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Sabrina Jenull, Bakk. rer. nat.

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A	Amoxicillin
ALK	Anaplastic lymphoma kinase
AP-1	Activator protein-1
AUG	Amoxicillin/Clavulanic acid
Bet	Beta-Isodona®
BMP	Bone morphogenetic protein
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CASO	Casein soy peptone agar
CDX	Cefadroxil
CFU	Colony forming unit
Chl	Chlorhexidin
CHS	Chondroitin sulfate
CI	Confidence interval
CIP	Ciprofloxacin
CLA	Clarythromycin
Co-Smad	Co-mediator Smad
Ctrl	Control
CTX	Cefotaxim
DF	Dilution factor
DMEM	Dublecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythimidine triphosphate
DXT	Doxycyclin

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GDF	Growth and differentiation factor
GM	Gentamicin
HIF-1	Hypoxia-inducible factor-1
IC	Inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IL-1	Interleukin-1
IMI	Imipenem
IMP	Inhibitor of metalloproteinases
IRES	Internal ribosome entry site
I-Smad	Inhibitory Smad
LEV	Levofloxacin
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharides
LZD	Linezolid
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan cancer foundation-7
MD	Mean difference
MIS	Muellerian inhibiting substance
MFX	Moxifloxacin
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MT-MMP	Membrane type matrix metalloproteinase
MTT	Methyl thiazole tetrazolium bromide

MTZ	Metronidazol
MY	Lincomycin
NF-κB	Nuclear factor-κB
O	Octenisept® diluted 1:2 with aqua dest. (sterile filtrated)
O+	Octenisept® diluted 1:2 with sterile 5 % glucose solution
OCT	Octenisept®
PA	Plasminogen activation
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PEA3	Polyomavirus enhancer activator 3
PDGF	Platelet-derived growth factor
PG	Penicillin G
PH	Phenoxyethanol
Plg	Plasminogen
PTZ	Piperacillin/Tazobactam
PV	Penicillin V
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute medium
R-Smad	Receptor-regulated Smad
RT	Reverse transcriptase
PEN-STREP	Penicillin-streptomycin
Tau	Taurolin®
TBE	Tris/Borate/EDTA
Tcf-4	Transcription factor-4
TGC	Tigecyclin
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-α

tPA	Tissue type plasminogen activator
TS	Cotrimoxazol
uPA	Urokinase-like plasminogen activator
uPAR	Urokinase-like plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VA	Vancomycin
VN	Vitronectin

1 Introduction

Antiseptics are compounds which are able to kill or inhibit the growth and development of microorganisms (Hubner et al, 2010). Clinically, these substances are used for both, intact skin and wounds (Atiyeh et al., 2009). They are applied topically on the surface of the body, in body cavities or on surgically exposed tissues (Stahl et al., 2010) in order to reduce the risk of colonization of microorganisms or to treat already existing infections of wounds (Hubner et al., 2010). Wound infections are associated with a delay of wound healing (Hirsch et al., 2009) and the development of severe morbidity up to sepsis (Steinstraesser et al., 2002). Therefore, if the bacterial colonisation and the bio burden of a wound could be decreased by topical antiseptics, antibiotic therapy might be not needed (Leaper and Durani, 2008). However, antiseptics may be cytotoxic to the wound itself (Hirsch et al., 2009). For this reason, antiseptic preparations should fulfil certain requirements in order to be the agent of choice. To chose the right antiseptic for wound management, overall data regarding toxicity, safety, efficiency, clinical application and contraindications are needed (Hubner et al., 2010).

In the last few decades, new compounds have been in clinical use. Among these, one is octenidine dihydrochloride (Hubner et al., 2010), which is, in combination with 2-phenoxyethanol (PH), a licensed product in European countries (Octenisept® (OCT) with 0,1 % octenidine and 2 % PH) (Stahl et al., 2010). It has become the antiseptic agent of first choice in many hospitals (Franz and Vogelein, 2011), where it is used for skin, mucous membrane and wound antisepsis (Hubner et al., 2010) (Gilbert and Moore, 2005). It has a broad antimicrobial spectrum against a variety of gram-positive and gram-negative bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA) (Kramer et al, 2008), skin microflora and fungi (Sedlock and Bailey, 1985). As antiseptics are applied on host tissue, the cytotoxic side effects should be minimal without adversely affecting the microbicidal properties of the compound. Due to this, the indications for local antiseptics should be carefully chosen (Marquardt et al., 2010) (Hirsch et al., 2009). Hence, the impact of octenidine on cell migration and proliferation was under investigation in the current work. Therefore, in vitro tests

using primary human fibroblasts which are the main cell type of the connective tissue and MCF-7 cells, a breast cancer cell line, were performed. Moreover, it was determined whether the application of octenidine causes any changes in the expression of genes involved in wound healing like the family of matrix metalloproteinases (MMPs), transforming growth factor (TGF)-β or vascular endothelial growth factor (VEGF) (Werner and Grose, 2003) (Lund et al., 1999). Another aspect of this work was to elucidate whether there are any interactions between commonly used antibiotics and selected antiseptics since they are often used in combination in clinical practise. However, the knowledge of possible interactions is limited which led us to the aim to further shed light on this issue.

All these investigations were also performed with a new OCT formulation where the compound is diluted 1:2 with a 5 % glucose solution. In case report studies, this formulation was better tolerated than OCT diluted with saline. Previous experiments in our working group showed an enhanced stability of cell adhesion upon application of the glucose formulation of OCT which reflects a stable cell-matrix association in vivo. This would explain the favorable results from case reports. Moreover, we tried to dig deeper into the paradox observation that OCT is quite toxic in the cell culture but well tolerated in clinical practise what is not explainable so far. Extracellular matrix components (ECM) like CHS are highly negatively charged and dermal fibroblasts are embedded therein. To elucidate we searched for experimental evidence that octenidine antiseptics are sequestered from the cellular membranes by CHS. The complex formation of octenidine and ECM components could substantially decrease the cytotoxicity of octenidine while the antimicrobial activity is sustained.

2 Literature review

2.1. Wound healing

2.1.1. Overview

The skin can be divided into two distinguishable tissue layers: a keratinized epidermis which consists of several cell layers and the underlying dermis separated by a basement membrane (Figure 1) (Fuchs and Raghavan, 2002). The dermis is a thick tissue layer comprised of collagen-rich dermal connective tissue which provides nutrients and support (Martin, 1997). The epidermis is the primary barrier of the external environment and the internal tissues (Kirker et al., 2009). It has various protective barrier functions against microbial pathogens, oxidant stress including ultraviolet light or mechanical insults (Elias, 2007). Consequently, any damage to it must be rapidly and efficiently repaired (Martin, 1997).

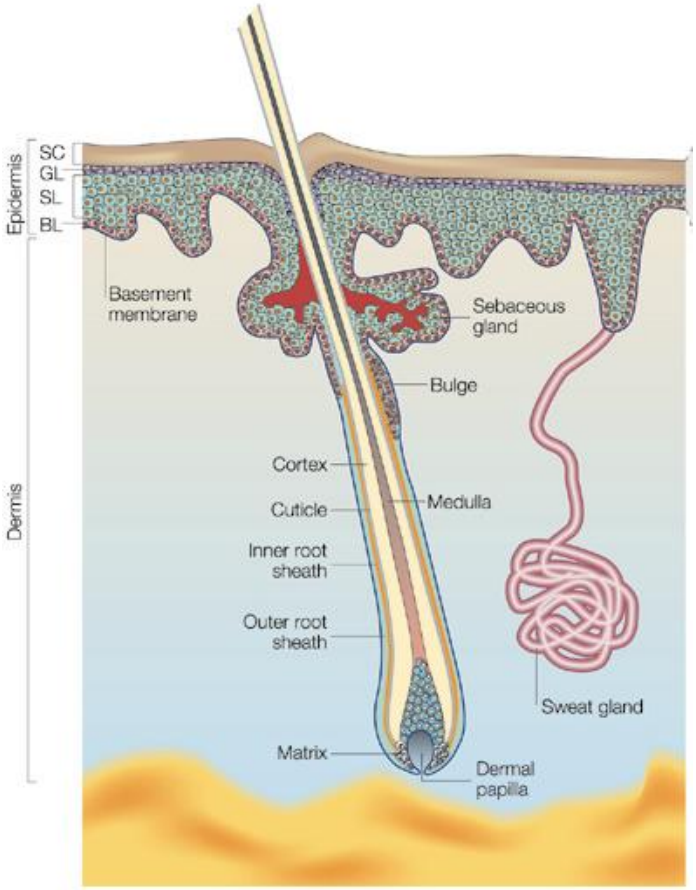


Figure 1. Overview of the skin layers (adapted from Fuch and Raghavan, 2003)

The healing of a cutaneous wound is a very complex, dynamic and well organized process. Several different cell types, including inflammatory cells, endothelial cells, keratinocytes and fibroblasts, and cellular pathways need to be coordinated in order to secure an efficient repair (Werner and Grose, 2003) (Gurtner et al., 2008) (Singer and Clark, 1999).

Usual mammal wound repair can be divided into three classical overlapping stages: inflammation, new tissue formation and remodeling. Immediately after an injury occurs, the inflammatory phase is initiated, which serves to remove dead tissue, to stop ongoing blood and fluid loss and to prevent infection (Singer and Clark, 1999). Wound repair is induced via the secretion of various growth factors and cytokines by injured blood vessels, activated platelets (Werner and Grose, 2003) and inflammatory cells (Kung et al., 2008). This is accompanied by the formation of a blood clot as a consequence of the disruption of blood vessels (Singer and Clark, 1999) . The blood clot is the result of the activated coagulation cascade and consists of a fibrin matrix which acts as a scaffold for the infiltrating inflammatory cells and fibroblasts.

The second stage, the proliferative phase, is characterized by the migration of inflammatory cells and fibroblasts which form the granulation tissue and replace the fibrin matrix. Fibroblasts produce large amounts of collagen-rich extracellular matrix that is deposited in the provisional matrix. Additionally, this cell type differentiates into myofibroblasts which are important for wound contraction.

Another important process during wound repair is the formation of new blood vessels (known as angiogenesis) (Gurtner et al., 2008) because they secure nutrient and oxygen supply (Kung et al., 2008).

Keratinocytes start to migrate from the edges of the wound to cover up the injured dermis until a monolayer is created. This is followed by the re-establishment of the epidermis and the basal lamina. It is believed that the stop of the proliferating keratinocytes is a consequence of contact inhibition (Martin, 1997). Eventually, the granulation tissue is replaced by scar tissue which mostly consists of extracellular matrix proteins and collagen and is mechanically insufficient (Werner and Grose, 2003).

2.1.2. Molecular mechanisms

The complex mechanisms of wound repair are navigated by the interplay of cell-cell/ cell-matrix interactions and by a plethora of cytokines and growth factors (Werner and Grose, 2003). One of the first signals is secreted by platelets which are located in the newly formed fibrin clot after wounding occurred. They secrete growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). These signalling molecules initiate the recruitment of inflammatory cells, the migration of fibroblasts to the healing wound and the stimulation of wound contraction (Martin, 1997) (Barrientos et al., 2008). Additionally, the fibroblast growth factor (FGF) family represents a well known protein family involved in wound healing. They are produced by various cell types such as fibroblasts, keratinocytes or endothelial cells and play an important role during re-epithelialisation and vascularisation (Barrientos et al., 2008). Another essential angiogenic factor which is released at the wound site by predominantly keratinocytes and macrophages is a member of the vascular endothelial growth factor (VEGF) family, VEGF-A (Werner and Grose, 2003). Its expression is induced in a paracrine manner by the afore mentioned growth factor families (Figure 2) (Barrientos et al., 2008).

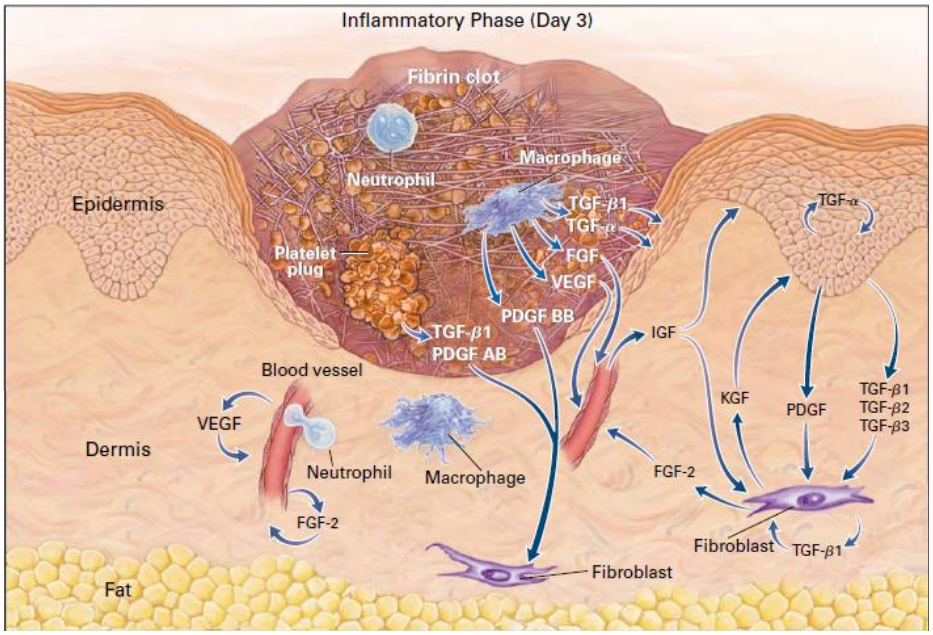


Figure 2. Inflammatory phase of a cutaneous wound: Growth factors implicated in cell migration into the wounded area are illustrated (Singer and Clark, 1999).

Adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and L-selectin are indispensable in the recruitment of leukocytes from the periphery to the site of inflammation. Thus, they are a fundamental component during tissue repair where the leukocytes, such as neutrophils and macrophages, infiltrate to the site of injury and form a first line defense against invading environmental pathogens. Moreover, these inflammatory cells secrete growth factors and cytokines which are needed for the initial fibroblast and keratinocyte activation and the removal of cell debris and foreign particles (Yukami et al., 2007).

The proteolytic degradation of extracellular matrix is considered to be an essential part of wound healing. Especially the concerted activity of family members of the matrix metalloproteinase (MMP) family and components of the plasminogen activation (PA) system are needed for the keratinocyte migration over the provisional matrix composed of the fibrin clot (Frossing et al., 2010). Therefore, the leading-edge keratinocytes express MMPs and plasminogen (Plg). These fibrinolytic enzymes enable the cells to cut their path through the fibrin clot during re-epithelialisation (Figure 3) (Barrientos et al., 2008).

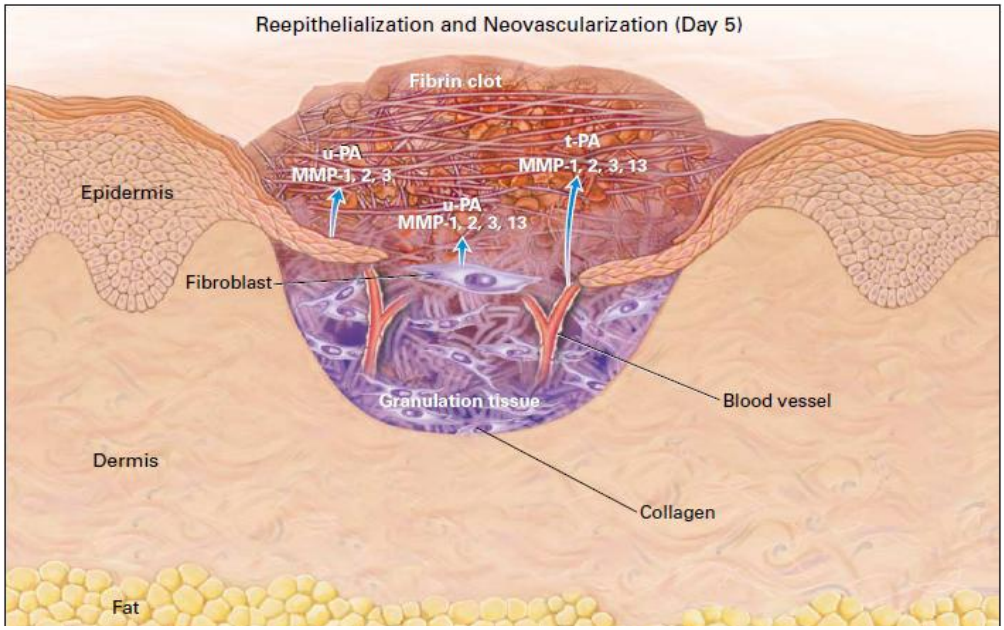


Figure 3. Re-epithelialisation and vascularisation of a cutaneous wound after injury. Proteinases suggested to be involved in cell movement are depicted (Singer and Clark, 1999)

The next section will provide more insight into the function and activation of some of these mentioned factors which were under investigation in the current work.

2.2. Transforming growth factor (TGF)-β family

The TGF-β family represents a superfamily of cytokines. In mammals the ligand members of the TGF-β superfamily are divided into two subfamilies: the TGF-β, Activin/Inhibin/Nodal and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/Muellerian inhibiting substance (MIS). They all share certain sequence and structural elements and they regulate diverse cellular processes, including cell proliferation, differentiation, apoptosis, bone morphogenesis and wound healing (Li and Verma, 2002; Shi and Massague, 2003). MIS and GDF are not expressed continuously, but only in a few cell types or for a limited period of time during development. In contrast, TGF-β1 and BMP appear prevalent during embryogenesis and in the adult organism (Massague et al., 2000).

2.2.1. TGF- β pathway

The TGF-β superfamily-initiated signal transduction is mediated through certain transmembrane receptor serine/threonine kinases termed as type I and type II receptors. The signal transduction begins with the ligand docking on the type II receptor which results in the formation of a heteromeric receptor complex of type I and type II receptor. Upon the ligand binding of type II receptor, it activates type I receptor through phosphorylation on serine and threonine sites in a highly conserved GS domain (SGSGSG sequence). Subsequently, receptor I transfers the signal by targeting family members of the Smad proteins (Massague et al., 2000; Wrana and Attisano, 2000), which encompass a family of transcription factors (Li et al., 2006).

There are three functional classes of Smad proteins with distinct functions: the receptor-regulated Smad (R-Smad: Smad 1, 2, 3, 5, 8), the Co-mediator Smad (Co-Smad: Smad 4) and the inhibitory Smad proteins (I-Smad: Smad 6, 7) (Shi and Massague, 2003). R-Smads are activated by the type I receptor kinases through phosphorylation of the two distal serines of the C-terminal SSXS motif (Derynck and Zhang, 2003). This subgroup of Smad proteins is important for the specificity of the biological response. Smad 2 and Smad 3 are induced by receptor docking of TGF-β and Activin. In contrast, Smad 1, 5 and 8 transmit the biological actions of BMP (Massague

et al., 2000; Wrana and Attisano, 2000) after the pathway is initiated by ligand docking to ALK1, ALK2 and ALK3. The activation of the Smad1/5/8 route seems to be cell type and differentiation stage specific (van der Kraan et al., 2009).

For interactions with DNA, Smad proteins need DNA binding cofactors, due to the fact that the affinity of Smads for their target DNA sequences is too low. The cell type dependent expression of these transcriptional cofactors contributes to a cell type specific response to the nuclear translocation of the Smad complex. Each subgroup of Smads has its own specific DNA binding cofactor (Massague et al., 2000). In addition, Smads are able to associate with either transcriptional co-repressors or co-activators. Transcriptional co-repressors act as negative regulators of Smad target genes and promote histone deacetylation (Figure 4). Co-activating factors are associated with histone acetylase transferase activity. Thus, dependent on the association partners of Smad proteins, transcription of target genes is either repressed or activated by chromatin remodeling effects (Massague et al., 2000).

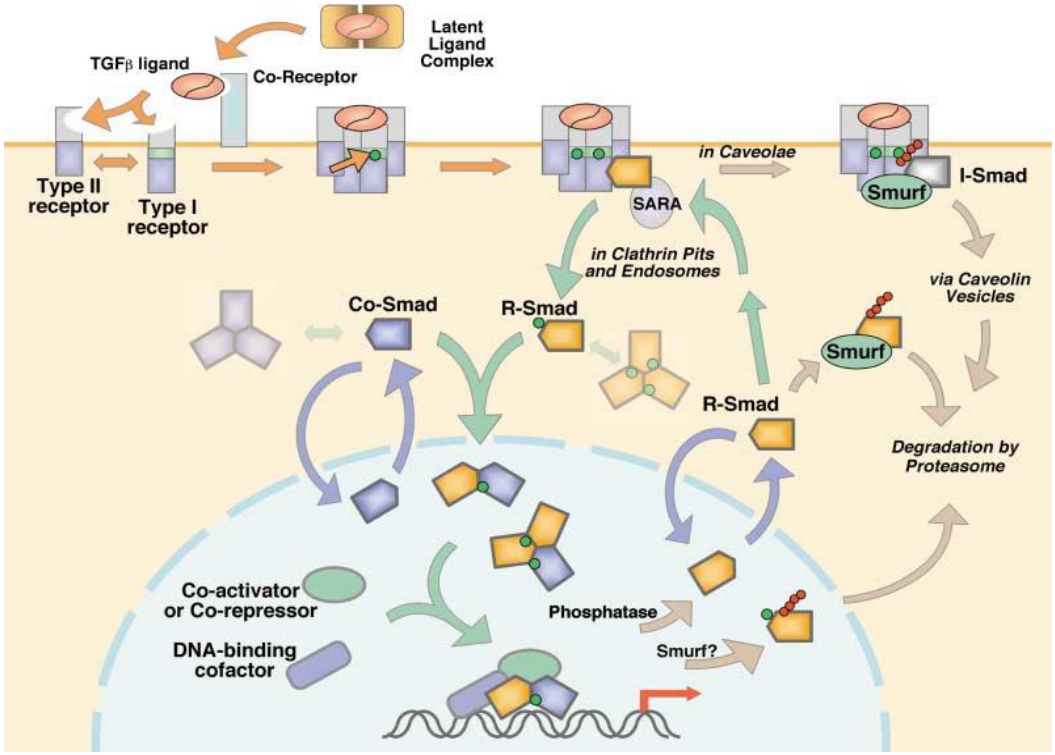


Figure 4. Schematic overview of the TGF-β signalling pathway (Shi and Massague, 2003).

During wound healing, TGF-β signalling is believed to be important for tissue remodeling and extracellular matrix deposition. Relevant sources during this physiological state are injured skin, macrophages, fibroblasts, endothelium and epithelia (Martinez-Ferrer et al., 2010). This protein is known to be involved in all three stages of wound repair. It acts as a chemotactic agent for infiltrating immune cells and stimulates the dermal collagen deposition by dermal fibroblasts as demonstrated by a transgenic mouse model (Martinez-Ferrer et al., 2010).

Especially the process of re-epithelialisation is a crucial step and TGF-β is known to influence the migration and proliferation of keratinocytes (Amendt et al., 2002). Because of conflictive study results, the distinct role of TGF-β during re-epithelialisation is still not fully understood. On the one hand, it inhibits keratinocyte proliferation in vitro and in vivo. For instance, Amendt et al. demonstrated that in mice lacking type II TGF-β receptor exclusively in keratinocytes, the re-epithelialisation process is accelerated (Amendt et al., 2002). Similar results could be gained by inhibiting the downstream element Smad-3 of the TGF-β signalling cascade in mice (Ashcroft et al., 1999) and in a conditional knock out of type II TGF-β receptor in dermal fibroblasts in mice (Martinez-Ferrer et al., 2010).

On the other hand, it could be shown that TGF-β enhances the expression of specific integrins, which results in a more sufficient migration of epidermal cells over the provisional wound matrix. However, it must be noted that the outcome of TGF-β signalling depends on the cell type and differentiation and in most studies it is not possible to distinguish between direct and indirect effects of TGF-β (Amendt et al., 2002). The antiproliferative effects of TGF-β are mediated by the activation of cyclin-dependent kinase (CDK) inhibitors, which inhibit a key player of cell cycle progression. It is known that TGF- β1 is a repressor of *c-myc* (Mukherjee et al., 2010) which leads to the activation of p15 and p21. These proteins are specific inhibitors of the early G1-phase of the cell cycle (Warner et al., 1999). *C-myc* encodes a transcription factor that is required for growth and proliferation. Overexpression of this gene is implicated in tumorigenesis due to uncontrolled DNA replication which leads to the accumulation of DNA damage within the cell (Dominguez-Sola et al., 2007).

