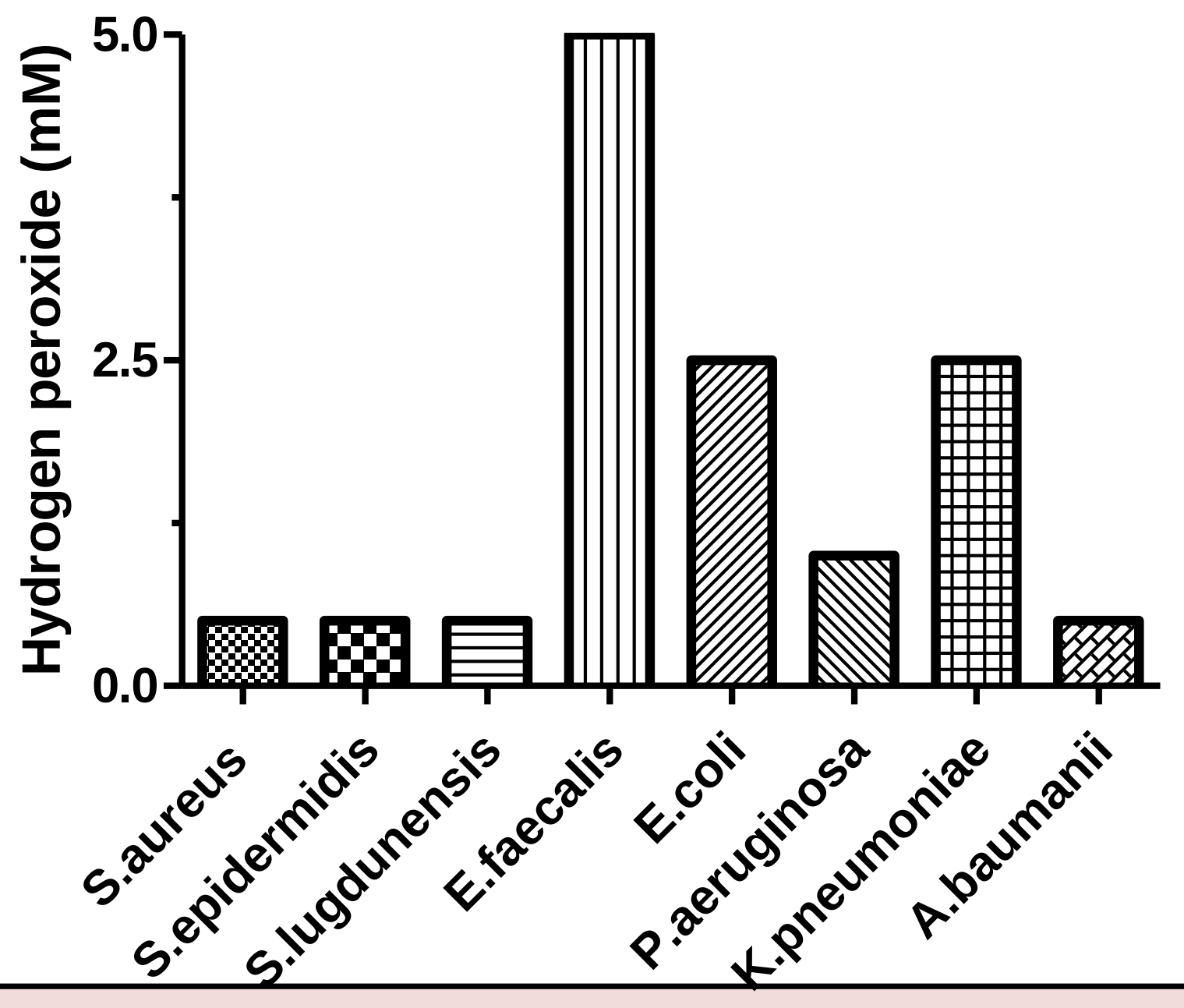
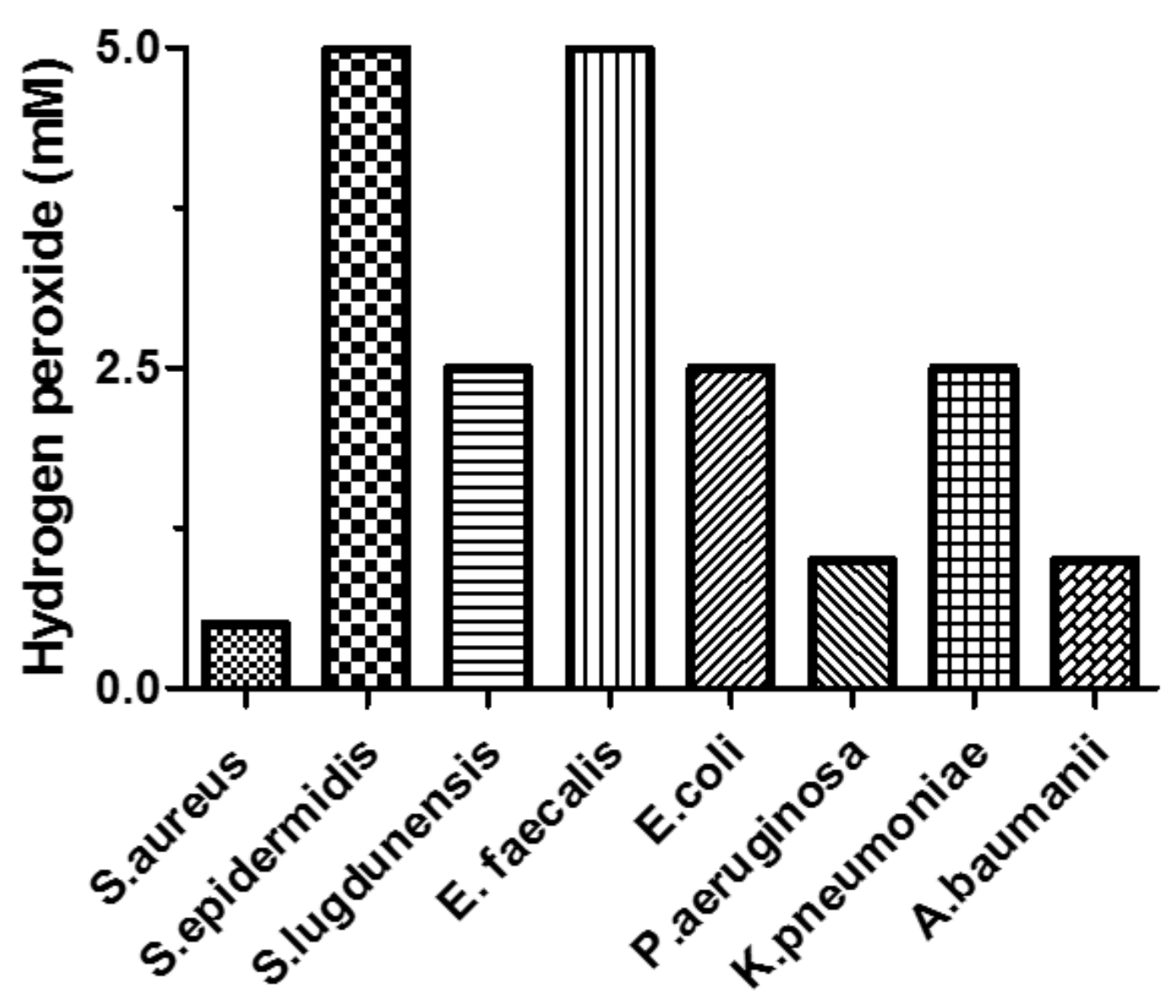