2.3. Extracellular matrix (ECM) degrading enzymes

Targeted proteolysis is required during cell migration and invasion through tissue barriers. Hence, it is an essential part during wound healing and other physiological processes like morphogenesis and embryonic development, as well as pathophysiological processes including cancer invasion and metastasis (Juncker-Jensen and Lund, 2011) (Collen and Lijnen, 2004). These events are dependent on the coordinated and tightly controlled activity of the plasminogen activation (PA) system and members of the matrix metalloproteinase (MMPs) family (Frossing et al., 2010).

2.3.1. Plasminogen activation (PA) system

The plasminogen activation (PA) system is a temporally and in regard of activation highly regulated extracellular proteolytic cascade. Activation of this system is induced by the secretion of either tissue type plasminogen activator (tPA) or urokinase-like PA (uPA) from cells after stimulation by growth factors, cytokines or hormones. This release usually occurs during tissue remodeling, inflammation and thrombosis (Collen and Lijnen, 2004) (Collen and Lijnen, 2004). Sites for uPA and tPA pathway initiations are uPA receptor (uPAR) expressing cells and fibrin deposits, respectively. PAs then convert the inactive pro-enzyme plasminogen (Plg) into the trypsin-like serine protease plasmin. This is achieved by the concomitant binding of PAs and Plg to cell surfaces or fibrin (Lund et al., 2006).

Plasmin has a broad proteolytic spectrum. It degrades extracellular matrix proteins, growth factor precursors (Blasi, 1993) and specifically, it removes fibrin clots from the vasculature. A process known as fibrinolysis (Figure 5) which has to be tightly regulated (Dellas and Loskutoff, 2005). This is partly achieved by plasminogen activator inhibitor-1 (PAI-1) and by the physiologic inactivator of plasmin, α2-antiplasmin (Loskutoff and Quigley, 2000), whereas plasmin activation control is primary conducted by inhibiting PAs via PAI-1 (Dellas and Loskutoff, 2005).

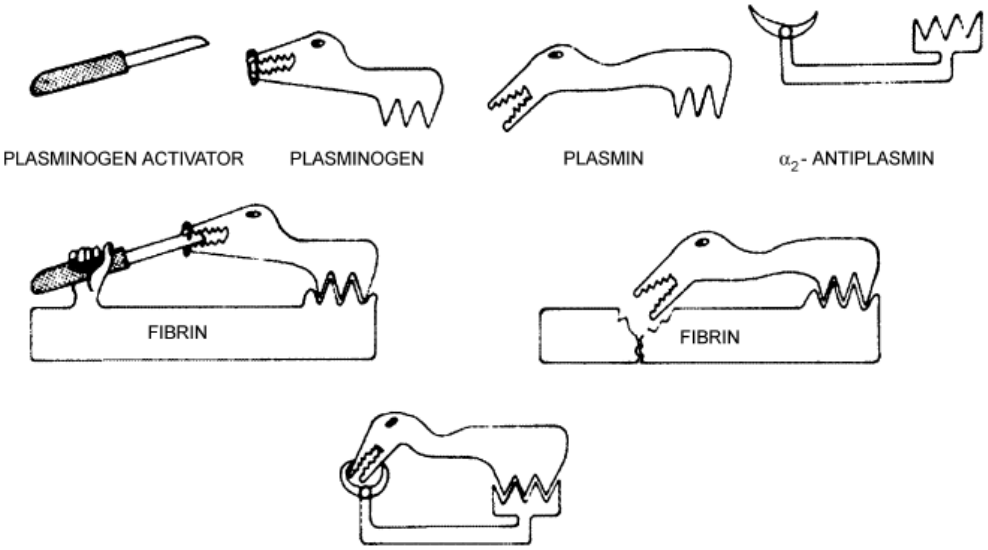


Figure 5. Schematic illustration of the activation of plasminogen and its inactivation: Plasminogen and plasminogen activator associate on fibrin where conversion of plasminogen into plasmin occurs. The legs of the animal represent lysine residues important for interactions with fibrin and α2-antiplasmin (Collen, 1982).

PAI-1 occurs in vitro as an active or latent state depending on the folding of the protein's reactive centre loop. The origin of circulating PAI-1 is less clarified. One important source may be the liver, where PAI-1 gene expression is increased during trauma, sepsis, stress and inflammation. Additionally, the vasculature and activated platelets release PAI-1 in order to inactivate tPA. Important regulators for PAI-1 are for instance cytokines such as tumor necrosis factor (TNF)-α and Interleukin-1 (IL-1) and grow factors like TGF-β which induces PAI-1 in many cell types (Slivka and Loskutoff, 1991)(Dellas and Loskutoff, 2005).

Injury leads to vasodilatation. Consequently, leakage of plasma proteins into the connective tissue leads to the activation of the coagulation cascade and fibrin is produced. As mentioned earlier, this fibrin matrix provides a road map to direct the infiltrating cells (Loskutoff and Quigley, 2000). For that, migrating leading edge keratinocytes express uPA and uPAR during re-epithelialisation in wound healing (Lund et al, 2006). Studies with Plg and PAI-1 deficient mice underlined the important role of proteolytic degradation of ECM during wound healing. It could be shown that wounds from animals lacking Plg have a significantly attenuated healing rate. In accordance to this, PAI-1 knock out results in accelerated wound healing. It is suggested that PAI-1 could influence wound healing by modulating the fibrinolytic environment at the

wounded site (Chan et al., 2001). It could be demonstrated that PAI-1 inhibits smooth muscle cell migration by blocking the binding of the integrin $\alpha_v\beta_3$ on the cellular surface to vitronectin (VN) (Stefansson and Lawrence, 1996). VN is an abundant circulating plasma protein which can be found in the ECM as part of the provisional matrix after tissue injury (Lopez-Guisa et al. 2011). Cells can attach to this ECM protein via integrins and uPAR (Czekay and Loskutoff, 2009). PAI-1 binds VN with high affinity as well. The binding sites on VN for uPAR and integrins are overlapping with the binding site of PAI-1. Thus, PAI-1 can block integrin- and uPAR-mediated cell attachment to VN. It is suggested that the presence of VN in a matrix or fibrin clot might enhance cell migration of smooth muscle cells and primary keratinocytes. Thus, the inhibitory effect of PAI-1 on cell migration is not dependent on the inactivation of PAs. The binding of PAs to PAI-1 results in loss of PAI-1 affinity to VN and restores cell migration (Stefansson and Lawrence, 1996). Moreover, PAI-1 can cause cell detachment from other ECM proteins which require the interaction with uPA and uPAR (Czekay and Loskutoff, 2009). The receptor localizes uPA on the cell surface of migrating cells. Additionally, this interaction causes a conformational change in the receptor resulting in the binding of matrix-associated integrins. Eventually, the addition of PAI-1 leads to the formation of a transient complex of these proteins which are then internalized. The endocytic clearance of the adhesion receptors contributes to the deadhesive property of PAI-1 (Czekay and Loskutoff, 2009) (Dellas and Loskutoff, 2005) (Czekay et al., 2003).

2.3.2 Matrixmetalloproteinases (MMPs)

The MMP family compromises a big family of zinc-dependent endo-peptidases that catalyze the degradation of ECM components and non-matrix proteins. They are involved in a plethora of physiological and pathophysiological processes such as embryonic development, tissue morphogenesis, wound healing, angiogenesis, inflammatory diseases and cancer.

The members share common structural and functional elements: an amino-terminal pro-peptide, a catalytic domain and a hemopexin-like domain. They are synthesized as inactive pro-enzymes (Sternlicht and Werb, 2001) (Massova et al., 1998) which are either membrane bound or secreted and subsequently processed and thus, activated (Yan and Boyd, 2007). The catalytic domain contains two zinc ions and 2-3 calcium ions. The pro-peptide domain features a conserved sequence harboring a cysteine which ligates the catalytic zinc ion (Nagase and Woessner, 1999). The removal of the pro-peptide via proteolysis results in the activation of the enzyme (Massova et al., 1998). Depending on the substrate specificity and localization, MMPs can be divided into gelatinases, stromelysines and membrane type MMPs (MT-MMPs).

The tight regulation of MMP activity is pivotal for normal tissue homeostasis. They must be activated (or inactivated) at the right time and must be located in the right cell type in an appropriate amount (Sternlicht and Werb, 2001). Therefore, these enzymes can be controlled at various levels including transcription, secretion, activation and inhibition. For instance, cell surface proteins such as the uPA/plasmin system and other plasma proteinases, as well as MT-MMPs are potent activators of pro-MMPs (Nagase and Woessner, 1999) (Chakraborti et al., 2003). Well known physiological inhibitors of the members of the MMP family are tissue inhibitors of metalloproteinases (TIMPS) (Yan and Boyd, 2007), inhibitors of metalloproteinases (IMPs) and α_2-macroglobulin which is a general proteinase inhibitor (Chakraborti et al., 2003).

Since MMP substrate specificities are partly overlapping, the unique function of an MMP family member is determined by different spatial and temporal expression patterns. With the exception of MMP-2 which is constitutively expressed (Sternlicht and Werb, 2001), normal tissue levels of MMPs are rather low. However, gene expression of MMPs is highly inducible by growth factors, cytokines, chemical agents, physical stress, oncogenic transformation (Nagase et al., 1999) or cell matrix contact (Parks, 1999). The promoters of MMP genes share common structural features that allow a co-regulation of their expression in some extent. These *cis*-elements allow the binding of various transcription factors including AP-1, PEA3, β-Catenin/Tcf-4 and NF-

κB. AP-1 and PEA3 are important for triggering signals from cytokines and growth factors such as TGF-β, PDGF, TNF-α, VEGF or EGF into the nucleus resulting in the *trans*-activation of MMP promoters. Enhanced induction of AP-1 and PEA3 is mostly accomplished by mitogen-activated protein kinases (MAPKs)-dependent phosphorylation. Beside transcriptional control, stabilization of MMP mRNA, translational regulation and epigenetic mechanisms such as DNA-methylation, histone acetylation and chromatin remodeling are implicated in monitoring MMP gene expression (Chakraborti et al., 2003) (Yan and Boyd, 2007).

During wound repair, a diverse set of MMPs are synthesized by multiple cell types in different compartments within the wound environment (Table 1) (Parks, 1999).

Table 1.: Expression of MMPs in different cell types during cutaneous wound repair (Parks, 1999)

		Cell type		
Enzyme	**MMP number**	**Keratinocytes**	**Dermal cells**	**Inflammatory cells**
Collagenase-1	MMP-1	+	+	+
Collagenase-2	MMP-8	–	–	+
Collagenase-3	MMP-13	–	+	–
Stromelysin-1	MMP-3	+	+	–
Stromelysin-2	MMP-10	+	–	–
Gelatinase-A	MMP-2	–	+	–
Gelatinase-B	MMP-9	+	–	+
Matrilysin	MMP-7	–	–	–
Macrophage metalloelastase	MMP-12	–	–	+
MT1-MMP	MMP-14	–	+	–

For instance, MMP-1 is expressed during the movement of keratinocytes across the provisional matrix as a consequence of integrin interactions on the cell surface with type I collagen (Saarialho-Kere et al., 1995). It was shown that MMP-2 mRNA levels are upregulated during wound healing in mice in the granulation tissue and dermal fibroblasts, as well as MMP-9. However, MMP-2 deficiency in these mice did not affect wound healing. Other wound healing studies in mice deficient for MMP-3, MMP-9, MMP-13 and MMP-14 demonstrated the same result. This could be due to redundant functions among MMPs in term of substrates and localization as well as the presence of other protease system like the PA system (Frossing et al. 2010) (Jensen and Lund, 2011). The latter assumption was strengthen by Lund *et al.* who observed that Plg knock out in mice treated with galardin, an inhibitor of a broad spectrum of MMPs, leads to a complete wound healing arrest (Lund et al., 1999). In addition to the breakdown of matrix barriers, Pilcher and co-workers claimed that MMP-1 contributes

to the right orientation of keratinocytes during re-epithelialisation on type I collagen by converting it into gelatin for which integrins of keratinocytes have a lower affinity. Consequently, the tight binding of integrins and type I collagen is loosened up and keratinocytes are allowed to move and are held in direction (Pilcher et al., 1997).

2.4. Vascular endothelial growth factor (VEGF)

The VEGF family currently encompasses five isoforms generated from one mRNA by alternative splicing: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PLGF). They transfer their biological functions via the binding to three distinguishable tyrosine kinase receptors, designated as VEGFR-1, VEGFR-2 and VEGFR-3, whereas the biological actions of VEGF-A and its receptors VEGFR-1 and VEGFR-2 are the best studied ones (Werner and Grose, 2002).

VEGF is an essential regulator of angiogenesis and vasculogenesis under physiological (e.g. embryogenesis and wound healing) and pathophysiological conditions (e.g. tumor growth and invasion). VEGF induction is achieved by a number of growth factors and cytokines. For instance, PDGF, FGF-4, TNF-α or TGF-β act as angiogenic factors via VEGF activation (Neufeld et al., 1999). Furthermore, hypoxia and nutrient stress are very potent stimulators of some VEGF family members. Hypoxia may promote VEGF transcription through the transcription factor hypoxia-inducible factor 1 (HIF-1). An additional mechanism is the stabilization of VEGF mRNA and via a cap-independent translation using the internal ribosome entry site (IRES) (Figure 6.) (Banerjee et al., 2007).

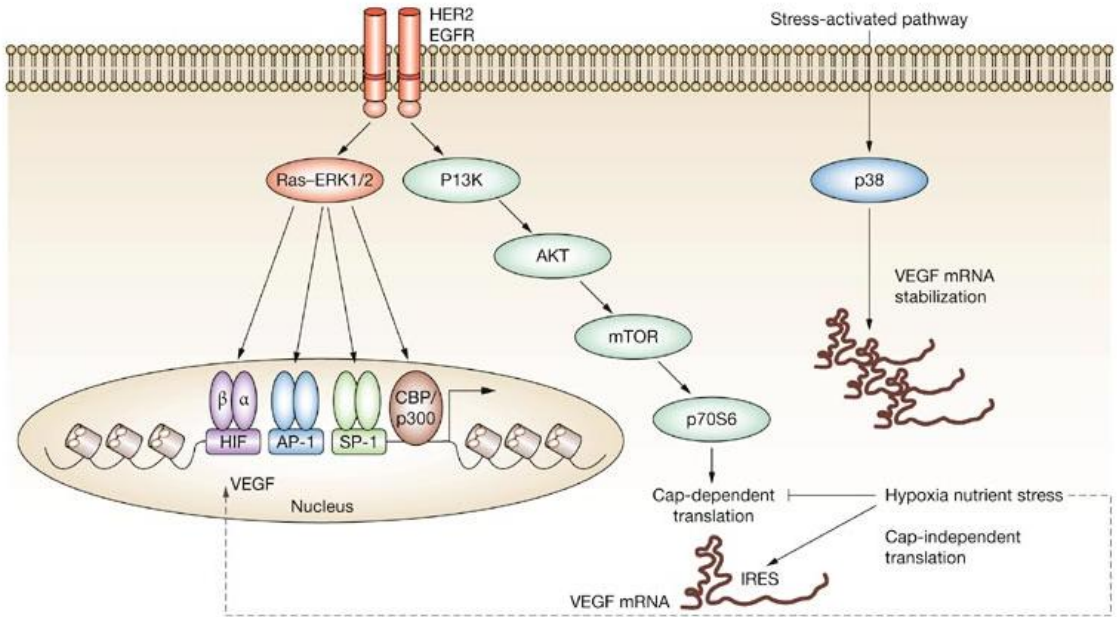


Figure 6. Pathways for VEGF stimulation: Growth factor signals initiate the phosphorylation of co-factors via MAPK-pathway and phosphatidylinositol 3´-Kinase/Akt signalling leading to VEGF transcription. Additionally, stress activated pathways stabilise the VEGF mRNA and mediate cap-independent translation of VEGF (Banarjee et al., 2007)

The finding of increased amounts of VEGF in skin wounds suggested a potential role for this protein during wound healing. Earlier studies accounted keratinocytes, fibroblasts and macrophages as the primary source of VEGF in wounds (Nissen et al., 1998). Also activated platelets, smooth muscle cells, neutrophils and endothelial cells contribute to VEGF release upon injury (Bao et al., 2009). Especially VEGF-A was shown to be an important angiogenic agent during wound healing as it was observed that the protein and its receptor are upregulated in an acute wound. Growth factors and hypoxia are very prominent in the wound environment. Hence, VEGF stimulation is secured (Barrientos et al., 2008) and is able to initiate angiogenesis by enhancing endothelial cell migration. This is mediated by promoting chemotaxis (e.g. stimulating the expression of uPA) and vasodilatation. Moreover, VEGF is known to be a mitogenic factor for endothelial cells (Bao et al., 2009). Eventually, the resulting angiogenesis recovers tissue perfusion (Barrientos et al., 2008).

2.5. Intercellular adhesion molecule-1 (ICAM-1)

Intercellular adhesion molecule-1 (ICAM-1) belongs to the Ig superfamily composed of five extracellular Ig-like domains, a transmembrane domain and a cytoplasmatic tail. The amount of the Ig-like domains can vary due to alternative mRNA splicing leading to the generation of different isoforms. It is a membrane glycoprotein and is an important factor during inflammation and immune responses as it acts as a co-stimulatory molecule during antigen presentation. Aside from this, ICAM-1 is highly upregulated during inflammation on endothelial cells as pro-inflammatory cytokines are released and mediates adhesion of leukocytes to the endothelium enabling them to migrate to sites of inflammation via interaction with leukocyte integrin lymphocyte function-associated antigen-1 (LFA-1) (Robledo et al., 2003) (Yang et al., 2005).

It is recognized that the later stages of wound repair depend on the initial inflammatory phase after an injury happens. Therefore, the recruitment of inflammatory cells from the circulation to the wounded site via the interactions of adhesion molecules on the immune cells and the endothelium is of great importance. The reason for this is, as stated earlier, that leukocytes like macrophages and neutrophils are crucial sources of growth factors and cytokines and are a defense system against invading pathogens in the wound environment. It was shown that, in mice lacking ICAM-1 expression wound healing, keratinocyte migration and formation of granulation tissue was inhibited. This effect was even more severe when LFA-1 was knocked out simultaneously. These observations were accompanied by decreased infiltration of neutrophils and macrophages which was suggested to be the cause of the disturbance in wound healing (Nagaoka et al., 2000) (Yukami et al., 2007).

2.7. Wound management and the role of antiseptics

A good quality of wound care is considered to be an essential part to facilitate healing of acute traumatic or chronic wounds. This is often associated with the prevention of wound contamination and infection (Atiyeh et al., 2009). The breakthrough of good wound care came in the 19th century with the discovery that bacteria are the cause of infection which led to the development of aseptic and antiseptic surgery (Leaper and Durani, 2008). Nowadays, it is well recognized that wound infection contributes to a delay of healing (Vermeulen et al., 2010). Open wounds, especially difficult-to-heal wounds like chronic leg ulcers or deep burns, are an attractive environment for bacterial colonization (Hirsch et al., 2009). Microbial contamination drives chronic inflammation and some bacterial strains like *Staphylococcus aureus* and *Pseudomonas aeruginosa* express very destructive virulence factors that interfere with the host's immune system and re-epithelialisation processes (Percival et al., 2010; Scales and Hufnagle, 2012). Therefore, most studies focus on the relationship of the facultative or aerobic bacteria like the afore mentioned ones. Nevertheless, anaerobic species form a significant population in wounds when considering that they are often within a hypoxic environment. These bacteria are not easy to isolate and cultivate and thus, are not often recognized in wound isolates (Bowler et al., 2001), but their metabolic products, like short chain fatty acids, are potent in inhibiting wound healing processes. For instance, butyrate was shown to inhibit neutrophil degranulation and lysozyme activity in vitro. In addition, these products inhibit re-epithelialisation via inducing cell cycle arrest in fibroblasts and keratinocytes (Wall et al., 2002). Furthermore, anaerobic bacteria express adhesion factors, tissue damaging exoenzymes, like collagenases and gelatinases, and anti-phagocytic factors (Percival et al., 2010).

Wound infection can occur when the microbial colonization reaches a critical level. However, recent findings showed that a correlation of exact microbial numbers (CFU/g of tissue) and wound healing does not exist. Nevertheless, wound colonization by microorganisms indeed influences wound healing as reasoned above. However, microbial numbers should not be interpreted alone when predicting wound infection.

In fact, the distinct bacteria species and their impulse to form biofilms should be considered as well (Percival et al., 2010).

One strategy to prevent and treat wound infections is the application of systemic antibiotics and topical antiseptics/antibiotics (Hirsch et al., 2009). However, the extensive use of antibiotics in the past has led to a dramatically increasing occurrence of resistant human pathogens which limits the therapeutic application of antibiotics. On the contrary, antiseptics that do not have a specific target, like antibiotics (e.g. cell wall biosynthesis or RNA transcription) (Andersson and Hughes, 2010), are unlikely to cause resistant pathogens.

Antiseptics are antimicrobial agents that kill or inhibit the growth of microorganisms in or on living tissues after topical administration (Hubner et al., 2010). They are applied on intact skin for hand washing by medical personnel, for prepping patients preoperatively or prior punctures. Additionally, these compounds are used as anti-infective prophylaxis for open wounds or to treat already infected wounds. Considering cytotoxic data, many authors disagree with the prophylactic treatment of open wounds using antiseptics, as their cytotoxicity may overweight the beneficial effects. However, if an acute wound should be inhibited to become chronic, the prevention of wound infection is the greatest concern. This is a present challenge of the National Health Service in order to secure life quality for the patients and to minimize hospital costs (Atiyeh et al., 2009). Even Alexander Fleming already stated in 1919 that the value of an antiseptic compound is rather measured by its effect on tissues than its antimicrobial actions (Hirsch et al, 2009).

The toxicity of local antiseptic agents is well assessed in cell culture studies. Since human tissue is less sensitive to antiseptic exposure, it tolerates it better than cells in tissue culture do. Due to this fact, the determined toxicity values should be considered to be rather a relative measure. Furthermore, cytotoxicity of an antiseptic agent also depends on the cell type used. For compounds which were shown to have harmful effects on tissue culture cells, a dose reduction might be considered without losing antimicrobial effectiveness. Of course, this has to be clarified in clinical studies (Muller and Kramer, 2008).

Thus, indications for local antiseptics should be carefully chosen (Marquardt et al., 2010). Sufficient data regarding toxicity, efficiency, tissue compatibility and contraindications are needed (Hubner et al., 2010). Many studies were performed in order to create new antiseptic substances. One result of these studies was the development of an alkanediylbis[pyridine] germicidal agent known as octenidine dihydrochloride (Stahl et al., 2010) (Sedlock and Bailey, 1985).

2.8. Octenidine dihydrochloride

The combination of 0,1 % octenidine dihydrochloride with 2 % phenoxyethanol (PH) [(Octenisept ® (OCT)] is an important antiseptic solution approved in several European countries as a medical substance (Kramer et al., 2008). It can be either applied on skin or for the antisepsis of mucous membranes (Hubner et al., 2010) and it is recommended for the treatment of acute wounds (Kramer et al., 2004). Its molecular weight is approximately 624 kDa, stability is given in pH ranges from 1,6 to 12,2 and its activity is not reduced upon contact with mucin or albumin.

Chemically, octenidine is a cationic surface active substance which has 2 non-interacting cationic centers within its molecule that are separated by a long aliphatic carbohydrate chain (Figure 7). Consequently, it binds onto negatively charged surfaces like bacterial cell envelopes and eukaryotic cell membranes (Hubner et al., 2010). Like for other cationic antiseptics, the main binding partners are assumed to be salts of fatty acids glycerol phosphates, which are found in the cell membranes (Gilbert and Moore, 2005) (Kramer et al., 2008).

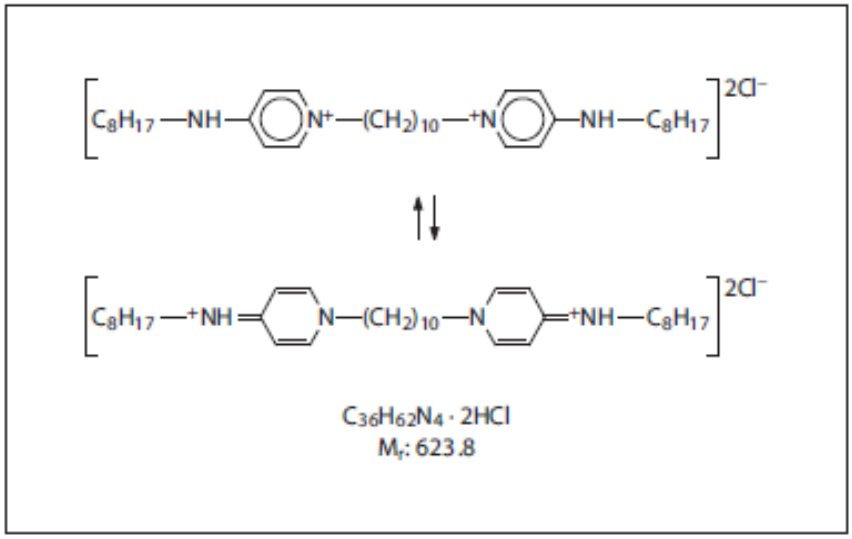


Figure 7. Chemical structure of octenidine dihydrochloride (Hubner et al., 2010)

Due to this strong and unspecific interaction with cell wall and cell membrane components, octenidine is effective against a wide range of microorganisms (Muller and Kramer, 2007).

It acts against gram-positive and gram-negative bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA) (Hubner et al., 2010), plaque forming bacteria (Slee and

O'Connor, 1983) and fungi (Sedlock and Bailey, 1985). For instance, OCT was shown to be superior to other commonly used antiseptics including triclosane, PVP- iodine and Chlorhexidine digluconate regarding the antimicrobial efficiency against *Candida albicans, Staphylococcus aureus and Pseudomonas aeroginosa*. Moreover, sufficient bacterial reduction could be achieved after 1 minute contact time. This short time efficacy is especially important, as antiseptics are often applied on skin and mucous membranes prior to invasive procedures (Koburger et al., 2010).

In clinical practise, antibiotics and antiseptics are commonly used in combination. Thus, possible interactions between these two anti-infective classes are clinically of special interest. First in vitro studies indicate that octenidine in combination with systemic antibiotics like Imipenem and Piperacillin + tazobactam could be synergistic in their antimicrobial efficiency against *Enterococcus sp.*, *P. aeruginosa* and *Escherichia coli*. However, this interesting finding needs further investigation (Hubner et al., 2010).

Beside a broad antimicrobial spectrum with rapid onset, antiseptic preparations should also have good tissue compatibility (Muller and Kramer, 2008). In vitro studies from cell culture showed that OCT has a rather high cytotoxic effect on human amnion cells, human primary fibroblasts and human primary keratinocytes (Kramer et al., 2004) (Hirsch et al. 2009). Furthermore, it was observed that OCT treatment leads to wound healing retardation in pigs in vitro (Kramer et al., 2004). In contrast, a few years later, Kramer and Muller purported that OCT and polyhexanide have the highest biocompatibility index. This means that these compounds are more effective against microorganisms than to cultured fibroblasts (Muller and Kramer, 2008). In accordance with this, Stahl et al. reported that treatment with OCT did not negatively influence the re-epithelialisation of wounded porcine skin explants (Stahl et al., 2010). Additionally, it was observed that it causes the slightest effect on microcirculation in comparison to other antiseptic agents. This is of great importance, since insufficient nutritional supply via the microcirculation of the skin may lead to inadequate wound healing and skin necrosis (Langer et al., 2004). Recently, a randomized double-blind controlled study with chronic leg ulcers patient could confirm the last mentioned results. In this study a

high tissue compatibility and tolerability was shown for OCT treatment over a 12-week period with several applications a week (Vanscheidt et al., 2012).

The discrepancy between the high cytotoxic effects in in vitro studies and the favorable clinical results might have several reasons. Firstly, most in vitro studies are performed under sterile conditions. Due to this fact, OCT was found to be inferior to Ringer solution which would not be the case in septic wounds (Kramer et al., 2004). Secondly, human tissue tolerates the exposure to antiseptic compounds better than tissue culture cells (Muller and Kramer, 2008). The reason for this is that in a clinical setting, the topically applied substance will first get in contact with fibrin, blood and debris. Harmful effects on this layer are tolerated as long as there is no damage to the deeper, more sensitive structures (Marquardt et al., 2010). Last but not least, it could be observed that octenidine dihydrochloride is able to adhere to cell membrane components. In these stable complexes, the cytotoxicity of octenidine is decreased, whereas the antiseptic efficiency is unchanged (Muller and Kramer, 2007). In summary, OCT is a promising antiseptic agent for topical skin and wound treatment (Stahl et al., 2010) which is also suitable for chronic wound management (Vanscheidt et al., 2012).

3 Materials and Methods

3.1 Cell culture

3.1.1 Used materials, devices and reagents

Laminar Flow Hood Biosafe 1	Ehret
Incubator Queue	Sanova
Microscope	Nikon TMS
Water bath Thermo-Boy	MGW Lauda
Drier	Binder
Cell culture flasks	Greiner
6-well microtiter plate	Greiner
12-well microtiter plate	Greiner
96-well microtiter plate	Greiner
Sterile filter (0,2 µm pore diameter)	Sartorius
RPMI 1640	Biochrom AG
DMEM	BioWhittaker, Lonza
FCS	GIBCO
L-Glutamine (200 mM in 0,85 % NaCl)	BioWhittaker, Lonza
PEN-STREP (10 000 U/ml)	BioWhittaker, Lonza
Trypsin/EDTA	PAA
Ethanol 70 %	
5 x PBS, pH 7,4; 1000 ml:	
• 40 g NaCl	Roth
• 14,5 g $Na_2HPO_4 \cdot 12\ H_2O$	MERCK
• 1 g KH_2PO_4	MERCK
• 1 g KCl	MERCK
• 1 M NaOH for adjustment of the pH value	MERCK
• filled up with aqua dest.	
Glucose 2,5 % (sterile filtrated)	Fresenius Kabi
Octenisept® (0,1 g Octenidine in 100 ml aqueous solution)	Schülke & Mayr

Chlorhexidine (1 g Chlorhexidine in 500 ml aqueous solution)	Raphael Donner Apotheke
Taurolin® (0,5 g Taurolidin in 100 ml aquous solution)	Chemomedica
Beta-Isodona® (10 g Povidin-Iodine complex in 100 ml aqueous solution)	Mundipharma
LPS from *Escherichia coli*	Sigma

3.1.2 Sterile working

All cell culture procedures were conducted in sterile laminar-airflow hood, which was disinfected with ultraviolet light over night. Only sterile or sterile filtrated solutions were used, to avoid microbial cell contamination. Furthermore, pipette tips were autoclaved at 120 °C for 30 minutes prior utilization. Reusable glasswares were sterilized at 180 °C over night. Last but not least all other materials (boxes for pipette tips, flasks, hands, etc.) were cleaned with 70% ethanol.

3.1.3 Cell lines

3.1.3.1 MCF-7 cells

The MCF-7 breast cancer cell line was developed by Dr. Herbert Soule at the Michigan Cancer Foundation and was taken from a pleural effusion of a metastatic breast cancer patient in 1970 (Levenson and Jordan, 1997). These cells express the estrogen-receptor (ER) on their surface and thus, are the first hormone-sensitive breast cancer cell line. Generally, MCF-7 cells are the longest studied breast cancer model systems (Simstein et al., 2003) and retain several characteristics of mammary epithelium (Lloyd et al., 1996).

3.1.3.2 Fibroblasts

Fibroblast used in this work were a gift from Johannes Berger (MUW, Center for Brain research), and were taken from a healthy male adult.

3.1.4 Preparation of the cell culture medium for MCF-7 cells and fibroblasts

MCF-7 cells were either grown in complemented Dublecco's modified Eagle Medium (C-DMEM) or complemented Roswell Park memorial institute (C-RPMI) medium. For the preparation of complemented media, 50 ml fetal calf serum (FCS), 5 ml 200mM glutamine and 5 ml 10 000 U/L Penicillin-Streptomycin (PEN-STREP) were added to 500 ml culture medium. FCS and glutamine serves as nutritional factors for cell growth whereas PEN-STREP inhibits unwanted bacterial growth in the medium. Additionally, an uncomplemented DMEM was set up by only administrating 5 ml 10 000 U/L PEN-STREP. The media are stored at 4 °C and heated up at 37 °C prior to usage.

3.1.5 Cultivation of MCF-7 cells and fibroblasts

In order to start a culture, cells, which were stored at -80 °C, were thawed and quickly transferred to 5 ml warm culture medium in a 25 cm^2 cell culture flask. After 12 to 24 hours the medium was changed to remove the remaining DMSO. Generally, the cells were grown at 37 °C, 5 % CO_2 atmosphere until they reached confluency (= ~ 90 % confluent) or otherwise the desired density.

3.1.6 Passaging (subculturing) MCF-7 cells and fibroblasts

The cell density was monitored under the microscope. At the confluent stadium, cells were split and diluted to ensure sufficient space and nutrition for further proliferation. Since MCF-7 and fibroblasts cells stick to the culture flask's surface (also called adherent cells), they first needed to be loosening up. Therefore the cell culture medium was aspirated. Afterwards 1,5 ml for 25 cm^2 cell culture flasks or 4 ml for 75 cm^2 cell culture flasks of warm Trypsin-EDTA were added and incubated at 37 °C for several minutes. Trypsin is a serine-protease and hydrolyzes the bonds between cells and the culture flask. This process can be watched under a light microscope. After the incubation time, the Trypsin was aspirated and the cell culture flask was gently tapped to detach the cells from the surface of the flask. Then the flask was washed several times usually with 5 ml (for 25 cm^2) or 10 ml (for 75 cm^2) of fresh culture medium in order to suspend the cells in the medium. The suspension was transferred into a

desired volume (depending on the desired dilution factor of the new subculture) of fresh medium, gently mixed and seeded into a new cell culture flask.

3.1.7 Freezing MCF-7 cells and fibroblasts

Freezing cells started with the same steps as passaging until trypsinisation. Afterwards the loosened cells were taken up with 2 ml cold, uncomplemented DMEM and 0,5 ml of the cell suspension was added to the 0,5 ml prepared ice cold cryo-solution (complemented medium: FCS: DMSO = 5:3:2). Subsequently, the cells were put into a styrofoam box and frozen at -80 °C. For long-term storage, cryovials were transferred in liquid nitrogen.

3.1.8 Cell seeding

MCF-7 cells were seeded when they reached 80 % confluency and fibroblasts at a confluent stadium. Depending on the experiment performed, cells were either seeded into a 96-well, 12-well or 6-well microtiter plate. For the first one 100 µl cell suspension per well were needed, for the 12-well 1 ml and for the latter one 2 ml.

3.1.9 Incubation

Incubation of MCF-7 cells and fibroblasts with the antiseptic agents was performed when they reached the desired density.

For assessing cell adhesion, incubation solutions were pipetted directly into the cell culture medium in the well, unless it is otherwise stated (see section 3.5. MTT-assay). Cells were incubated with different solutions in several dilutions of Octenisept ® - either diluted 1:2 in sterile distilled water (O) or sterile 5% glucose solution (O+), Chlorhexidine, Taurolidin® and Beta-Isodona®.

3.2 Cell lysation

3.2.1 Used materials, devices and reagents

Ice

5 x PBS, pH 7,4; 1000 ml:

- 40 g NaCl — Roth
- 14,5 g $Na_2HPO_4 \cdot 12\ H_2O$ — MERCK
- 1 g KH_2PO_4 — MERCK
- 1 g KCl — MERCK
- 1 M NaOH for adjustment of the pH value — MERCK
- filled up with aqua dest.

peqGOLD TriFast™ — PEQLAB

3.2.2 Cell lysation

After the incubation time ended, the cells were immediately placed on ice to stop all ongoing metabolic events. The upcoming working steps were no longer performed under a laminar airflow hood, but at the laboratory bench. The cell culture medium was aspirated and the cells were washed twice with 2 ml/well of ice cold 1 x PBS. Then the cells were incubated with 0,5 ml/well peqGOLD TriFast™ for 5 to 10 minutes at room temperature. Finally, the cells were scratched with a sterile pipette tip from the well's surface and transferred into Eppendorf-tubes. The cell lysate could be stored at -20 °C.

3.3 RNA Isolation and reverse transcription

3.3.1 Used materials, devices and reagents

Centrifuge	Eppendorf 5417R
Mini centrifuge	ROTH
Thermo Cycler	PEQLAB Primus 25 advanced
peqGOLD TriFast™	PEQLAB
Phase lock gel heavy	5 Prime
DEPC	Sigma
DEPC-water RNasefree • 100 µl DEPC • diluted in 1 litre aqua dest. for 3 h under the hood • 2 h autoclaved at 120 °C	
DEPC-pipette tips, -reactiontubes RNasefree • soak in DEPC-water over night • autoclaving at 120 °C for 2 h; dry at 70 °C	
Chloroform	MERCK
Isopropanol	MERCK
Ethanol 75 % in DEPC water	MERCK
Ethanol 70 %	
Random hexamer primer 0,2 µg/µl	Fermentas
Reaction Buffer (5x)	Fermentas
dNTP Mix 10mM; 100 µl • 5 µl dATP, dGTP, dCTP, dTTP 100 mM each • diluted in 80 µl sterile filtrated aqua dest.	Fermentas
RiboLock™ RNase inhibitor 40 U/µl	Fermentas
RevertAid™ M-MuLV reverse transcriptase 200 U/µl	Fermentas

3.3.2 RNA isolation

Before starting RNA isolation, all surfaces in use were cleaned with 70% Ethanol. The cells were lysed and thawed under the mini lamina at room temperature and the

centrifuge was cooled down up to 4 °C. In the meanwhile the phase lock gel tubes were spinned down 20 to 30 seconds in the mini centrifuge. Then 250 µl of cold chloroform and 500 µl of the cell lysate were pipetted into the tubes, which were afterwards thoroughly shaken in order to form a transiently homogenous suspension of the aqueous sample phase and the organic chloroform phase in order to maximize the diffusion area. The samples were centrifuged (5 minutes, 16 000 g, 4 °C), which lead to a separation of the protein-containing organic phase, the DNA enriched interphase and the RNA harboring aqueous phase. The following procedures were exclusively performed on ice. After that, the upper aqueous phase was added to 250 µl of cold isopropanol, prepared in new DEPC-treated reaction tubes during sample centrifugation. The tubes were stored 24 h at -20 °C. During this time, RNA precipitation took place.

Then the tubes were centrifuged at 4 °C, 12 000 g for 10 minutes. This step usually leads to the retrieval of a RNA pellet. The pellet was washed with 1 ml 75 % cold ethanol. For that, the isopropanol was discarded, the ethanol was added and the afore vortexed sample was centrifuged at 4 °C, 12 000 g for 10 minutes to recollect the RNA pellet. This step was repeated another time. Finally, the ethanol was removed as much as possible and the pellet was air-dried under the hood.

3.3.3 Reverse transcription (RT)/ cDNA synthesis

The dry RNA pellet was resuspended in 14 µl DEPC water and dissolved at 55 °C in the water bath for 10 minutes. 12 µl of the dissolved RNA sample was transferred into PCR tubes and 1 µl random Hexamer primer (2µg/µl) was added. Afterwards, the tubes were incubated for 10 minutes at 70 °C in a Thermocycler. Meanwhile, a mastermix for the following cDNA synthesis was prepared:

Table 2. Pipetting scheme for the preparation of the mastermix used for cDNA synthesis.

Reagent	Conc. of stock solution	Volume	Endconcentration
RT Buffer	5x	4 µl	1x
dNTPs	100mM	2 µl	10mM
RNase Inhibitor	40 U/µl	0,5 µl	1 U/µl
Reverse Transcriptase	200 U/µl	0,5 µl	5 U/µl

7,5 µl of the mastermix were then mixed with the sample, which were incubated for 1 h at 37 °C and afterwards 10 minutes at 60 °C in a thermo cycler. The newly synthesized cDNA was stored at -20 °C.

3.4 Polymerase chain reaction (PCR)

3.4.1 Used materials, devices and reagents

Thermocycler	PEQLAB primus 25 advanced
Electrophoresis chamber	Bio-Rad
Power supply	Bio-Rad
Microwave	Moulinex
Sterile filter (0,2 µm pore diameter)	Sartorius
Sterile filtrated aqua dest.	
Taq buffer + KCl 10x	Fermentas
$MgCl_2$ 25 mM	Fermentas
dNTP mix 10 mM; 100 µl	
• 5 µl dATP, dGTP, dCTP, dTTP each 100 mM	Fermentas
• diluted in 80 µl aqua dest. sterile filtrated	
Taq polymerase recombinant 1 U/µl	Fermentas
Taq polymerase recombinant 5 U/µl	Fermentas
Gene specific Primer 100 µM	VBC-Genomics
• primerdesign via PRIMER 3 SOFTWARE	
• diluted 1 :10 in sterile filtrated aqua dest.	
Agarose powder	MERCK
Ethidium bromide	MERCK
Gene Ruler™ 1kb DNA ladder 0,5 µg/µl	Fermentas
5 x TBE Buffer; 1000 ml :	
• 54 g tris(hydroxymethyl)aminomethane	MERCK
• 27,5 g boric acid H_3BO_3	MERCK
• 20 ml 0,5 M EDTA solution	
0,5 M EDTA solution; pH 8, 500 ml:	
• 93 g titriplex	MERCK
• dissolved in 400 ml Aqua dest.	
• 10 N NaOH for adjustment of the pH value	MERCK
• filled up with Aqua dest.	

6 x loading dye	Fermentas
2 UV transilluminator	UVP
ChemiImager^{TM} 4400	Biozym
AlphaEaseFC-ChemiImager 4400	Alpha Innotec

3.4.2 Procedure

The polymerase chain reaction serves as a method for sequence-specific exponentially amplification of a DNA or cDNA section. For that, all reagents, which were stored at -20 °C, were thawed on ice and thoroughly mixed. This was followed by the preparation of a mastermix:

Table 3. Pipetting scheme for the preparation of the mastermix used for PCR.

Reagent	Conc. of stock solution	Volume	Endconcentration
Aqua dest. steril filtrated		31 µl	
Buffer	10 x	5 µl	1 x
$MgCl_2$	25 mM	5 µl	2,5 mM
dNTPs	10 mM	4 µl	0,8 mM
Primer	10 µM		
(forward/reverse)		1,5 µl each	0,3 µl each
Taq polymerase	1 U/µl or 5 U/µl	1 µl or 0,2 µl	0,02 U/µl

1 µl of the cDNA sample was mixed with 49 µl mastermix in a PCR tube, vortexed and incubated in a thermo cycler. During this process, the machine runs through a defined cyclic temperature program, which included the following steps:

1. Initial denaturation: the DNA doublestrand is separated by heat at 94 °C (3 minutes).
2. Denaturation at 94 °C (40 seconds).
3. Annealing: the temperature is lowered to a primer-specific temperature (Table 4) in order to allow their hybridisation to the single stranded DNA template. Primers mark the starting point of the newly synthesized DNA strand, which is complementary to the template strand (60 seconds).

4. Elongation: in this step the temperature is raised on the temperature optimum of the Taq polymerase (72 °C) and a second DNA strand, complementary to the template strand, is synthesized by the enzyme. Thereby, the primers determine the starting point of this process (60 seconds)

5. Final elongation at 72 °C. (5 minutes)

6. Storage at 4 °C.

The so amplified cDNA fragments were then separated by size and identified using an agarose gel electrophoresis. For that, a 1,5 % agarose gel was prepared by mixing 0,45 g agarose powder with 30 ml 1 x TBE buffer in a glass beaker. Then the mixture was boiled in the microwave until the solution was clear. After the viscous mass was cooled down at approximately 60 °C, 0,5 mg/ml ethidium bromide was added. Ethidium bromide is able to fluoresce in ultra-violet light when it intercalates into the DNA. Therefore, this substance facilitates the visualization of the separated DNA fragments in an agarose gel. The gel was poured into a gel sled with inserted 8-well comb, which was removed after the agarose was solidified. The gel on the sled was then transferred into an electrophoresis chamber. The chamber was filled with 1 x TBE buffer. The construct was now ready for the loading of the PCR-samples.

The samples were mixed with the 6 x loading dye in a 1:6 ratio (15 µl sample plus 3 µl loading dye) and then pipetted into the slots of the gel. A DNA-ladder served as a size marker and was applied into the gel slots as well (2,5 µl). The DNA was separated with constant 100 V and 50-60 mA for 20 minutes. Finally, the DNA bands were visualized under ultraviolet light in a transilluminator and photographed. The intensity of the signal was analyzed with AlphaEaseFC Chemilmager 4400 software.

Table 4. Overview of the nucletoidesequences of the forward and reverse primer of the corresponding amplified genes. Additionally, the specific annealing temperature (°C AT), the optimal number of cycles (cyc.) and the size of the PCR product (bp) are depicted.

Gene	Nucleotidesequenz: Forward Primer Reverse Primer	°C AT	cyc .	bp
GAPDH	5'-GGA GCC AAA AGG GTC ATC ATC TC-3' 5'-GTC ATG AGT CCT TCC ACG ATA CC-3'	62	23	185
PAI-1	5'-TGC TGG TGA ATG CCC TCT ACT-3' 5'-CGG TCA TTC CCA GGT TCT CTA-3'	61	30	399
uPA	5'-CCA AGG AGG GCA GGT GTG CG-3' 5'-CGG GTG GTG CCC GTT TCC TC-3'	67	30	480
ICAM-1	5'-CGT GTC CTG TAT GGC CCC CG -3' 5'-GGG AGG CGT GGC TTG TGT GT -3'	70	30	426
MMP-1	5'-AAG GTT AGC TTA CTG TCA CAC GCT T -3' 5'-GTG CTG AAG GAC ACA CTA AAG AAG A -3'	61,4	30	787
MMP-2	5`-GTG CTG AAG GAC ACA CTA AAG AAG A-3` 5`- TTG CCA TCC TTC TCA AAG TTG TAG G-3`	61	30	605
MMP-3	5'-GAA ATG CAG AAG TTC CTT GG-3' 5'-GTG AAA GAG ACC CAG GGA GTG-3'	60	30	489
MMP-9	5'-ATT CAG GGA GAC GCC CAT TT-3' 5'-GTG CAG GCG GAG TAG GAT TG-3'	63	30	317
TGF-β1	5'-GCT GCA CTT GCA GGA GCG CAC -3' 5'-GGA CTC AGC TCT GGT TGG TG -3'	65,8	32	336
TGF-β RI	5'-GGG GCC ATG TAC CTT TTT GT-3' 5'-ACG GAG TTG GGG AAA CAT ACT-3'	61	30	325
VEGF-A	5'-GGA CAT CTT CCA GGA GTA-3' 5'-TGC AAC GCG AGT CTG TGT -3'	60	30	413

3.5 MTT- assay

3.5.1 Used materials, devices and reagents

Microplate reader	Bio-Rad
Microscope	Nikon TMS
96-well microtiter plate	Greiner
MTT (3-3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	Invitrogen
DMSO	MERCK
5 x PBS, pH 7,4; 1000 ml:	
• 40 g NaCl	Roth
• 14,5 g $Na_2HPO_4 \cdot 12\ H_2O$	MERCK
• 1 g KH_2PO_4	MERCK
• 1 g KCl	MERCK
• 1 M NaOH for adjustment of the pH value	MERCK
• fill up with aqua dest.	
Prism.3.02	GraphPad

3.5.2 Procedure

The MTT-cell proliferation assay is a method for detecting cell survival and proliferation within cell population after incubation with certain substances. The basis of this test is the reduction of the yellow MTT (3-3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple formazan product in living cells (Figure 8) (Mosmann, 1983).

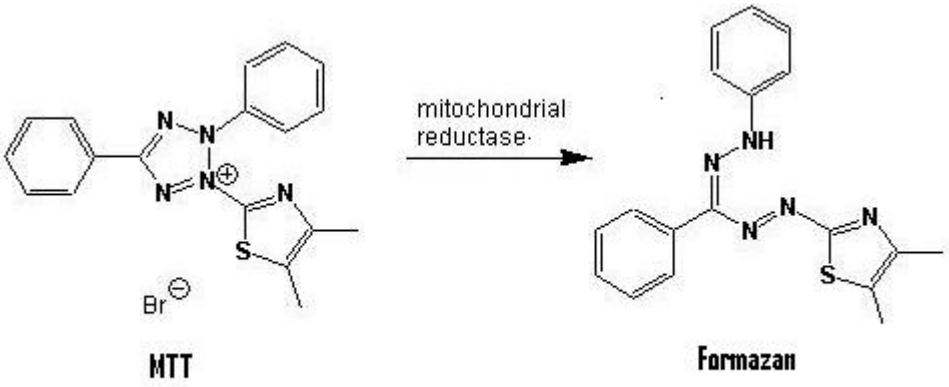


Figure 8. Formation of the purple formazan product by mitochondrial reductase of viable cells (Mosmann, 1983).