Figure 5: MBC of H_2O_2 against different bacterial species



Results & Conclusion: Results (Figure 3, 4 and 5) showed different MIC and MBC values of H_2O_2 for different bacterial species indicating their differences in susceptibility to treatment. There was no clear preference between Gram positive and Gram negative bacteria. Among the tested bacteria, *S. aureus* showed 99.9% bacterial reduction at the concentration of 0.5 mM, while *E. faecalis* showed the highest MIC value of 5 mM. H_2O_2 was found most effective against *S. aureus* (one of the most pathogenic bacteria) and less effective against *E. faecalis*. *Acinetobacter baumanii* being one of the most pathogenic bacteria involved in serious skin wound infections also showed the same MIC value (0.5 mM) as *S. aureus*.

Cytotoxicity results (figure 1 and 2) showed two distinct patterns in our experimental set-up: the highest concentrations rapidly induced cell death characterized by morphological evidence and plasma membrane damage as compared to the concentrations of 1 mM and 100 µM where the cytotoxic effect only gradually increased with time. Results showed that 10 µM concentration did not show cytotoxicity. This data also indicated the concentration dependent distinct pathways of H_2O_2 -induced cytotoxicity. Also, we speculated that the cytotoxic effects would differ depending on "at once" H_2O_2 exposure or exposure to "gradually" produced H_2O_2 by glucose oxidase and glucose entrapped into a matrix. Different H_2O_2 administration influences the ability of the cells to eliminate and detoxify H_2O_2 and needs further investigation.

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