Depending on the aim of the tests, two different procedures were performed:

(1) Assessment of cell proliferation: A 96-well microtiter plate was incubated with various dilutions of O, O+ or chondroitin sulfate (CHS) at 37 °C for 1 h. During this time, the chemical agents were allowed to adsorb on the well surface. This was followed by washing twice with sterile 1x PBS and drying over night at 37 °C. On the next day cells were seeded into the pre-incubated 96-well plate and the proliferation rate was determined after 24 h using the MTT-assay for which the procedure is described below.

(2) Assessment of cell adhesion: Cells were seeded into a 96-well plate and placed into the incubator at 37 °C until they reach ~80 % confluency. Afterwards they were incubated for 30 minutes with several dilutions of the antiseptic agent. Then the cells were washed twice with 150 µl warm and fresh culture medium. For the MTT-assay, 5 mg MTT were dissolved in 1 ml sterile filtrated 1 x PBS, which served as a MTT stock solution. From this, a 10 % MTT solution in complemented DMEM was prepared and 100 µl were added to each well. The plate was then incubated for one and a half hour at 37 °C. The incorporation process of the purple formazan product into living cells could be monitored under the microscope. The incubation period was followed by two washing steps with 150 µl warm 1 x PBS. The non-soluble formazan was dissolved in 50 µl DMSO and the extinction was measured at 595 nm. The analysis of the data was achieved with “Prism3.20”. The proliferation rate was estimated by the quotient of the extinction value of the treatment cells and the extinction value of the reference cells.

3.6 Scratch assay/ Migration test

3.6.1 Used materials, devices and reagents

Microscope	Zeiss Axiovert 135
6-well microtiter plate	Greiner
12-well microtiter plate	Greiner
RPMI 1640	Biochrom AG
FCS	GIBCO
L-Glutamine (200 mM in 0,85 % NaCl)	BioWhittaker, Lonza
PEN-STREP (10 000 U/ml)	BioWhittaker, Lonza
Glucose 2,5 % (sterile filtrated)	Fresenius Kabi
Octenisept® (0,1 g Octenidine in 100 ml aqueous solution)	Schülke & Mayr
Chlorhexidine (1 g Chlorhexidine in 500 ml aqueous solution)	Raphael Donner Apotheke
Taurolin® (0,5 g Taurolidin in 100 ml aquous solution)	Chemomedica
Beta-Isodona® (10 g Povidin-Iodine complex in 100 ml aqueous solution)	Mundipharma

3.6.2. Procedure

Cells were seeded evenly into 6-well or 12-well microtiter plates and grown in complemented medium until the reach almost confluency (fibroblasts 4 days, MCF-7 cells 2 days). Then the cells were carefully scratched in the middle of the well surface with a yellow pipette tip and the culture medium was replaced with fresh one. This was followed by taking a 12-bit picture with a spot camera marking the time point “0h”. Afterwards the cells were treated with the different antiseptic agents under investigation for 2 minutes (Table 5). Subsequently, each well was washed once with fresh culture medium and the microtiter plates are further incubated at 37 °C to assess the cell migration for up to 48 h.

Table 5. Illustration of the applied concentrations of the antiseptic agents in the scratch assay: Each compound was diluted 1:75 or 1:30 in 2 ml of the cell culture medium.

Compound	**Endconcentration (1:75/1:30)**
Octenisept	7/17 mg/L
Octeniplus	7/17 mg/L
Chlorhexidine	26/67 mg/L
Taurolin	67/ 167 mg/L
Betaisodona	1,3/ 3 g/L

3.7 Cooperation testing of antiseptics and antibiotics

3.7.1 Used materials, devices and reagents

Laminar Flow Hood Biosafe 1	Ehret
Incubator	Binder
Petri Dish	Sterilin
0,9 % NaCl solution, 2000 ml	
• 18 g NaCl	ROTH
• filled up with aqua dest.	
Trypticase Soy Broth, 1000 ml; autoclaved	
• 30 g Trypticase Soy Broth	
• 5 g Yeast Extract	
• 15 g Agar	
• filled up with aqua dest.	
CASO-Agar ready for use; 1000 ml; autoclaved	ROTH
• 5g Peptone from Casein	
• 5g Peptone from Soy	
• 5g NaCl	
• 15g Agar	
• filled up with aqua dest.	
Ethanol 70 %	ROTH
Glucose 5 %	Fresenius Kabi
Octenisept® (0,1 g Octenidine in 100 ml aqueous solution)	Schülke & Mayr
Chlorhexidine (1 g Chlorhexidine in 500 ml aqueous solution)	Raphael Donner Apotheke
Taurolin® (0,5 g Taurolidin in 100 ml aquous solution)	Chemomedica
Beta-Isodona® (10 g Povidin-Iodine complex in 100 ml aqueous solution)	Mundipharma
Antibiotic filter discs (table 6)	Mast Diagnostica

Table 6. Overview of the used antibiotics. The concentration of each compound is indicated as mg per antibiotic paper disc. Moreover, the corresponding abbreviation (abbr.) and mode of action are depicted.

Antibiotic	Abbr.	[mg/disc]	Mode of action
Linezolid	LZD	30	Inh. of proteinsynthesis
Vancomycin	VA	30	Inh. of cell wall synthesis
Metronidazol	MTZ	5	DNA ds breaks
Cotrimoxazol	TS	35	Inh. of DNA synthesis
Ciprofloxacin	CIP	5	Gyrase Inhibitor
Levofloxacin	LEV	5	Gyrase Inhibitor
Moxifloxacin	MFX	5	Gyrase Inhibitor
Gentamicin	GM	10	Proteinsynthese
Clarythromycin	CLA	15	Inh. of proteinsynthesis
Tigecyclin	TGC	15	Inh. of proteinsynthesis
Doxycyclin	DXT	30	Inh. of proteinsynthesis
Cefotaxim	CTX	30	Inh. of cell wall synthesis
Imipenem	IMI	10	Inh. of cell wall synthesis
Piperacillin/Tazobactam	PTZ	36	Inh. of cell wall synthesis
Amoxicillin/Clavulansäure	AUG	30	Inh. of cell wall synthesis
Amoxicillin	A	10	Inh. of cell wall synthesis
Penicillin G	PG	10	Inh. of cell wall synthesis
Penicillin V	PV	10	Inh. of cell wall synthesis
Cefadroxil	CDX	30	Inh. of cell wall synthesis
Lincomycin	MY	15	Inh. of proteinsynthesis

3.7.2 Bacterial strain and culturing

The investigations were carried out with the non-human pathogenic *Bordetella petrii* (DSM 12804) and *Staphylococcus aureus* (DSM 20231). *B. petrii* were grown on CASO-Agar plates at 27,5 °C until a bacterial lawn has formed. *S. aureus* were cultivated on Trypticase Soy Broth and were incubated at 37 °C. Plates were stored at 4 °C until usage; if the cultures were not needed immediately. Bi- to three-weekly, a fresh Agar plate was inoculated with a colony from the precursor plate. This was performed by streaking a sterile bacteria-laden inoculation loop across the surface of the agar plate under a laminar flow.

3.7.3 Procedure

Antibiotic sensitivity was tested by performing the agar diffusion test. Therefore, bacteria were transformed to 5 ml of sterile NaCl-solution with a bacteria-laden inoculation loop. The bacterial suspension was densely spread on a CASO-Agar or Trypticase Soy Broth-Agar (depending on the bacterial strain to be tested) plate with a sterile cotton bud. Afterwards, antibiotic discs (Table 6) were placed on the streaked bacterial suspension with forceps, which were flamed with ethanol before usage. For cooperation testing with the chosen antiseptics, 10µl of the antiseptic agents were dropped directly onto the antibiotic discs. Additionally, the effects of bacterial growth of the antiseptics alone were estimated as well. For this, 5µl, 10µl or 20µl of the substance under investigation were dropped on sterile discs, which were placed on the bacterial suspension like the ones with antibiotics. The whole procedure was performed under semi-sterile conditions (flame). The plates were evaluated after 48 h at 27,5 °C (*B. petrii*) or 48 h at 37 °C (*S. aureus)* by measuring the diameter of the formed zone of inhibition, which reflects the degree of bacterial sensitivity against the applied compound.

4 Results

The usage of a 1:1 OCT dilution with a physiological sodium chloride (NaCl) solution is clinically common practise. Previous studies in the working group of Prof. Hüttinger showed that OCT in dilution with NaCl induces crystallization which could impair the microbicidal efficacy and tissue tolerability of octenidine. Unpublished data from a case study suggest that the dilution of OCT with a 5 % glucose solution has a beneficial effect on wound healing without affecting the antiseptic properties of OCT. Our working group could demonstrate that the addition of glucose leads to an enhanced attachment of human glioblastoma cells in comparison to OCT with NaCl. This parameter indicates the formation of a stable cell-matrix connection in vivo which is a favorable effect during wound healing.

The current work further investigated the effects of glucose in combination with octenidine (Octeniplus, O^+) in comparison to the "classical" OCT formulation (O) and other commonly used antiseptics including Chlorhexidine (Chl), Beta-Isodona® (Bet) and Taurolin® (Tau). Thereby, the focus was on the impact on cell proliferation, cell migration and cell adhesion, gene expression and interactions with various antibiotics. These parameters where assessed by using the MTT-assay, for the determination of cell proliferation and cell adhesion; reverse transcription polymerase chain reaction (rt-PCR), for the effects on gene expression; the in vitro Scratch assay, for the investigation of cell migration; and the agar diffusion test which shed light on the antimicrobial efficiency and possible interactions with antibiotics. With these tests, we wanted to look behind the paradox observations of the high cytotoxic properties of O in vitro and the good tolerability in clinical practise.

All tests, except the agar diffusion test, where performed with primary human fibroblasts and MCF-7 cells. It is necessary to cause as less damage as possible to the upper epithelial layer upon antiseptic application in order to support wound closure. Moreover, the migration of fibroblasts into the wounded area is essential for depositing extracellular matrix proteins in order to establish the granulation tissue

(Gurtner et al., 2008). Due to this fact, it was examined whether O+ had any beneficial effect on these cells in comparison to O which could explain the positive results from case report studies.

The agar diffusion test was conducted with two different bacterial strains: (1) *Bordetella petrii* which is not pathogenic to humans (Gross et al., 2008) and (2) *Staphylococcus aureus* which is a frequent isolate from acute and chronic wounds (Kirker et al., 2009).

The following graphs, tables and pictures represent the impact of O and O+ in various concentrations on fibroblasts and MCF-7 cells considering cell proliferation, adhesion, migration and gene expression. These data which are the outcome of at least 2 independent experiments were statistically analyzed using Prism 3.0 (significance $p < 0,05$).

4.1. Effects of O, O+ and chondroitin sulfate on cell proliferation and cell adhesion in MCF-7 cells

4.1.1. Impact of O in comparison to O+ on MCF-7 cell proliferation

Since the conservation of cell proliferation during wounding is indispensable, an ideal antiseptic should not interfere with this process. For testing, MCF-7 cells were grown on microtiter plates coated with different concentrations of O and O+ for 24 h which resulted in highly significant growth retardation ($p < 0,001$) (see Table 7). Even at a higher dilution factor (1:1024 i.e. 0,5 mg/L octenidine), the proliferation rate upon O+ and O application was only 40 % and 50 % of untreated cells, respectively. However, in the presence of 0,012 mg/L, the average growth rate reached more than 100 % of control cells. At this concentration, O+ allowed greater proliferation than O ($p < 0,001$) (Table 8, Figure 9A).

Chondroitin sulfate (CHS) is a sulfated glycosaminoglycan and is distributed as side chains of proteoglycans in the ECM and on cellular surfaces. It is highly negatively charged (Ogawa et al. 2012). Hence, it is likely that CHS and octenidine interact with each other and most of the applied octenidine molecules are retained in the ECM of the tissue. In such complexes, the cytotoxicity of octenidine may be decreased while the antimicrobial efficiency is maintained. This would partly explain the different tolerability of octenidine in vitro and in vivo studies. The addition of 1 g/L CHS to the antiseptic stock solutions did not ameliorate the cytotoxic effects of O and O+. A rather more severe inhibition of cell proliferation could be detected in the course of the concentration gradient. At 125 mg/L O/O+ and 250 mg/L CHS, the proliferation rate was less than 10 % in comparison to cells solely grown in 10 % FCS. The maximal growth rate (approximately 85 %) was measured at the lowest antiseptic and CHS concentration (Figure 9B).

Table 7. Effect of O (vs ctrl) and O+ (vs ctrl) on MCF-7 cell proliferation. Dishes were coated with whether O and O+ or O and O+ supplemented with CHS and a serial dilution thereof. Cells were grown in medium on these coats for 24 hours and then subjected to the MTT-assay. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O/O+ and control (= 100 %), CI confidence interval.

	Conc. O/O+[mg/L]	**125**	**31,25**	**7,81**	**1,95**	**0,5**	**0,012**
	Conc. CHS [mg/L]	**250**	**62,5**	**15,6**	**3,9**	**0,9**	**0,24**
	DF	**4**	**16**	**64**	**256**	**1024**	**4096**
O	MD	-73,75	-74,69	-70,3	-65,5	-43,48	14,46
	P-value	P < 0.001	P < 0.001	P < 0.01	P < 0.01	P > 0.05	P > 0.05
	95 % CI	-121.3 to -26.19	-122.2 to -27.13	-117.9 to -22.74	-113.1 to -17.94	-91.03 to 4.083	-33.10 to 62.02
O+	MD	-68,63	-62,37	-61,22	-67,69	-60,28	82,93
	P-value	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.01	P < 0.001
	95 % CI	-116.2 to -21.07	-109.9 to -14.81	-108.8 to -13.66	-115.3 to -20.13	-107.8 to -12.72	35.37 to 130.5
O w CHS	MD	-97,86	-78,86	-64,14	-57,05	-49,74	-16,13
	P-value	P < 0.001	P < 0.001	P < 0.01	P < 0.05	P < 0.05	P > 0.05
	95 % CI	-145.9 to -49.87	-126.9 to -30.87	-112.1 to -16.15	-105.0 to -9.054	-97.73 to -1.747	-64.12 to 31.86
O+ w CH	MD	-91,39	-68,22	-60,8	-63	-36,8	-13,26
	P-value	P < 0.001	P < 0.01	P < 0.01	P < 0.01	P > 0.05	P > 0.05
	95 % CI	-139.4 to -43.40	-116.2 to -20.22	-108.8 to -12.81	-111.0 to -15.00	-84.79 to 11.20	-66.91 to 40.40

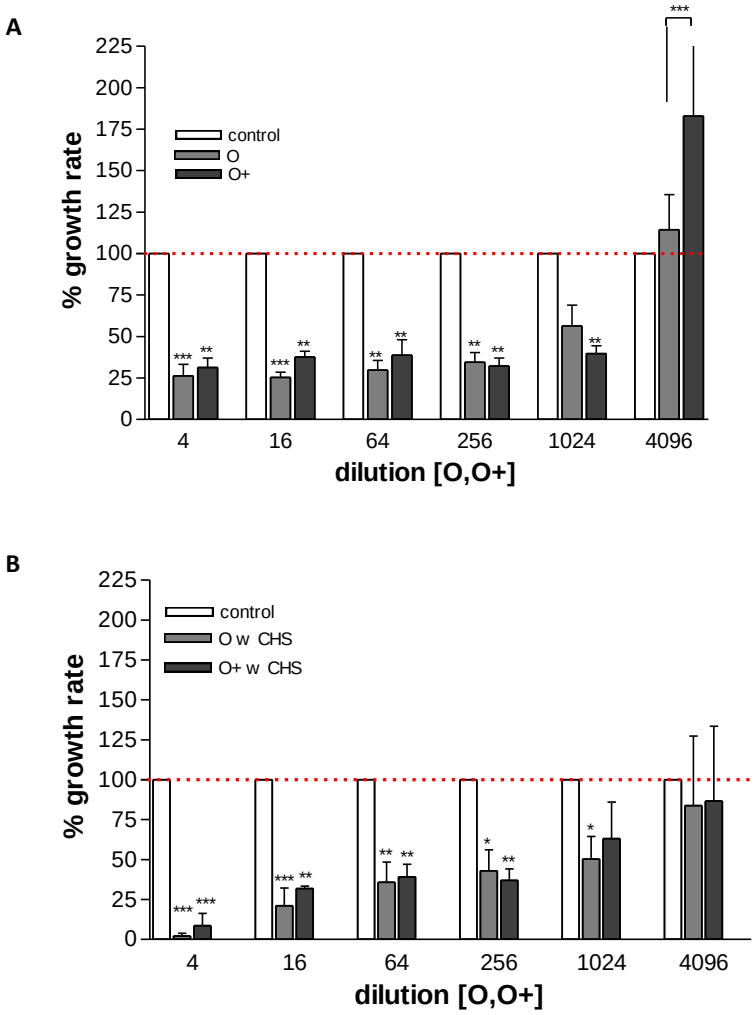


Figure 9. Effect of O and O+ (with or without CHS) on MCF-7 cell proliferation after 24h incubation. (A) There was a concentration dependent inhibition of cell proliferation, whereas at the highest dilution, O+ had a higher biocompatibility in comparison to O. (B) The addition of CHS (1 g/L) to the O and O+ stock solution led to a significant proliferation inhibition in the course of the concentration gradient with no significant differences between the two Octenisept® formulations. The calculation of the growth rates [%] were done in covering to cells grown for 24 h in the absence of antiseptic agents and CHS which were set to 100 % (represented by the red dotted line). Error bars indicate the SD from three measurements ($p < 0,05$ *, $p < 0,01$ < **, $p < 0,001$ ***).

Table 8. Effect of O vs O+ on MCF-7 cell proliferation. Dishes were coated with whether O and O+ or O and O+ supplemented with CHS and a serial dilution thereof. Cells were grown in medium on these coats for 24 hours and then subjected to the MTT-assay. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O and O+, CI confidence interval.

	Conc. O/O+ [mg/L]	**125**	**31,25**	**7,81**	**1,95**	**0,5**	**0,012**
	Conc. CHS [mg/L]	**250**	**62,5**	**15,6**	**3,9**	**0,9**	**0,24**
	DF	**14**	**16**	**64**	**256**	**1024**	**4096**
O vs O+	MD	-5,115	-12,32	-9,081	2,192	16,81	-68,48
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001
	95 % CI	-48.35 to 38.12	-55.55 to 30.92	-52.32 to 34.15	-41.04 to 45.43	-26.43 to 60.04	-111.7 to -25.24
O w CHS vs O+ w CHS	MD	-6,472	-10,65	-3,34	5,95	-12,94	-2,871
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-50.08 to 37.13	-54.25 to 32.96	-46.94 to 40.26	-37.65 to 49.55	-56.55 to 30.66	-51.62 to 45.88

To examine how MCF-7 cells better proliferate in the presence of O or O+ and how CHS influences such proliferation, MCF-7 cells were grown in wells coated with 1 g/L CHS constantly and with different O/O+ concentration. Therefore, only the concentration of octenidine decreased but CHS concentration was unaltered in the course of dilution. Table 9 illustrates that in the presence of 1 g/L CHS and up to 1,95 mg/L octenidine significant growth retardation occurs ($p < 0,001$). At the lowest O+ concentration, the proliferation rate increased significantly by 60 % in comparison to control cells (Table 10). On the contrary, with the same O concentration, the proliferation rate did not exceed the level of control cells. Instead, O had a more positive effect on cell proliferation at 0,5 mg/L in comparison to O+ ($p < 0,05$) (Figure 10).

Table 9. Effect of several O and O+ concentrations with CHS (vs ctrl) on MCF-7 cell proliferation. Dishes were coated with 1 g/L CHS and a serial dilution of O or O+. Cells were grown in medium for 24 hours and then subjected to the MTT-assay. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O/O+ and control (= 100 %), CI confidence interval.

	Conc. O/O+ [mg/L]	125	31,25	7,81	1,95	0,5	0,012
	DF	**4**	**16**	**64**	**256**	**1024**	**4096**
O w CHS coat	MD	-78,43	-78,57	-71,63	-70,07	-14,67	-6,022
	P-value	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P > 0.05	P > 0.05
	95 % CI	-112.6 to -44.25	-112.7 to -44.40	-105.8 to -37.45	-104.2 to -35.89	-48.84 to 19.51	-40.19 to 28.15
O+ w CHS coat	MD	-81,83	-77,29	-77,15	-75,74	-49,81	66,67
	P-value	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.01	P < 0.001
	95 % CI	-116.0 to -47.66	-111.5 to -43.12	-111.3 to -42.98	-109.9 to -41.56	-83.98 to -15.63	32.49 to 100.8

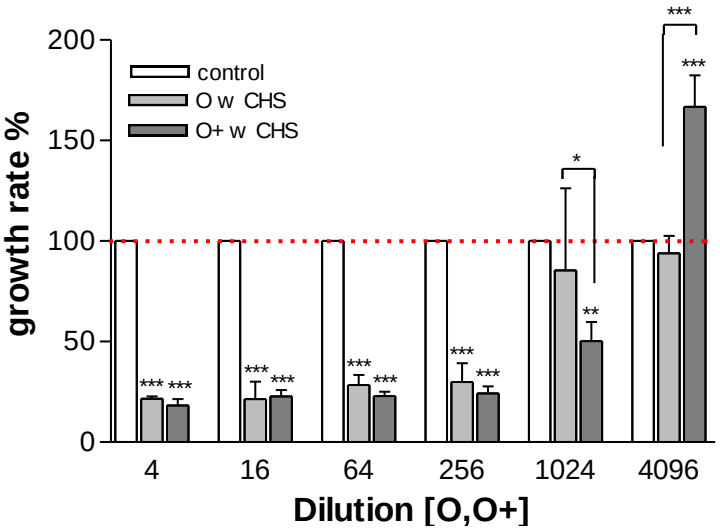


Figure 10. Effect of O and O+ complemented with 1 g/L CHS on MCF-7 cell proliferation after 24h incubation. Cell proliferation was significantly inhibited in the presence of CHS and octenidine whereby O+ had a higher biocompatibility in comparison to O at the highest dilution factor. The calculation of the growth rates [%] were done in covering to cells grown for 24 h in the absence of antiseptic agents and CHS which were set to 100 % (represented by the red dotted line). Error bars indicate the SD from three measurements (p < 0,05 *, p < 0,01 ** p < 0,001 ***).

Table 10. Effect of several O and O+ concentrations with CHS on MCF-7 cell proliferation. Dishes were coated with 1 g/L CHS and a serial dilution of O or O+. Cells were grown in medium for 24 hours and then subjected to the MTT-assay. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O and O+, CI confidence interval.

	Conc. O/O+ [mg/L]	125	31,25	7,81	1,95	0,5	0,012
	DF	**4**	**16**	**64**	**256**	**1024**	**4096**
O w CHS vs O+ w CHS	MD	3,401	-1,275	5,526	5,668	35,14	-72,69
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05	P < 0.001
	95 % CI	-27.66 to 34.47	-32.34 to 29.79	-25.54 to 36.59	-25.40 to 36.73	4.075 to 66.20	-103.8 to -41.62

In the next assay, 96-well plates were pre-incubated (coated) with a serially diluted 1 g/L CHS solution and with 0,5 g/L O and O+. Cell proliferation was dramatically decreased upon this treatment in comparison to control cells (max. growth rate 10 %) (Figure 11). CHS coat with O+ was less cytotoxic than O (Table 11), but significant growth inhibition was induced by both preparations. However, 0,24 mg/L of CHS coat could ameliorate the highly cytotoxic effect of O+. Cell proliferation was 60 % of reference cells in response to this combination (Figure 11).

Table 11. Ratios of growth rates of MCF-7 cells cultured in 0,5 g/L O vs O+. Cells were grown on different concentrations of CHS coats (ranging from 250 to 0,24 mg/L). The Growth rate is the ratio of the extinction value of O with CHS and the extinction value of O+ with CHS. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O with CHS and % of O+ with CHS, CI confidence interval

	Conc. CHS [mg/L]	**250**	**62,5**	**15,6**	**3,9**	**0,9**	**0,24**
	DF	**4**	**16**	**64**	**256**	**1024**	**4096**
O w CHS vs O+ w CHS	MD	-38,81	-32,38	-23,1	-21,19	-12,62	-51,19
	P-value	P < 0.001	P < 0.001	P < 0.05	P < 0.05	P > 0.05	P < 0.001
	95 % CI	-58.96 to -18.66	-52.53 to -12.23	-43.25 to -2.94	-41.34 to -1.04	-32.77 to 7.53	-71.34 to -31.04

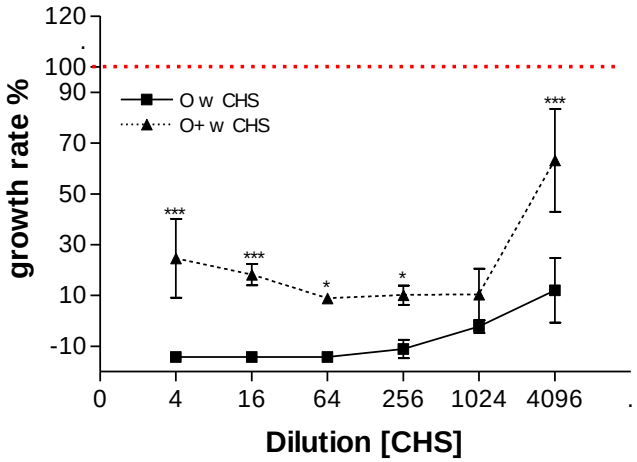


Figure 11. Effect of 0,5 g/L O and O+ (with several CHS concentrations) on MCF-7 cell proliferation after 24h incubation. Cell proliferation was significantly inhibited in the presence of different CHS concentrations and octenidine. CHS in combination with O+ had a better biocompatibility than O. The calculation of the growth rates [%] were done in covering to cells grown for 24 h in the absence of antiseptic agents and CHS which were set to 100 % (represented by the red dotted line). Error bars indicate the SD from three measurements ($p < 0,05$ *, $p < 0,01$ **, $p < 0,001$ ***).

In summary, high concentrations of O and O+ had severe effects on cell proliferation. However, in lower amounts, O+ was better tolerated than O. Moreover, the presence

of CHS seemed to have a beneficial effect when it was applied in very small amounts. This result further supports the hypothesis of a subtle role of CHS in the presentation of octenidine to bacteria and cells. At a certain low concentration, it ameliorated the cytotoxic effects of 0,5 g/L O+ (Figure 11).

4.1.2. Impact on MCF-7 cell adhesion of O in comparison to O+

Beside proliferation, stable cell adhesion to the extracellular matrix is postulated to be an important factor during wound repair. Due to this fact, the effect of O and O+ incubation (30 minutes) on MCF-7 cell adhesion was investigated. After the incubation, the cells were washed stringently in a normalized procedure with PBS and the remaining attached cells to the microtiter plate surface were then subjected to the MTT-assay. The absorbance at 595nm correlates with the cell number that is still attached to the well surface after the procedure. O and O+ had no negative effect on cell adhesion when it was diluted 1:100 and higher (Figure 12). Below this mark, a concentration dependent loss of cell adhesion was observed with no significant differences between O and O+ (Table 12).

Table 12. MCF-7 cell adhesion after 30 minutes incubation with various O concentrations in comparison to O+. Serial dilutions of O and O+ stock solution were done directly in the cell culture medium. Cells were incubated 30 minutes and then subjected to the MTT-assay. No significant differences between O and O+ were detected. The statistical analysis was conducted using Bonferroni-Test. DF Dilution Factor, MD mean difference between % of O and % of O+, CI confidence interval.

	Conc. O/O+ [mg/L]	**50**	**5**	**0,5**	**0,05**	**0,005**
	DF	**10**	**100**	**1000**	**10000**	**100000**
O vs O+	MD	2,288	5,72	5,529	-1,716	24
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05
	95 % CI	-36.70 to 41.28	-33.27 to 44.71	-33.46 to 44.52	-40.70 to 37.27	-14.99 to 62.99

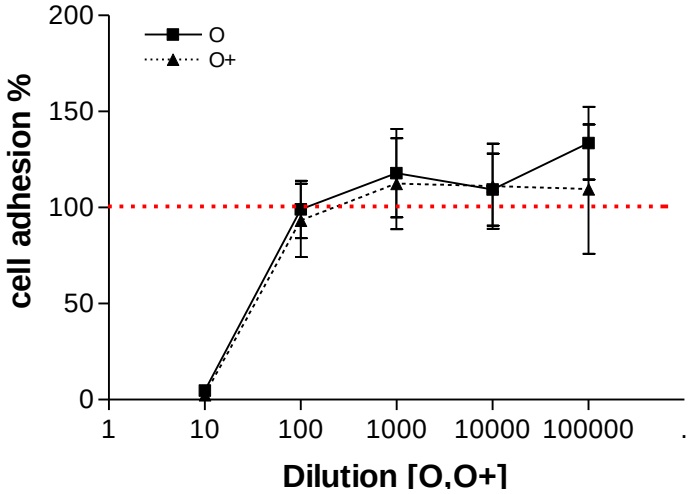


Figure 12. Effect of O and O+ on MCF-7 cell adhesion after 30 minutes incubation. Incubation with 50 mg/L O and O+ leads to a complete loss of cell adhesion. However, this effect is abrogated with decreasing concentrations of the antiseptic agents. Then, the level of cell adhesion is comparable to that of control cells. The quotient of the extinction value of treatment cells and the extinction value of the reference cells represents the % of cell adhesion. Reference cells were taken as 100 % (represented by the red dotted line). Error bars indicate the SD from three measurements.

It is apparent from Figure 12 that the IC50 value for octenidine ranges between 50 mg/ml and 5 mg/ml of O and O+. The IC50 value describes the concentration of a compound at which 50 % of the cell population survives. To determine this value, the dilution factors were adjusted and the assay was repeated under the same conditions. This led to the result that at 15 ± 1 mg/L (i.e. 1:33 dilution) O/O+ 50 % of cell adhesion were lost (Figure 13). The presence of glucose did not influence this value. From this graphic, a value for IC10 (concentration at which 10 % of cell adhesion is lost) and IC90 (concentration at which 90 % of the cells loose their adhesion) was calculated as well (not shown in the graphic). The IC10 was measured at 7 ± 0,3 mg/L O/O+ (i.e. 1:75 dilution) and the IC90 at 30 ± 5 mg/L O/O+ (i.e. 1:15 dilution).

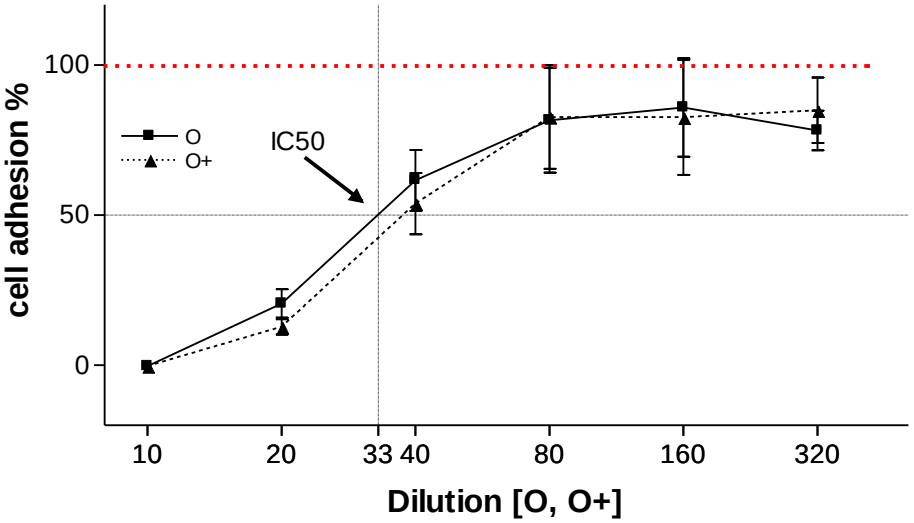


Figure 13. Determination of the IC50 value of O and O+ in MCF-7 cells. Cells were incubated with different O and O+ concentrations (ranging from 1:10 to 1:320 dilution) for 30 minutes and then subjected to the MTT-assay. After 30 minutes incubation with a 1:33 dilution of O and O+, 50 % loss of cell adhesion was detected with no differences between O and O+. The % of adhesion was calculated by the ratio of the extinction value of treatment cells and the extinction value of reference cells. Reference cells were set to 100 % (represented by the red dotted line). Error bars indicate the SD from three independent experiments, measured as triplicates.

The calculated IC values for O and O+ were used to further analyse the optimal contact time of the antiseptic compound. The optimal contact time of octenidine allows clinical efficiency with minimal disturbance of cell adhesion. MCF-7 cells were incubated with different O and O+ concentrations (IC10, IC50 and IC90 value) for 1 s, 5 s, 10 s, 20 s, 60 s, 120 s, 300 s and 600 s and the residual attached cells were investigated by the MTT-assay. Incubation with 30 mg/L O and O+ (i.e. IC90, 90 % loss of cell adhesion) resulted in a striking decrease of cell adhesion after 600 s (10 minutes). Furthermore, even when the contact time was reduced to 1 s, approximately 50 % of cell attachment was lost (Figure 14A). Cell adhesion was never lower than 50 % at any time point in response to 15 mg/L (i.e. IC50) of the antiseptic agents in comparison to control cells. Incubation for 300 s (5 minutes) and shorter always resulted in a cell number between 70 % and 80 % (Figure 14B) of reference cells. As expected, the concentration corresponding to the IC 10 showed a good biocompatibility in vitro. This amount of O and O+ hardly caused severe cell damage since cell adhesion was at the same level as control cells at any given contact time. Additionally, O+ had a better tissue compatibility after 60 s and 5 s exposure time in comparison to O ($p < 0,001$ and $p <$

0,01 respectively) (Figure 14C). In all other settings, glucose addition did not provide any advantage (Table 13).

Table 13. Cell adhesion in response to the IC values of O in comparison to O+ after the indicated contact times in MCF-7 cells. Serial dilutions of O and O+ stock solution were done directly in the cell culture medium. Cells were incubated for the indicated periods and then subjected to the MTT-assay. No significant differences between O and O+ were detected. The statistical analysis was conducted using Bonferroni-Test. MD mean difference between % of adhesion of O and % of adhesion of O+, CI confidence interval.

	Contact time [s]	600	300	120	60	20	10	5	1
O vs O+ IC90	MD	-7,317	-13,77	-10,08	3,46	-10,66	-2,863	-11,14	7,00
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-29.71 to 15.07	-36.16 to 8.62	-32.47 to 12.31	-18.93 to 25.84	-33.05 to 11.73	-25.25 to 19.53	-33.53 to 11.25	-15.38 to 29.40
O vs O+ IC50	MD	4,561	4,489	-11,32	-2,56	-11,62	-11,52	-6,03	-17,79
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-16.14 to 25.26	-16.21 to 25.19	-32.02 to 9.384	-23.26 to 18.14	-32.33 to 9.078	-32.22 to 9.184	-26.73 to 14.67	-38.49 to 2.912
O vs O+ IC10	MD	-21,96	-14,54	-1,95	-44,37	-19,81	-15,59	-36,83	-22,05
	P-value	P > 0.05	P > 0.05	P > 0.05	P < 0.001	P > 0.05	P > 0.05	P < 0.01	P > 0.05
	95 % CI	-51.44 to 7.529	-41.45 to 12.38	-28.87 to 24.97	-71.28 to -17.45	-46.72 to 7.110	-42.51 to 11.33	-63.75 to -9.917	-48.97 to 4.862

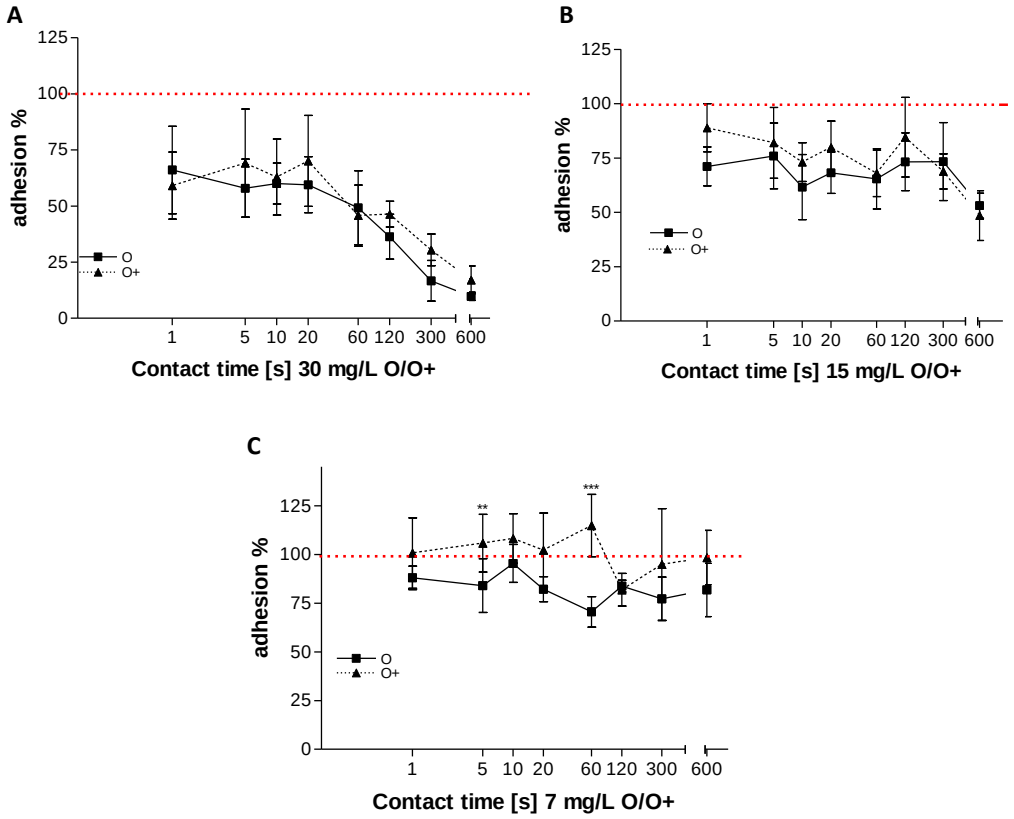


Figure 14. Effect of different concentrations of O in comparison to O+ on MCF-7 cell adhesion. O and O+ were diluted directly in the cell culture medium. Cells were incubated for the indicated time points with 30 (A), 15 (B) or 7 mg/L (C) of O/O+ and then subjected to the MTT-assay. The % of adhesion was calculated by the ratio of the extinction value of treatment cells and the extinction value of reference cells. Reference cells were set to 100 % (represented by the red dotted line). Error bars indicate the results from two independent experiments, measured as triplicates ($p < 0.01$ **, $p < 0.001$ ***).

Again, for elucidation of the toxicity paradox these experiments allow the assumption that the sequestration of octenidine is to be achieved within 10 minutes to avoid toxicity for the cell but to keep bactericidal efficiency.

In these short-time application experiments, the earlier observed advantages of the O+ formulation in comparison to the classical O preparation were only reproducible when the concentration of the antiseptics were rather low (7 mg/L). At higher dosage rates (15 mg/L and 30 mg/L) O+ was not less cytotoxic than O.

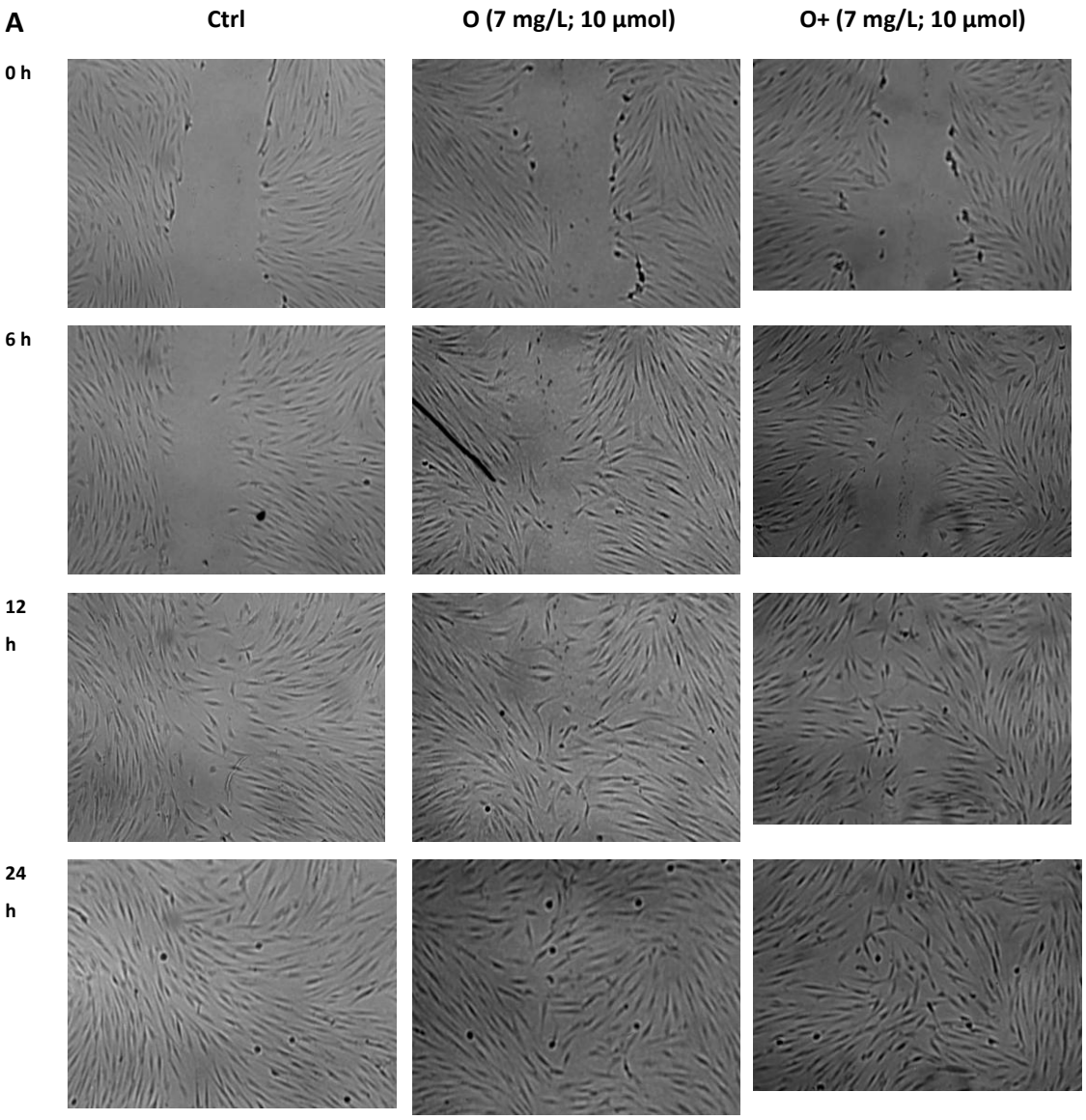
In accordance with the manufacturer's instructions, the contact time of Octenisept® is ideally between 30 seconds and 2 minutes when applied topically. For this reason, the incubation time for the following experiments was chosen to be 2 minutes (if not otherwise stated) regardless of the used O and O+ concentration.

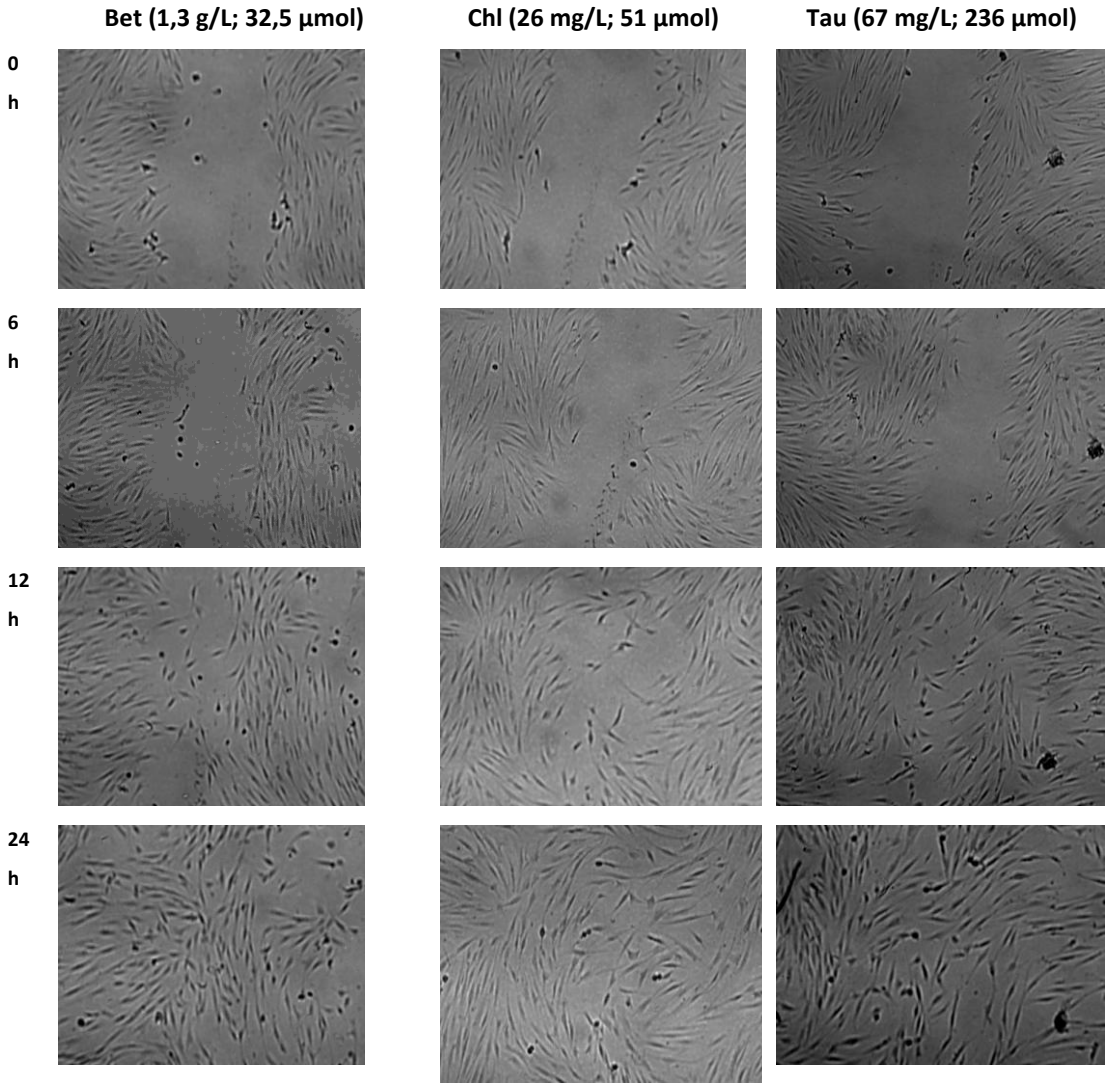
4.2. Influence of O and O+ on fibroblast and MCF-7 cell migration in comparison to other commonly used antiseptics

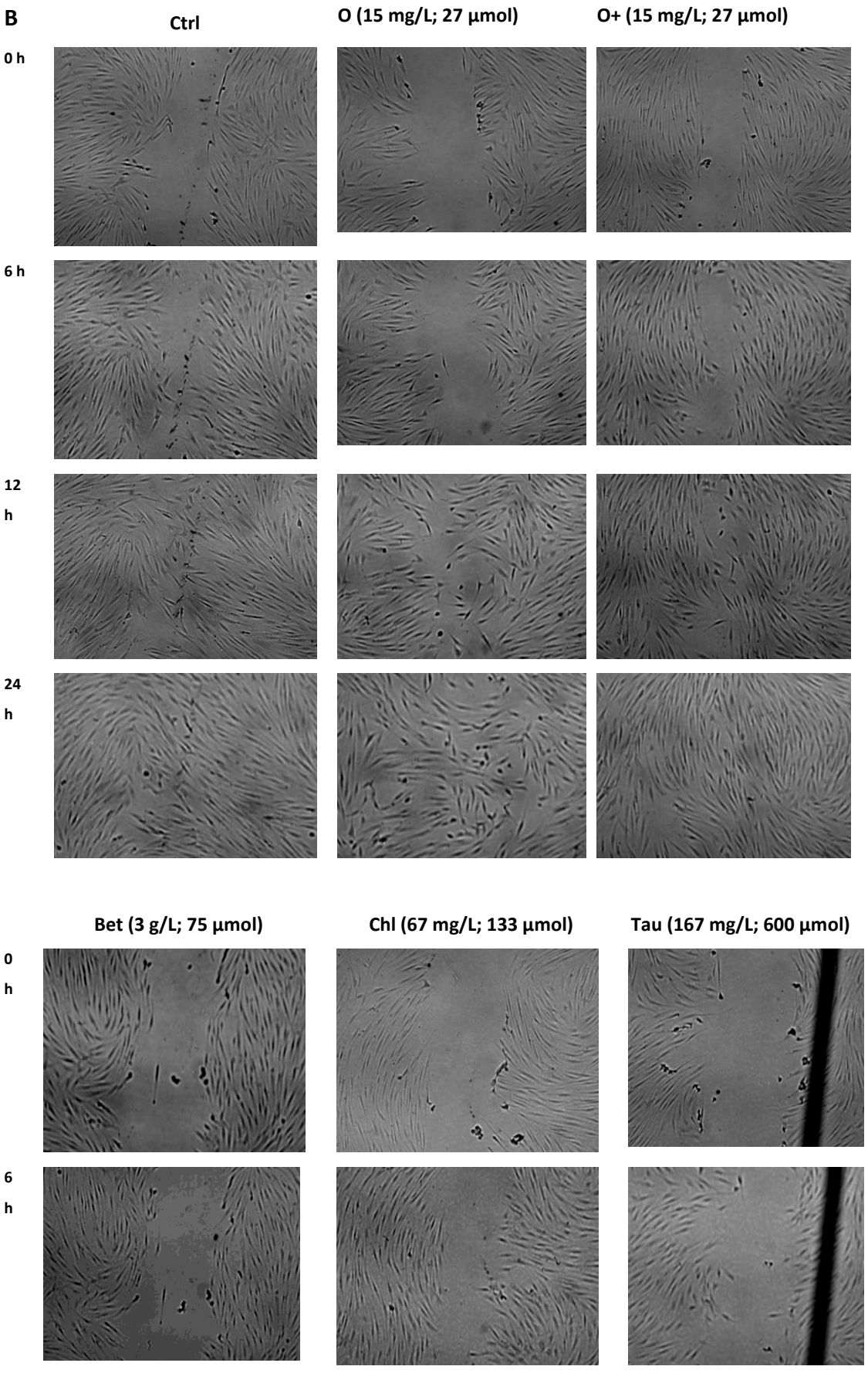
Next, the effect on cell migration of O and O+ was assessed by using the in vitro laceration assay. In this assay, the cell monolayer was scratched and cell migration into the cell-free areas was estimated by visual inspection. It was shown that only Beta-Isodona® impaired (1:75 dilution of the stock solution) and even inhibited (1:30) cell migration of primary human fibroblasts and wound closure. All other antiseptic preparations did not interfere with this process (Figure 15). However, to some extent cell detachment could be observed at the application point of Chl, O, O+ and Bet in some cases (data not shown). The application point refers to the site where the stock solution of the antiseptic compounds was administered and resulted from the addition of a stock solution volume to the culture medium volume.

The same assay was now performed using MCF-7 cells. It turned out that these cells need more time to migrate into the artificially introduced wounds. Due to this, the wound closure was monitored up to 98 h. Nevertheless, full re-establishment of a continuous cell monolayer was not achieved even by untreated cells serving as a reference. Unless cell migration occurred rather slowly, cell proliferation could be estimated easily. MCF-7 cells treated with O, Chl, Bet and Tau proliferated in the same extent than untreated ones as the cell density of the non-lacerated area increased, deduced from visual inspection. The wound area decreased after O, Chl, Bet and Tau treatment, but less obvious for O treated cells. On the contrary, the proliferation rate after O+ incubation seemed to be decreased in comparison to the other antiseptics and to control cells in MCF-7 cells (Figure 16).

In literature full wound closure was achieved with another clone of MCF-7 cells (Kang et al., 2009). Therefore, our MCF-7 cell clone seemed not to be the optimal cell type to assess cell migration via the in vitro laceration assay.


A
Ctrl
O (7 mg/L; 10 µmol)
O+ (7 mg/L; 10 µmol)
0 h
6 h
12 h
24 h


Bet (1,3 g/L; 32,5 µmol)
Chl (26 mg/L; 51 µmol)
Tau (67 mg/L; 236 µmol)
0 h
6 h
12 h
24 h


B
Ctrl
O (15 mg/L; 27 µmol)
O+ (15 mg/L; 27 µmol)
0 h
6 h
12 h
24 h
Bet (3 g/L; 75 µmol)
Chl (67 mg/L; 133 µmol)
Tau (167 mg/L; 600 µmol)
0 h
6 h

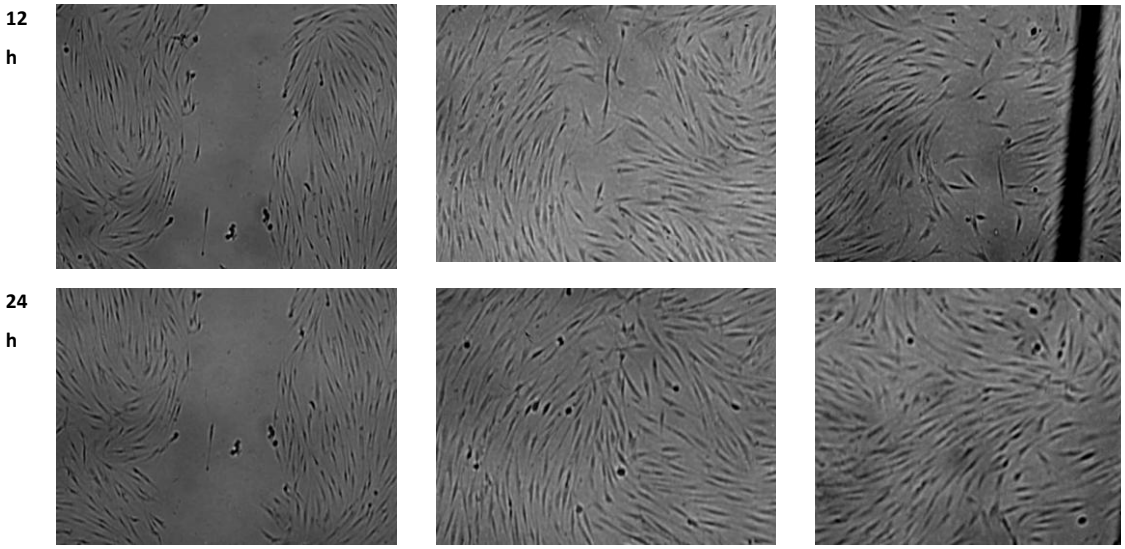
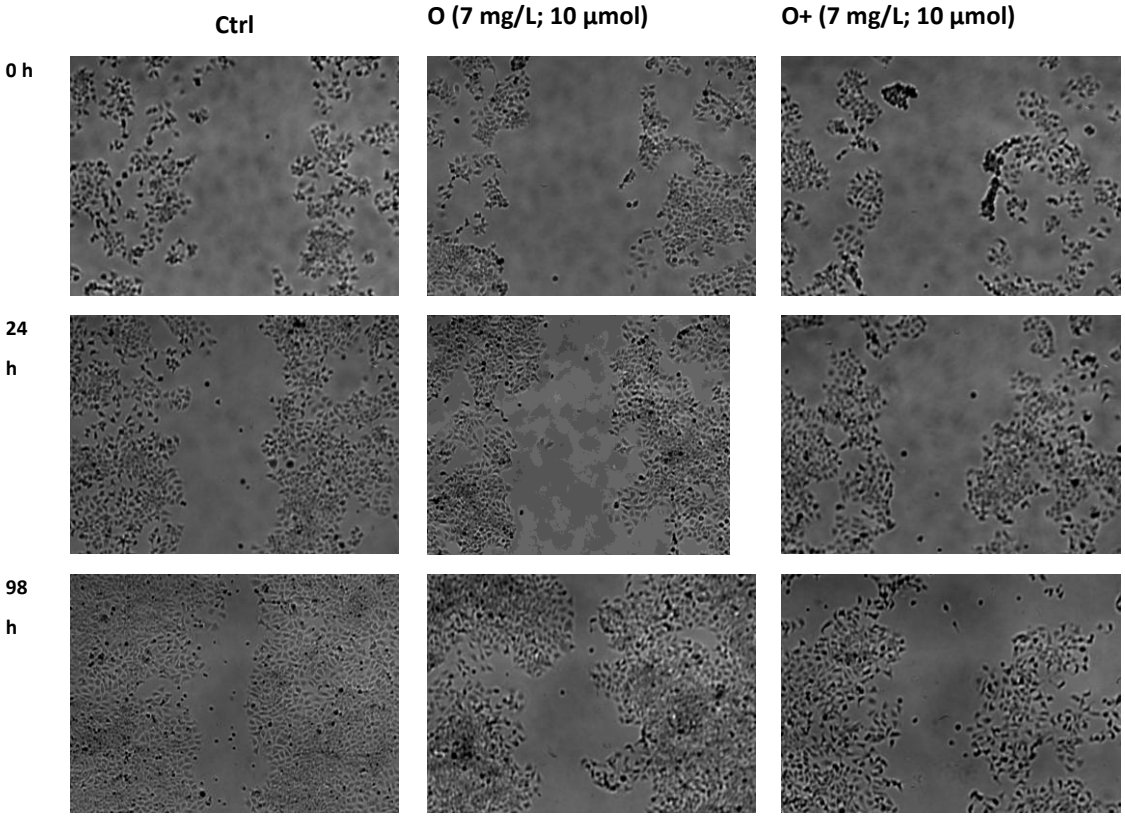


Figure 15. Representative pictures of the in vitro laceration assay with fibroblasts. Primary human fibroblasts were grown to confluency and at time point 0 a scratch-cross was introduced into the cellular monolayer. Incubations with indicated antiseptics or sterile filtrated aqua dest. (control) were then performed. The investigated antiseptic agents were either diluted 1:75 (A) or 1:30 (B). The experiment was repeated 4 times. Ctrl control, Bet Betaisodona, Chl Chlorhexidine, Tau Taurolin®.



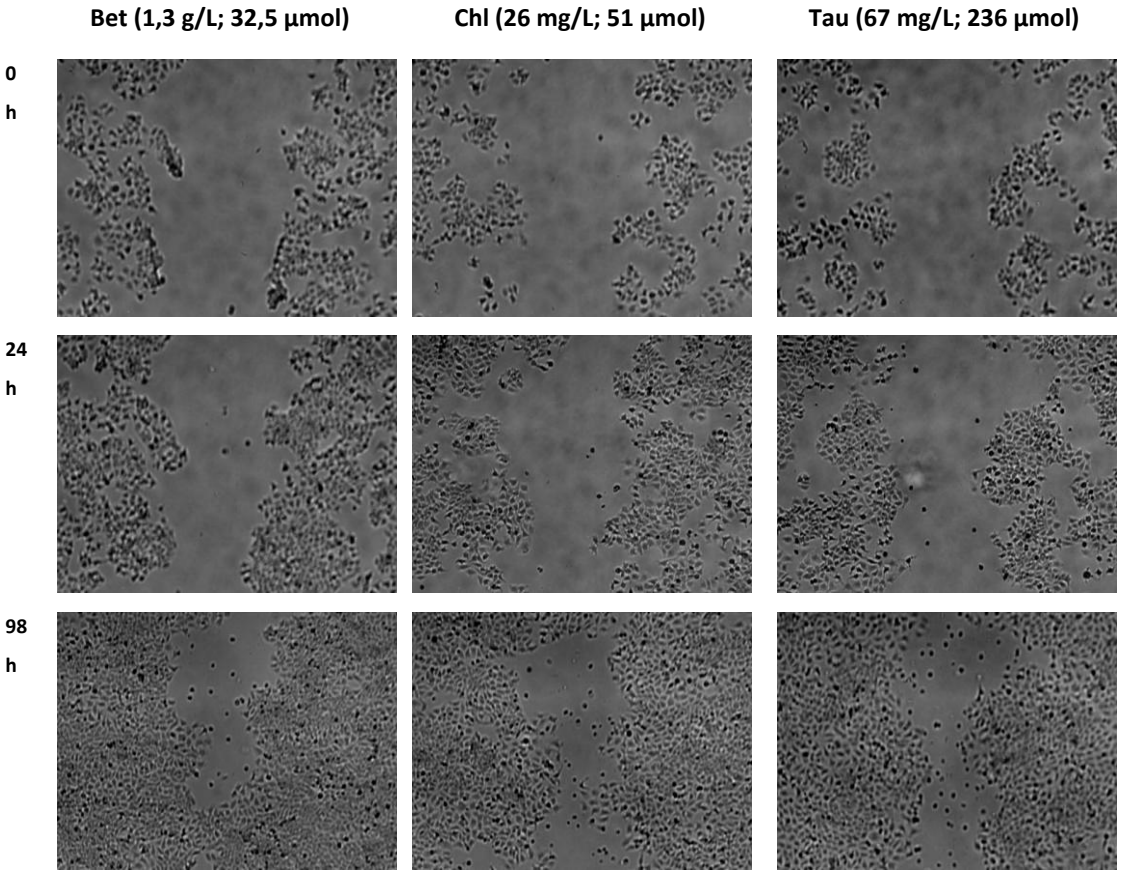


Figure 16. Representative pictures of the in vitro laceration assay with MCF-7 cells. MCF-7 cells were grown to confluency and at time point 0 a scratch-cross was introduced into the cellular monolayer. Incubations with indicated antiseptics or sterile filtrated aqua dest. (control) were then performed. The investigated antiseptic agents were diluted 1:75. The experiment was repeated twice. Ctrl control, Bet Betaisodona, Chl Chlorhexidine, Tau Taurolin®.

These tests showed that O and O+ did not negatively influence migration of fibroblasts in vitro after 2 minutes contact time. However, MCF-7 cell proliferation was slightly slowed down after O+ application.

There is still discrepancy between this tissue culture model and the in vivo situation whereby the effect was not so pronounced in fibroblasts. Fibroblasts started to migrate 6 hours after the antiseptic treatment and the cell monolayer was established after 24 hours. On the contrary, MCF-7 cells did not achieve a complete wound closure after 98 hours (regardless of the treatment).

4.3. Impact of O and O+ on the expression of selected genes involved in wound healing

Wound repair is a highly complex, dynamic and well regulated process which involves the interplay of various signalling molecules. Therefore, it was analyzed whether the application of O or O+ causes any changes in gene expression in primary human fibroblasts and MCF-7 cells after laceration and in normally growing fibroblasts. Additionally, it was determined whether O and O+ treatment causes any differences in gene expression in the presence of bacterial cell wall components. For that, cells were incubated for 2 hours with lipopolysaccharides (LPS, 10 µg/ml) prior to antiseptic treatment.

In unwounded fibroblasts, treatment with 7 mg/L (i.e. 10 µM = IC10) O led to a significant increase of MMP-1 and MMP-3 levels ($p < 0,05$ and $p < 0,001$ respectively) in comparison to control cells. Furthermore, the MMP-3 expression was significantly enhanced in comparison to 7 mg/L (i.e. 10 µM = IC10) O+ ($p < 0,01$) (Figure 17A, Table 14).

Laceration and LPS pre-incubation did not cause any significant changes in gene expression of MMPs in comparison to control cells and between O and O+ (Figure 17B-C). MMP-2 levels were increased after O+ application in unwounded fibroblasts but, this result was not significant (Figure 17C, Table 14). MMP-9 gene expression changes could not be analyzed statistically due to big variations between the distinct experiments.

Table 14. Relative gene expression levels of MMP family members in fibroblasts after O incubation in comparison to O+. Gene expression changes upon 7 mg/L O or O+ were tested under three conditions in fibroblasts: (i) no laceration = unwounded; (ii) lacerated = several scratches were introduced into the cell monolayer (#); (iii) 2 h pre-incubation with 10 µg/ml LPS and #. The depicted values are derived from two to four independent experiments. The statistical analysis was conducted using Bonferroni-Test. CI confidence interval MD mean difference between relative gene expression change of O and O+ LPS lipopolysaccharides.

	Gene	MMP-1	MMP-2	MMP-3
O vs O+	**MD**	1,86	-4,02	1,654
	P-value	P > 0.05	P > 0.05	P < 0.01
	95 % CI	-0.7297 to 4.450	-9.722 to 1.682	0.450 to 2.859
O# vs O+#	**MD**	-0,0131	0,0316	-0,436
	P-value	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-2.435 to 2.409	-4.624 to 4.687	-1.479 to 0.607
O#LPS vs O+#LPS	**MD**	-0,063	0,257	0,200
	P-value	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-2.809 to 2.684	-4.399 to 4.912	-1.005 to 1.404

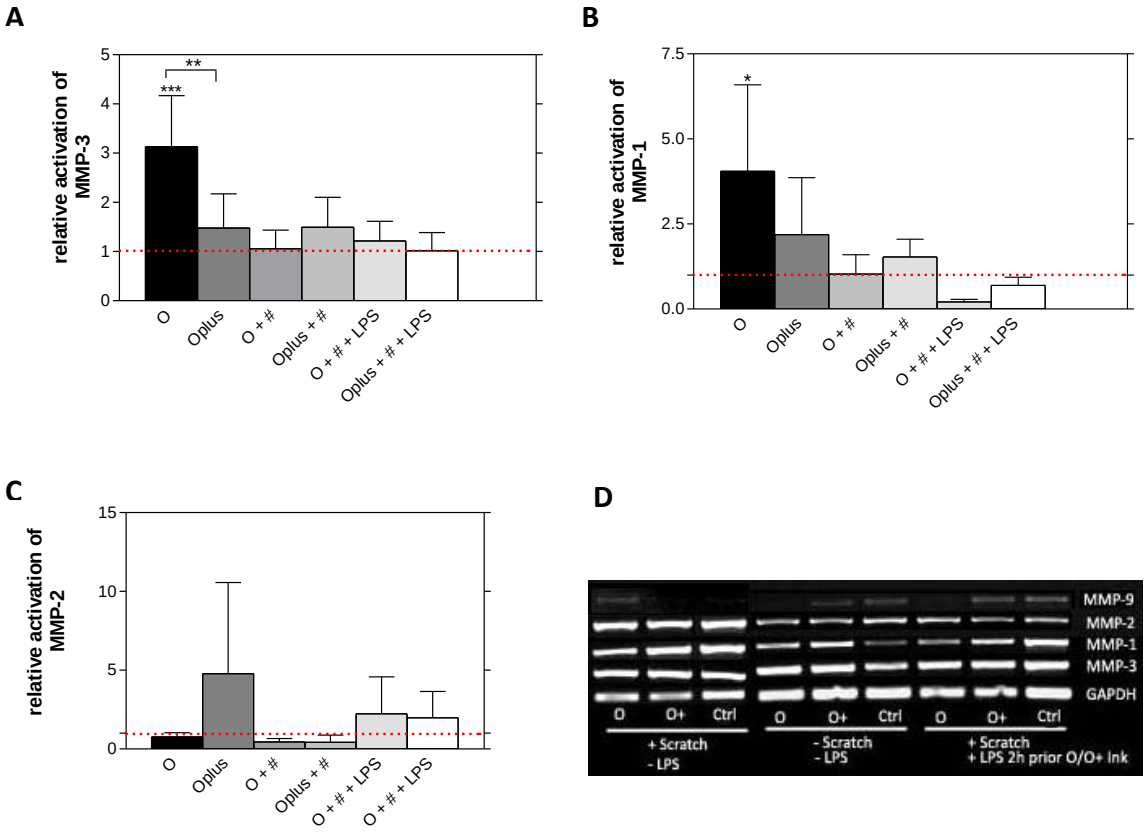


Figure 17. PCR analysis of MMP family members in primary human fibroblasts after O treatment in comparison to control cells and O+. Fibroblasts were incubated with 7 mg/L O or O+ under three different conditions (described at Table 14) for 2 minutes. Control cells were only incubated with sterile aqua dest. The cells were then washed with PBS and grown for 24 hours in fresh cell culture medium in the absence of the antiseptics. Gene expression changes were estimated by PCR using primers for MMP-3 (A), MMP-1 (B) and MMP-2 (C) and MMP-9. Representative agarose gel picture of the PCR products (D). GAPDH was used as house keeping gene. Relative activation was calculated by the quotient of the intensity from the PCR signal of treatment cells and reference cells. Reference cells were set as 1 (red dotted line). All data were normalized to GAPDH. Error bars indicate SD from two to four independent experiments (* p < 0,05; ** p < 0,01; *** p < 0,001).

As summarized in Figure 18, the different incubation procedures had no significant influence on gene expression of PAI-1, TGF-β1, VEGF-A, ICAM-1 and uPA. Only in lacerated fibroblasts treated with O+, TGF-β1 expression was totally abolished (Figure 18B). VEGF-A expression was more induced in non-lacerated fibroblasts after O administration than after O+ ($p < 0,05$). Two isoforms of VEGF-A were detected. Statistical analysis was only conducted for the smaller VEGF-A variant which was designated as VEGF-A1 (analogously, the bigger isoform was designated VEGF-A2) (Figure 18C). Also PAI-1 (Figure 18A) and ICAM-1 (Figure 18D) seemed to be upregulated in response to O in unwounded fibroblasts. However, a high variation between the independent experiments was detected. Therefore, these observations were not significant (Table 15). Especially, uPA duplicates were not matching after O+, scratch and LPS treatment (Figure 18E, second last column).

Table 15. Relative gene expression levels of genes in fibroblasts after O incubation in comparison to O. Gene expression changes upon 7 mg/L O or O+ were tested under three conditions in fibroblasts: (i) no laceration = unwounded; (ii) lacerated = several scratches were introduced into the cell monolayer (#); (iii) 2 h pre-incubation with 10 µg/ml LPS and #. The depicted values are derived from two to four independent experiments. The statistical analysis was conducted using Bonferroni-Test. CI confidence interval MD mean difference between relative gene expression change of O and O+ LPS lipopolysaccharides.

	Gene	PAI-1	TGFβ-1	VEGF-A	ICAM-1	uPA
O vs O+	**MD**	0,91	-0,30	0,878	1,772	0,003
	P-value	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05
	95 % CI	-0.602 to 2.432	-1.012 to 0.413	0.107 to 1.649	-0.982 to 4.525	-6.604 to 6.610
O# vs O+#	**MD**	0,028	0,732	0,341	-0,452	-1,073
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-1.392 to 1.447	-0.141 to 1.606	-0.684 to 1.367	-2.700 to 1.796	-6.468 to 4.322
O#LPS vs O+#LPS	**MD**	0,231	0,062	0,186	0,083	5,022
	P-value	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
	95 % CI	-1.286 to 1.748	-0.812 to 0.935	-0.937 to 1.309	-2.670 to 2.837	-1.585 to 11.63

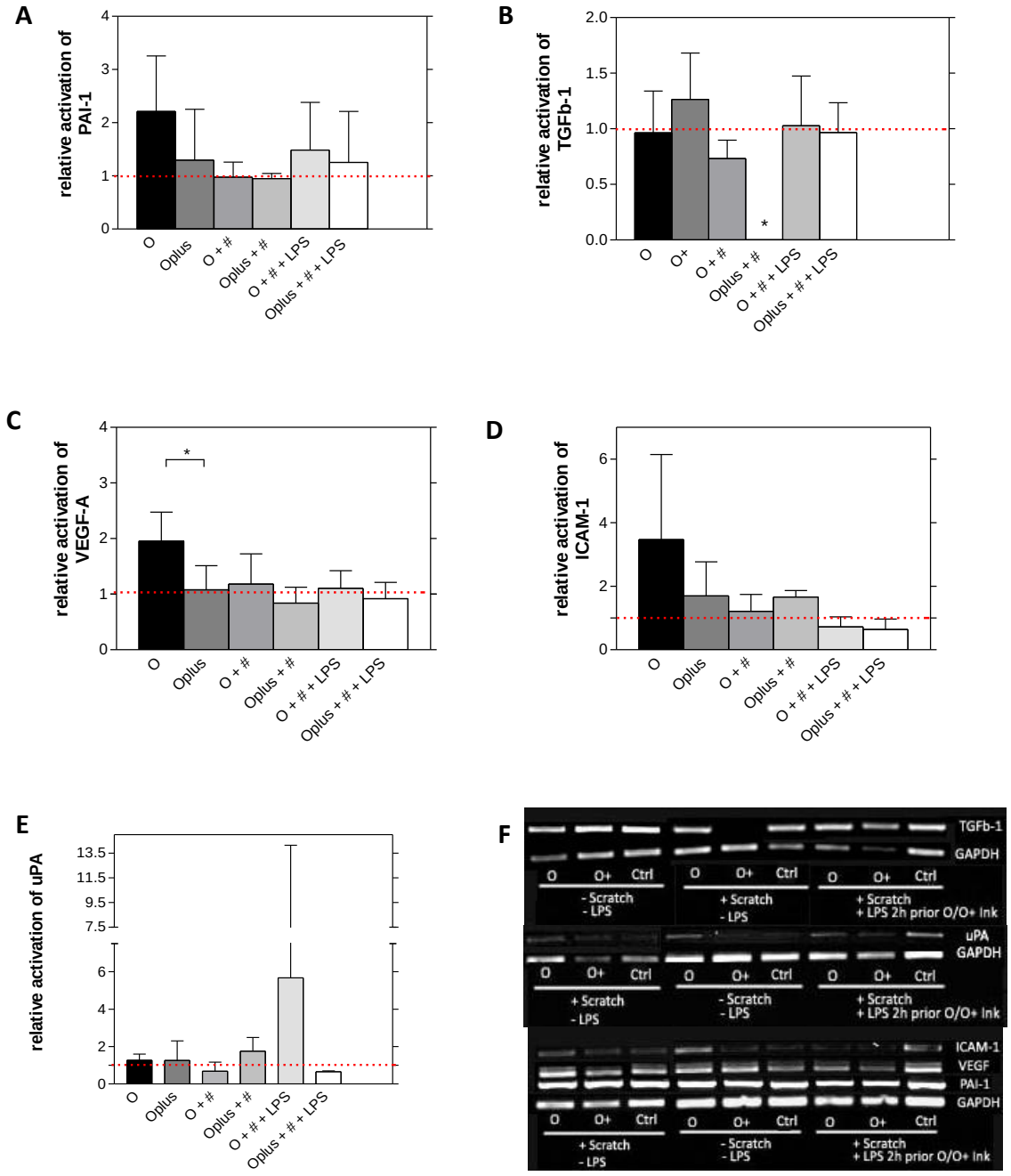


Figure 18. PCR analysis of the indicated genes after O treatment in comparison to control cells and O+. Fibroblasts were incubated with 7 mg/L O or O+ under three different conditions (described at Table 15) for 2 minutes. Control cells were only incubated with sterile aqua dest. The cells were then washed with PBS and grown for 24 hours in fresh cell culture medium in the absence of the antiseptics. Gene expression changes were estimated by PCR using primers for PAI-1 (A), TGFβ-1 (B), VEGF-A (C), ICAM-1 (D) and uPA (E). Representative agarose gel picture of the PCR products (F). GAPDH was used as house keeping gene. Relative activation was calculated by the quotient of the intensity from the PCR signal of treatment cells and reference cells. Reference cells were set as 1 (red dotted line). All data were normalized to GAPDH. Error bars indicate SD from two to four independent experiments (* $p < 0,05$).

In addition, it was analyzed whether higher concentrations of O and O+ cause more severe changes in gene expression of the investigated genes. Therefore, lacerated fibroblasts were treated with 17 mg/L (i.e. IC50) O and O+ and then subjected to rt-PCR.

Upon O+ application, increased levels of genes associated with matrix degradation were detected. Especially, transcription of MMP-3, MMP-2 and PAI-1 seemed to be highly activated in comparison to control cells. Certainly these 3 genes are worth to further investigate and to reduce the big standard deviation as well. On the contrary, O treatment did not induce such dramatic effects. Consequently, the differences between O and O+ were not significant either (Table 16). Definitely, none of the analyzed genes involved in ECM degradation was strongly inhibited in response to antiseptic treatment (Figure 19A). In addition, it was observed that O+ treatment caused a striking increase in VEGF-A1 expression by 2,5 fold (Figure 19C, first column pair). This result might be significant as it was achieved with a perfect duplicate. Furthermore, elevated levels of VEGF-A2, the bigger isoforms of VEGF-A, were measured after O+ application. The VEGF-A2 enhancement might be questionable due to the high standard deviation. ICAM-1 was induced after O and O+ incubation. However, the variation among the independent experiments entailed no significant results (Figure 19C, last column pair). Additionally, O, as well as O+, provoked a decrease in TGF-β1 levels by 60 and 35 %, respectively ($p < 0,05$ for O, non-significant for O+) (Figure 19C, second last column pair).

In summary, it could be shown that antiseptic treatment has generally no dramatic influences on gene expression with only few exceptions. Lacerated fibroblasts produced increased mRNA levels of MMP-3 and MMP-1 in response to O treatment whereas these effects were not detected in wounded and LPS pre-incubated cells. In addition, higher amounts of O and O+ did not induce significant gene activation. It is intriguing that O+ elevated mRNA levels more than O in most cases. Only TGF-β1 was slightly inhibited by O and O+ when increased concentrations (17 mg/L) were applied.

Table 16. Relative gene expression levels of selected genes in lacerated fibroblasts after O incubation in comparison to O+. Gene expression changes were tested upon 17 mg/L O or O+ in lacerated fibroblasts. The depicted values are derived from two independent experiments. The statistical analysis was conducted using Bonferroni-Test. CI confidence interval MD mean difference between relative gene expression change of O and O+.

O vs O+			
Gene	**MD**	**P-value**	**95 % CI**
MMP-1	-0,17	P > 0.05	-2.49 to 2.14
MMP-2	-6,18	P > 0.05	-22.68 to 10.33
MMP-3	-1,18	P > 0.05	-5.66 to 3.30
PAI-1	-5,20	P > 0.05	-17.60 to 7.20
uPA	-0,04	P > 0.05	-1.37 to 1.29
VEGF-A1	-1,35	P > 0.05	-3.51 to 0.81
VEGF-A2	-3,56	P > 0.05	-10.70 to 3.57
TGFβ-1	-0,26	P > 0.05	-0.64 to 0.12
ICAM-1	1,29	P > 0.05	-5.13 to 7.73

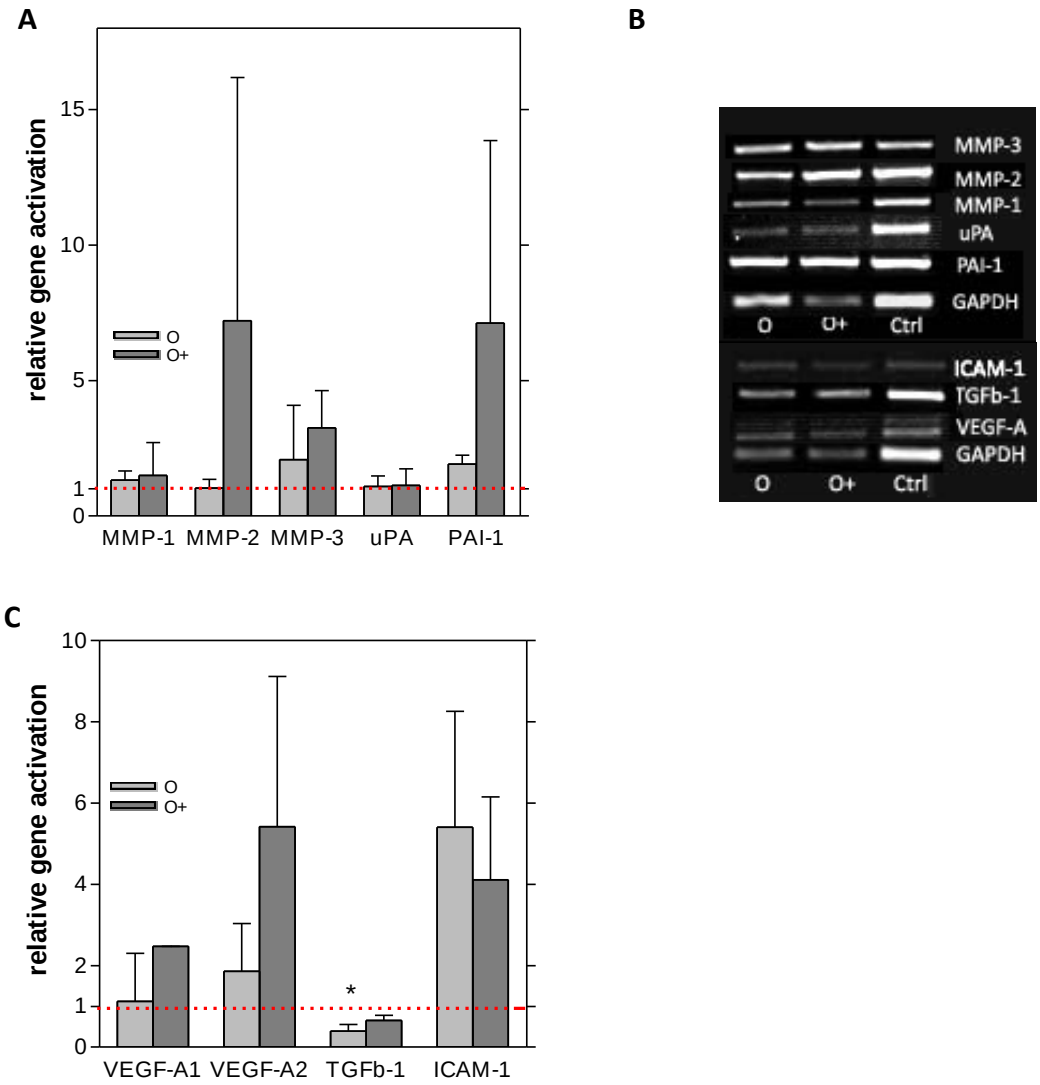


Figure 19. PCR analysis of indicated genes after high concentration treatment with O in comparison to control cells and O+ in wounded fibroblasts. Scratched fibroblasts were incubated with 17 mg/L O or O+ for 2 minutes. The cells were then washed with PBS and grown for 24 hours in fresh cell culture medium in the absence of the antiseptics. Gene expression changes were estimated by PCR using primers for genes involved in ECM degradation (A) and TGFβ-1, VEGF-A and ICAM-1 (C). Representative agarose gel picture of the PCR products (B). GAPDH was used as house keeping gene. Relative activation was calculated by the quotient of the intensity from the PCR signal of treatment cells and reference cells. Reference cells were set as 1 (red dotted line). All data were normalized to GAPDH. Error bars indicate SD from two to four independent experiments (* $p < 0,05$).

The results from Figure 19 should be considered with caution due to the high variation between the duplicates. Nevertheless, these primary results purport that O and especially O+ are potent to induce genes involved in ECM degradation (MMPs and PAI-1), cell-cell interaction (ICAM-1) and angiogenesis (VEGF-A). Therefore, it is worth to substantiate these results by repeating these experiments.

Additionally, the impact of O and O+ on gene expression was analyzed in MCF-7 cells. Therefore, the cell monolayer was lacerated several times, incubated with 7 mg/L of the antiseptic preparations for 2 minutes and were allowed to re-epithelialise for 24 hours. Cells were lysed and then subjected to rt-PCR. In addition, the effect of 1,85 mM glucose, which is the concentration that is administered with 7 mg/L O+, was assessed as well. Neither in untreated control cells nor in O and O+ treated cells, expression of MMP-1, MMP-3, MMP-2 and PAI-1 was observed (data not shown). The presence of 1,85 mM glucose did not cause any severe changes of mRNA level of the depicted genes (Figure 20A, black column). Although the measured mean values of the relative gene expression rate of VEGF-A1, VEGF-A2 and ICAM-1 was smaller after glucose incubation than after O and O+ treatment. O and O+ application did not provoke significant gene activation or inhibition as the relative expression levels were not significantly different than from control cells (Table 17). Only MMP-9 was shown to be decreased by 50 % after O incubation (Figure 20A), but without significance.

All in all, gene expression levels remained unaffected upon the different incubation procedures in lacerated MCF-7 cells.

Table 17. Relative gene expression levels of investigated genes in MCF-7 cells after O incubation in comparison to O+ and control cells. Gene expression changes were tested upon 7 mg/L O or O+ and 1,85 mM glucose in lacerated MCF-7 cells. The depicted values are derived from two to three independent experiments. The statistical analysis was conducted using Bonferroni-Test. CI confidence interval MD mean difference between relative gene expression change of O and O+ Ctrl Control.

	O vs O+			O vs Ctrl			O+ vs Ctrl		
Gene	**MD**	**P-value**	**95 % CI**	**MD**	**P-value**	**95 % CI**	**MD**	**P-value**	**95 % CI**
TGFβ-1	-0,188	P > 0.05	-0.420 to 0.045	-0,172	P > 0.05	-0.451 to 0.107	0,016	P > 0.05	-0.264 to 0.295
TGFβR-1	0,258	P > 0.05	-0.396 to 0.911	0,402	P > 0.05	-0.420 to 1.224	0,144	P > 0.05	-0.678 to 0.967
VEGF-A1	0,083	P > 0.05	-0.516 to 0.681	0,159	P > 0.05	-0.632 to 0.950	0,076	P > 0.05	-0.715 to 0.868
VEGF-A2	0,042	P > 0.05	-0.485 to 0.569	0,063	P > 0.05	-0.633 to 0.760	0,021	P > 0.05	-0.675 to 0.718
ICAM-1	0,013	P > 0.05	-1.354 to 1.381	0,311	P > 0.05	-1.639 to 2.262	0,298	P > 0.05	-1.653 to 2.249
MMP-9	-0,568	P > 0.05	-1.140 to 0.004	-0,505	P > 0.05	-1.256 to 0.246	0,063	P > 0.05	-0.688 to 0.814

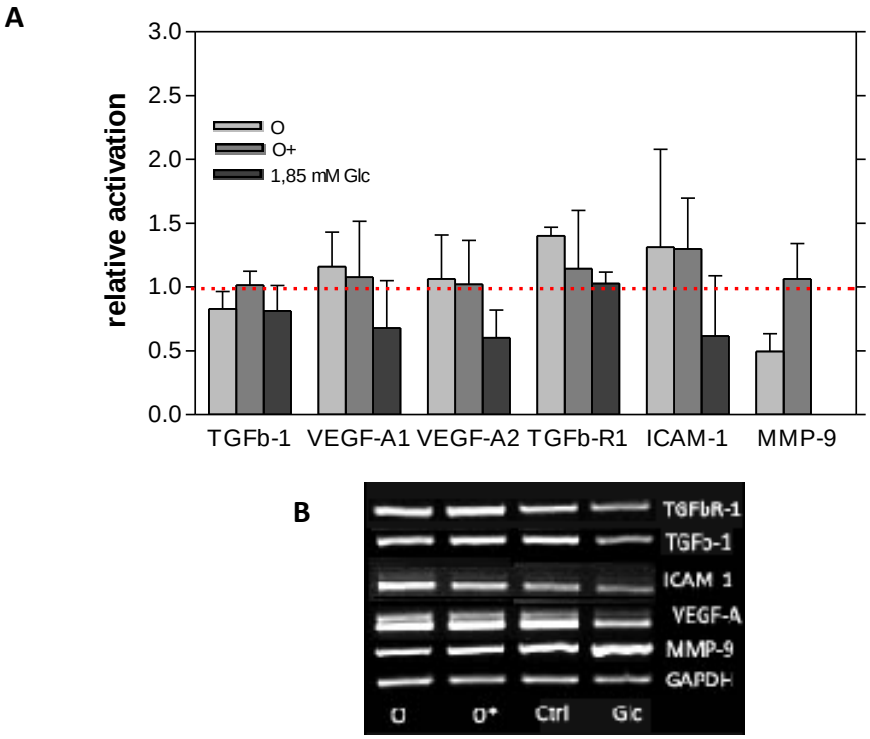


Figure 20. PCR analysis of investigated genes after O treatment in comparison to control cells and O+ in wounded MCF-7 cells. Scratched MCF-7 cells were incubated with 7 mg/L O or O+ or 1,85 mM glucose for 2 minutes. The cells were then washed with PBS and grown for 24 hours in fresh cell culture medium in the absence of the antiseptics. Gene expression changes were estimated by PCR using primers for the indicated genes (A). Representative agarose gel picture of the PCR products (B). GAPDH was used as house keeping gene. Relative activation was calculated by the quotient of the intensity from the PCR signal of treatment cells and reference cells. Reference cells were set as 1 (red dotted line). All data were normalized to GAPDH. Error bars indicate SD from two to four independent experiments.

4.4. Cooperativity between antibiotics and antiseptics

Antiseptics and antibiotics are commonly used in combination in clinical practise. However, little is known about possible interactions between those two antimicrobial agents. Therefore, the effects of a combined application of antibiotics and antiseptics were assessed using the agar diffusion test. We applied antiseptics, antibiotics and both in combination onto paper discs mounted on the agar and measured the diameter of the inhibited zone created from diffusion of the antimicrobials. To give positive cooperation a combined application must result in a greater diameter than the antibiotics alone.

As summarized in Table 18, some antibiotics (e.g. Linezolid, Vancomycin, Metronidazol or Penicillin G and V) were not able to inhibit bacterial growth. Similarly, O and O+ did not have bactericidal efficiency when only 5 µl and 10 µl were applied. However, the formation of a zone of inhibition was observed when O/O+ were combined with the antibiotics. This indicates a positive cooperation between those compounds. Synergistic effects between O and O+ and antibiotics were observed for 13 out of the 20 (2/3) tested combinations (in *B. petrii)* (Figure 21). Moreover, the simultaneous application of O/O+ and antibiotics did not led to significant negative cooperations. Only 5 weak antagonistic effects were measured. In comparison, concomitant application of Beta-Isodona® or Taurolin® and antibiotics caused a decrease of bactericidal efficiency of the antibiotic in more cases than O or O+. Additionally, Taurolin® in combination with the antibiotics did never result in a positive cooperativity. Only Chlorhexidine together with the antibiotic compounds acted in a more synergistic manner than O or O+ against *B. petrii*.

Table 18. Results from the agar diffusion test with *B. petrii*. For cooperativity testing, 10 µl of the antiseptic agents were applied directly onto the antibiotic paper discs. Additionally, antibiotics and antiseptics were tested as single compounds. The mean value ± SD (O, O+, antibiotics alone: n =3; Chl, Bet, Tau: n =2) of the diameter of the formed zone of inhibition are illustrated.

Combination:	O [mm]	O+ [mm]	Chl [mm]	Bet [mm]	Tau [mm]	only antibiotics [mm]
- 5 µl (antiseptic only)	0 ± 0	0 ± 0	17,5 ± 0,7	0 ± 0	0 ± 0	-
- 10 µl (antiseptic only	0 ± 0	0 ± 0	24 ± 0	10 ± 0	0 ± 0	-
- 20 µl (antiseptic only)	10 ± 1	10,0 ± 0,6	26 ± 0	12,5 ± 0,7	0 ± 0	-
Linezolid	9,3 ± 5,3	9 ± 5,5	26 ± 1,4	10 ± 0	0 ± 0	0 ± 0
Vancomycin	9 ± 5,8	4 ± 5,3	26 ± 0	10,5 ± 0,7	0 ± 0	0 ± 0
Metronidazol	9,5 ± 1,7	7,7 ± 5,2	26,5 ± 2,1	10,5 ± 0,7	0 ± 0	0 ± 0
Cotrimoxazol	10 ± 1,5	9 ± 0,6	27 ± 2,8	10 ± 0	0 ± 0	0 ± 0
Ciprofloxacin	23 ± 4,7	26,3 ± 1	27 ± 2,8	12,5 ± 3,5	13,5 ± 2,1	23 ± 6,2
Levofloxacin	28,6 ± 3,6	32,3 ± 5,2	30,5 ± 2,1	23 ± 4,2	22 ± 1,4	34,7 ± 4,9
Moxifloxacin	31,3 ± 2,1	30,7 ± 2,6	29,5 ± 2,1	24,5 ± 3,5	25 ± 1,4	30,7 ± 8,3
Gentamicin	17,7 ± 5,0	17,3 ± 4,6	26 ± 1,4	18 ± 2,8	21 ± 1,4	19 ± 3,6
Clarythromycin	25,5 ± 5,7	24,7 ± 0,6	26,5 ± 2,1	16 ± 8,5	24,5 ± 2,1	25,3 ± 9,3
Tigecyclin	38,3 ± 4,5	38,3 ± 3,6	36 ± 0	23,5 ± 5,0	32 ± 2,8	38,3 ± 2,5
Doxycyclin	48,3 ± 7,2	43 ± 9,2	43,5 ± 3,5	44 ± 2,8	40 ± 2,8	38 ± 5,7
Cefotaxim	9,7 ± 1	9,7 ± 1,2	25 ± 1,4	10 ± 0	0 ± 0	0 ± 0
Imipenem	39 ± 4,6	40 ± 5,1	38 ± 0	36,5 ± 2,1	38 ± 2,8	36 ± 1,4
Piperacillin/Tazobactam	38,7 ± 4,2	39 ± 12,7	39,5 ± 0,7	34 ± 5,7	34,5 ± 0,7	39,3 ± 4,6
Amoxicillin/Clavulanic acid	22,3 ± 2,5	22,7 ± 3,8	24,5 ±2,1	19,5 ± 0,7	20 ± 0	23 ± 5,2
Amoxicillin	8,7 ± 1	10 ± 1,5	25 ± 1,4	9 ± 0	10 ± 7	0 ± 0
Penicillin G	9 ± 1	10,3 ± 1,5	24,5 ± 2,1	10,5 ± 0,7	0 ± 0	0 ± 0
Penicillin V	9,3 ±1,5	10 ± 2	24,5 ± 2,1	9 ± 0	0 ± 0	0 ± 0
Cefadroxil	8 ± 1,5	10,3 ± 2,1	24 ± 1,4	10 ± 0	0 ± 0	0 ± 0
Lincomycin	7,7 ± 1,2	8 ± 1,7	23 ± 2,8	9,5 ± 0,7	0 ± 0	0 ± 0

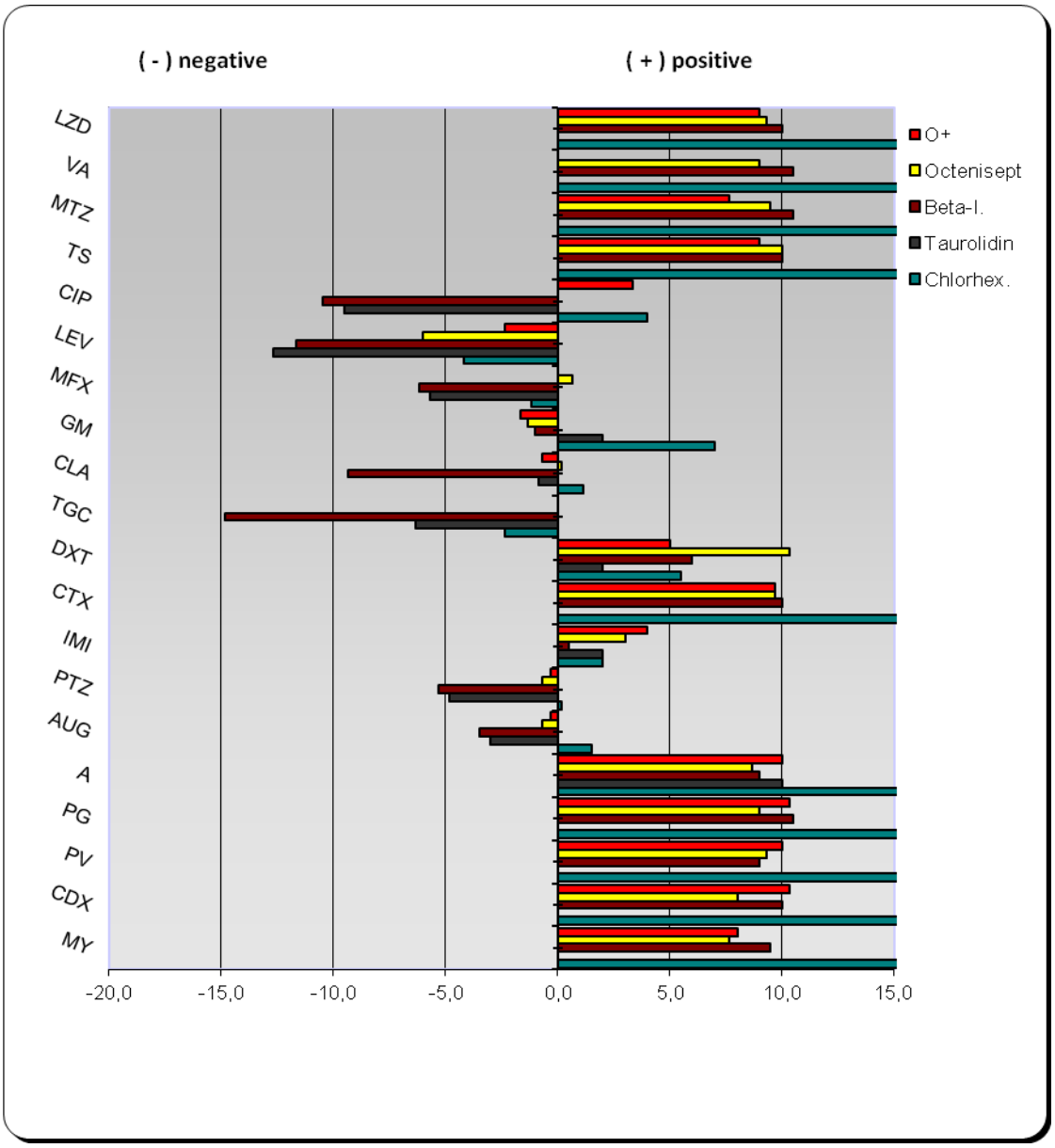


Figure 21. Cooperativity between antibiotics and antiseptics against *B. petrii*. Cooperativity between antibiotics and antiseptics was assessed by the agar diffusion test. Antiseptics, antibiotics and both in combination were applied. For that, 10 µl of the antiseptics were dropped onto the antibiotic paper disc mounted on the agar. After 48 hours, the diameter of the zone of inhibition was measured. Positive cooperativity was given when the diameter of zone of inhibition after the combined treatment was greater than that after application of the antibiotic alone. The presented data are the result from three independent experiments. Taurolidin = Taurolin®

These tests with *B. petrii* pointed toward the potency of combined application of bactericidal principles. Therefore, the experiments were repeated with *S. aureus.* Thereby, the results showed strong strain specificity of antibiotic sensitivity. Substantially, as illustrated in Table 19, the antiseptic agents themselves were able to

inhibit bacterial growth at all applied volumina as measured by the diameter of the zone of inhibition. Only Taurolin® was not potent against *S. aureus* if applied alone. O and O+ were more efficient against *S. aureus* than against *B. petrii* (Table 19). In this setting, O+ caused the most synergistic (9 out of 20) and the least antagonistic effects (7 out of 20) in combination with the antibiotics. Antibiotic application together with the other antiseptics under investigation led to negative cooperations in more than half of the tested combinations (Figure 22). One distinctive observation during these experiments was that *S. aureus* developed antibiotic resistance. After 2 days in the zone of inhibition re-growth of colonies were observed, but never with O+ (data not shown).

Table 19. Results from the agar diffusion test with S. aureus. For cooperativity testing, 10 µl of the antiseptic agents were applied directly onto the antibiotic paper discs. Additionally, antibiotics and antiseptics were tested as single compounds. The mean value ± SD (O, O+, antibiotics alone: n =3; Chl, Bet, Tau: n =2) of the diameter of the formed zone of inhibition are illustrated.

Combination:	O [mm]	O+ [mm]	Chl [mm]	Bet [mm]	Tau [mm]	only antibiotics [mm]
- 5 µl (antiseptic only)	3,7 ± 5,2	15 ± 0	10 ± 0	10 ± 0	0 ± 0	-
- 10 µl (antiseptic only	10 ± 0	14 ± 2,8	12 ± 0	12 ± 0	0 ± 0	-
- 20 µl (antiseptic only)	15 ± 0	15 ± 0	15 ± 0	15 ± 0	0 ± 0	-
Linezolid	10 ± 0	24 ± 0	20 ± 0	25 ± 0	20 ± 0	23,5 ± 2,1
Vancomycin	15 ± 0	15 ± 0	15 ± 0	15 ± 0	15 ± 0	15,5 ± 0,7
Metronidazol	12 ± 0	11 ± 0	15 ± 0	13 ± 0	0 ± 0	0 ±0
Cotrimoxazol	10 ± 0	11 ± 0	16 ± 0	13 ± 0	0 ± 0	0 ± 0
Ciprofloxacin	21 ± 0	22 ± 0	20 ± 0	23 ± 0	21 ± 0	23,5 ± 2,1
Levofloxacin	21 ± 0	24 ± 0	23 ± 0	25 ± 0	22 ± 0	24,5 ± 3,5
Moxifloxacin	25 ± 0	27 ± 0	27 ± 0	25 ± 0	25 ± 0	28 ± 4,2
Gentamicin	11 ± 0	11 ± 0	15 ± 0	10 ± 0	0 ± 0	0 ± 0
Clarythromycin	20 ± 0	20 ± 0	20 ± 0	21 ± 0	22 ± 0	22,5 ± 2,12
Tigecyclin	15 ± 0	15 ± 0	17 ± 0	15 ± 0	17 ± 0	20,5 ± 0,7
Doxycyclin	20 ± 0	20 ± 0	21 ± 0	22 ± 0	20 ± 0	24,5 ± 3,5
Cefotaxim	25 ± 0	25 ± 0	25 ± 0	25 ± 0	25 ± 0	25,5 ± 0,7
Imipenem	40 ± 0	36 ± 0	40 ± 0	36 ± 0	40 ± 0	45 ± 1,4
Piperacillin/Tazobactam	25 ± 0	30 ± 0	25 ± 0	26 ± 0	28 ± 0	30 ± 0
Amoxicillin/Clavulanic acid	20 ± 0	30 ± 0	22 ± 0	18 ± 0	24 ± 0	37 ± 4,2
Amoxicillin	15 ± 0	30 ± 0	18 ± 0	14 ± 0	24 ± 0	31 ± 1,4
Penicillin G	11 ± 0	30 ± 0	18 ± 0	10 ± 0	0 ± 0	39 ± 1,4
Penicillin V	10 ± 0	40 ± 0	19 ± 0	10 ± 0	0 ± 0	38 ± 2,8
Cefadroxil	20 ± 0	22 ± 0	20 ± 0	18 ± 0	19 ± 0	27,5 ± 3,5
Lincomycin	10 ± 0	20 ± 0	20 ± 0	10 ± 0	0 ± 0	21,5 ± 2,1

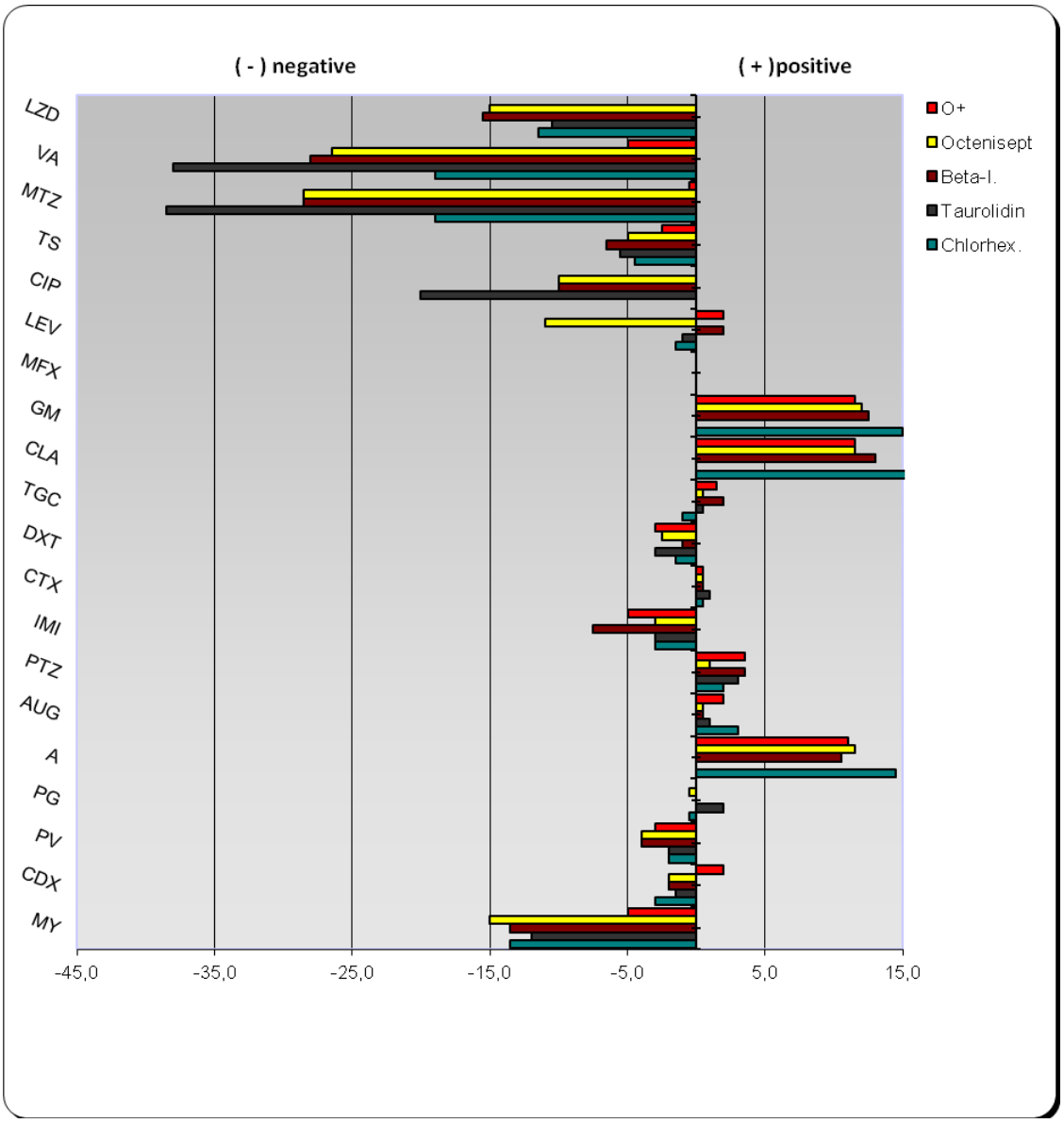


Figure 22. Cooperativity between antibiotics and antiseptics against *S. aureus*. Cooperativity between antibiotics and antiseptics was assessed by the agar diffusion test. Antiseptics, antibiotics and both in combination were applied. For that, 10 µl of the antiseptics were dropped onto the antibiotic paper disc mounted on the agar. After 48 hours, the diameter of the zone of inhibition was measured. Positive cooperativity was given when the diameter of zone of inhibition after the combined treatment was greater than that after application of the antibiotic alone. The presented data are the result from three independent experiments. Taurolidin = Taurolin®

Taken together, O+ was the antiseptic that had the most positive effect on bactericidal properties of antibiotics against *S. aureus* and hardly led to negative cooperations in combination with antibiotics. Chlorhexidine enhanced the potency of the tested antibiotics the most against *B. petrii* in comparison to the other antiseptics. These tests demonstrated that indeed, strong interactions between antibiotics and antiseptics are possible which can both, enhance and reduce the bactericidal efficiency of an antibiotic compound.

5 Discussion

All open wounds are contaminated by bacteria and hence, are at some risk of becoming infected. Since wound infection is associated with a prolonged healing time, it is the wound care practitioner's goal to prevent infection in order to reduce the suffering of the patient and hospital costs (Leaper and Durani, 2008, Atiyeh et al., 2009). Infection of a post surgical wound is associated with approximately 4000 $ hospital cost per infected patient. The application of systemic antibiotics and topical antiseptics is one of the first strategies to prevent or treat already existing wound infections. Considering the developing bacterial resistance against antibiotics and the burden of treatment, their usage should be carefully chosen. Therefore, the use of antiseptic agents is becoming increasingly important in wound management (Hirsch et al., 2010) because no danger of developing resistance has been reported yet (Hubner et al., 2010). However, concomitant with the toxicity of antiseptics against microorganisms, they may harm host tissue as well. Due to this fact, choice of agents and mode of applications must be optimized to yield an excellent wound care (Marquardt et al., 2010). Additionally, antiseptics and antibiotics are often administered concomitantly although not much is known about possible synergistic or antagonistic interactions between those compounds (Hubner et al. 2010).

In this study, Octenisept® (O) and a new Octenisept® formulation, which is diluted 1:2 with a 5 % glucose solution (O+), were tested in regard to cell proliferation, migration and interactions with antibiotics and compared to Chlorhexidine, Beta-Isodona® and Taurolin®. In in vitro studies it was demonstrated that Octenisept® has higher microbicidal efficiency and a better biocompatibility than Chlorhexidine and Beta-Isodona® (Koburger et al. 2010; Muller and Kramer, 2008). No studies comparing Octenisept® and Taurolin® were found.

So far, Octenisept® was diluted with saline. The main idea behind this was to reduce pain by decreasing osmotic provocation when O was applied alone. Some observations in clinical practise support the idea not to dilute with saline. This led to test glucose. In a few case reports, it was observed that O+ is superior to O with respect to wound healing. These observations were validated in our working group where we

demonstrated that the presence of glucose has a favorable effect on cell adhesion. In order to go deeper into the molecular mechanisms, it was assessed whether O and O+ affect gene expression pathways involved in wound healing and re-epithelialisation and cell proliferation as outcome of these was measured. Obviously, due to the fact, that the here presented results were performed in vitro, the reflection to the in vivo situation needs further testing in organ culture. Most obviously the paradox observation that O is extremely cytotoxic in tissue culture but no noticeable effect is observed in wounded skin cells, calls for more insight and tells a story of our hazy knowledge of the mechanism behind this. Speculations are that binding of Octenisept® to ECM components, like CHS, forms an active layer. This layer retains the antimicrobial efficiency of O while the rest of the applied substance is sequestered from the cells.

Indeed, MTT-test assays demonstrated that incubations with various O and O+ concentrations for 24 hours substantially inhibit MCF-7 cell proliferation, whereby no significant difference between the two formulations was observed. Even a dilution of 1:1024 caused a decrease by 50 % of MCF-7 cell proliferation rate, but with 1:4096 dilution of O+ growth rates were restored to normal and moreover cell proliferation was even enhanced (60 % over control cells) (Figure 9A). This was not observed so with O.

Sulfated glycosaminoglycans (GAGs) like CHS are highly negatively charged and abundant in the ECM and on cellular surfaces (Ogawa et al. 2012). Apart from being a structural element, it can bind growth factors like FGF and thus CHS regulates wound healing and cell migration (Malmström et al., 2012). Furthermore, negatively charged GAGs are likely to interact with cationic antimicrobials like octenidine. Dermal fibroblasts are embedded in the ECM and it is not clear whether octenidine antiseptics are sequestered from the cellular membranes by CHS in the ECM. The complex formation of octenidine and ECM components could substantially decrease the cytotoxicity of octenidine while the antimicrobial activity is maintained. This would

explain the paradox toxicity divergences of octenidine between in vitro and in vivo studies. Our experiments here serve as a starting point to investigate and understand the action of the antiseptic octenidine. Two different application strategies were tested: 1. adding CHS to the antseptic stock solution and 2. coating the dish bottom with CHS. The first should unveil effects of interaction whereas the latter come within reach to mimic ECM embedding effects of O and O+.

Hence we scouted for effects in the presence of 250 mg/L CHS and 125 mg/L (= 1:4 dilution) O/O+. Cell proliferation was only 5-10 % of control cells (Figure 9B) and the same O/O+ concentrations, without CHS, allowed cell proliferation 25-30 % of control cells. A 1:4096 dilution (as above in MCF-7) in the presence of CHS did not significantly interfere with normal cell proliferation, but the growth rate was never better than that of control cells.

Indeed, the addition of CHS to the antiseptic stock solutions rather elevated the cytotoxic potential of O and O+. However, when microtiter plates were coated with CHS by pre-incubation with 1 g/L CHS and then serial dilutions of O and O+ were added, the result looked like the one from the experiment illustrated in Figure 9A where growth rates were restored to normal. Again at 1:4096 dilution O+ enhanced cell proliferation significantly ($p < 0,001$) by 50 % in comparison to control cells (Figure 10) when grown on CHS coat.

This indicated that it makes a difference whether CHS is added directly to the antiseptic stock solutions or CHS and O/O+ are added successively to the microtiter plate and is the first experimental indication towards a matrix dependent action of CHS. With the latter procedure it was observed that small amounts of CHS (0,24 mg/L) decreased the cytotoxicity of high concentrations of O+ (0,5 g/L) by approximately 40 %. For comparison, 0,5 g/L of O or O+ without CHS caused a complete loss of cell proliferation under the same test conditions (data not shown). The combination of CHS and O+ is significantly better tolerated than with O (Figure 11). When reflecting these results to the in vivo situation, where dermal fibroblasts are embedded in the

ECM, it can be speculated that O+ is better sequestered from the cellular membranes by CHS in the ECM than O and thus, is less cytotoxic.

Furthermore, our results showed that the toxicity of O and O+ can be ameliorated when the contact time and the concentration of the antiseptics are modulated. When the 24 hours exposure time was reduced to 30 minutes, the observed harmful effects were decreased. Since here the MTT-test was performed directly after the 30 minutes incubations, these values measure the impact on cell adhesion and not cell proliferation. For adhesion inhibition the IC50 value of O and O+ was determined to be at 15 mg/L (i.e. 1:30 dilution) after 30 minutes contact time (Figure 13). In accordance with the manufacturer's instructions, a contact time of 2 minutes is sufficient in order to yield an optimal wound treatment.

In our test system, 15 mg/L O or O+ reduced adherent cell number by only 30 % after 2 minutes incubation time (Figure 14B). In comparison, the same concentration caused 50 % loss of cell adhesion after 30 minutes. Lower concentrations (7 mg/L i.e. 1:75 dilution, regarded as IC10 value, which signifies that 90 % of the cells adhere after 30 minutes incubation) did not harm MCF-7 cells after an exposure time for up to 10 minutes. Here, the O+ formulation resulted in better adhesion than O (Figure 14C).

Additionally, a 1:30 or 1:75 dilution of O and O+ did not impair or slow down fibroblast cell migration in our in vitro laceration assay which is a standard method for investigating wound closure after scratching the cellular monolayer. As comparison, Beta-Isodona® application decelerated fibroblast migration and inhibited wound closure (Figure 15A and B).

In summary, from these experiments it is obvious that octenidine can exert a very high cytotoxic potential. Especially if it is taken into account that the octenidine concentration of Octenisept® is 1 g/L and the stock solution is usually not more diluted than 1:2. However, we assume that if an antiseptic agent is topically applied onto a wound it becomes diluted by an unknown extent by the wound exudate which is a consequence of the inflammatory mediators released by leukocytes (Barrientos et al.

2008). These events lead to increased permeability of the capillaries (Singer and Clark, 1999). Consequently, leakage of fluid – the exudate – into the open wound occurs and gets in touch with the administered antiseptic compound and dilutes it. In addition, it is important that it is not inactivated by interactions with components of the exudate. In contrast to Chlorhexidine, octenidine is known to have no reduction in activity upon albumin or mucin contact and is not sensitive to hydrolysis (Stahl et al., 2010). Moreover, at the cellular level the concentration can be substantially reduced as most of the octenidine molecules are kept in the mesh of the ECM. Only a fraction of the applied substance reaches the cellular membrane which would cause cytotoxic effects. Concomitantly, it is suggested that octenidine could be continuously released from the ECM to sustain its antimicrobial activity (Muller and Kramer, 2007). Essentially, cells within human tissues embedded in ECM components better tolerate the exposure to antiseptic compounds (Muller and Kramer, 2008). Upon topical application onto a wound, antiseptics first get in contact with cell debris, fibrin or blood and the deeper more sensitive tissue layers are not damaged (Marquardt et al. 2010). Indeed, octenidine has proven its efficiency and safety in wounded tissue samples (Stahl et al., 2010)) and in clinical practise (Vanscheidt et al., 2012, Eisenbeiß et al., 2012). Especially the latter report emphasizes the good tissue tolerance with concomitant high antimicrobial efficiency in burns.

Until now, Octenisept® is not recommended for intra-operation in the abdominal surgery because ascites and chemical peritonitis occurred upon application (Hubner et al. 2010). Our results allow the suggestion that upon optimizing concentration and contact time, the intra-abdominal application must be safe. The here investigated glucose addition to the classical formulation was reported to have no harmful effects at this application area and also other beneficial results in case reports were observed. MTT-test analysis showed that O+ allows indeed, higher proliferation rates than O in long-term application (24 hours). Moreover, in our previous experiments O+ led to less cell detachment than O after 30 minutes contact time.

Next we tried to scout how O and O+ did influence cell proliferation and migration at the molecular level. We measured changes in expression of genes implicated in the re-epithelialisation process after incubation with 7 mg/L O and O+ for 2 minutes on confluent and on lacerated fibroblasts. This concentration, which corresponds to the IC10 value, was chosen to minimize cytotoxic effects. We found that in non-lacerated fibroblasts O leads to a significant induction of MMP-3 and MMP-1. Especially, MMP-3 levels were significantly (three fold) higher after O incubation than with O+. PAI-1 and uPA levels were not significantly altered after antiseptic treatment and the laceration procedure. In addition, the IC50 value of the antiseptic compounds was tested on lacerated fibroblasts as well. At these higher concentrations (IC50 value i.e. 27 mg/L), O+ caused enhanced levels of MMP-2, PAI-1 and MMP-3. However, there were high variations among the different experiments. MMPs, uPA and PAI-1 belong to the group of extracellular matrix degrading enzymes, with partly overlapping functions during wound healing (Lund et al., 1999). MMP-3 has a broad spectrum of substrate specificity and cleaves different types of collagen, fibronectin, gelatin and pro-MMPs like MMP-1 (Ye et al., 1996). Both enzymes, as well as other members of the MMP family and the PA-system, are known to be upregulated during repair processes (Parks, 1999). However, after disrupting the cellular monolayer and subsequent antiseptic treatment, no significant changes in expression levels of the investigated genes were detected. Such changes may occur only in the lacerated zone only and there is no possibility to select for cells that are from the wounded area only. In order to detect a significant change of gene expression levels, the signal must be very strong or act in a paracrine manner over the entire cellular layer.

Gene expression analysis of MMPs must be clarified also on the activity level as zymogens need activation by proteolytic cleavage to be functional (Massova et al., 1998). Therefore we performed zymogram analysis and we observed that the active form of MMP-2 and MMP-9 are generated but without detectable changes among the different treatments (data not shown). The sensitivity of this assay might be too low to detect subtle changes. Thomas et al. demonstrated that Chlorhexidine is a potent inhibitor of pro-MMP-2 and pro-MMP-9 release in dermal fibroblasts stimulated with

TNF-α and TGF-β1. On the contrary, Beta-Isodona® enhanced pro-MMP-9 secretion at low dosage rate, whereas higher-dosed exposure completely abrogated pro-MMP release (Thomas et al., 2009). In our laceration assay, dilutions of 1:75 and 1:30 of the Chlorhexidine stock solution did not inhibit cell migration. Beta-Isodona® (1:30) was a potent inhibitor of cell migration. Our laceration assay confirms that a certain pattern of MMPs is not essential at this level. The absence of a specific member of the MMP family will not necessarily lead to impaired wound healing as demonstrated by several mouse models. A reason for this is the redundant function of MMP family members and the presence of other protease systems including the PA system (Frossing et al. 2010, Lund et al. 1999).

TGF-β1 expression was not affected by the antiseptics when applied at lower (7 mg/L) concentrations. The exceptional observation was the complete TGF-β1 mRNA absence after O+ application on wounded fibroblasts while untreated and also O treated cells exhibited measurable TGF-β1 levels. However, target genes for TGF-β involved in migration, like VEGF-A, PAI-1 or MMPs (Dallas and Loskutoff 2005, Chakraborti et al., 2003, Neufeld et al. 1999), were not affected by these conditions. Higher amounts (IC50 = 15 mg/L) of O and O+ led to decreased TGF-β1 levels. Here, O was a more potent inhibitor than O+. This is remarkable, as 7 mg/L of O+ caused a complete loss of TGF-β1 expression. This result should be estimated with proper caution. Although TGF-β1 was decreased after higher O+ concentration, VEGF-A1 was increased (2,5 fold) after the same O+ treatment. The result was achieved by a perfect duplicate but upon statistical analysis it was without significance. The exact role of TGF-β in wound healing, though investigated with great effort, is still not well understood in full detail. It was reported that the cytokine inhibits keratinocyte proliferation. As underlined by several mice models, TGF-β therefore acts as negative regulator for re-epithelialisation. On the contrary, it was demonstrated that TGF-β enhances cells migration due to increased expression of integrins (Amendth et al., 2002) and acts as chemotactic factor for fibroblasts, macrophages and neutrophils. Moreover, it stimulates collagen deposition from fibroblasts and thus, is important for the

formation of the granulation tissue (Bowler et al., 2001). From these reports, it can be concluded that the outcome of TGF-β signalling is dependent on the cell type and the stage of wound healing. If octenidine is indeed an inhibitor of TGF-β1, one can speculate that the antiseptic can therefore enhance re-epithelialisation but the strength of a wound might be decreased. This would be a consequence of reduced collagen deposition by fibroblasts and a delay in the formation of new tissue. This assumption is worth for further investigation whereby it should be kept in mind that wound healing process is very complex and needs the interplay of many signalling molecules.

As mentioned earlier, O+ was shown to enhance cell adhesion in vitro, reflecting the formation of stable cell-matrix complexes. Therefore, ICAM-1 expression was analyzed and no significant alterations were observed. Only after O incubation (in non-lacerated fibroblasts) and higher O+ concentrations (17 mg/L; in lacerated fibroblasts), ICAM-1 levels were increased in comparison to control cells but without significance. Due to the fact that ICAM-1 is rather responsible for cell-cell interactions between endothelial cells and leukocytes (Robledo et al., 2003), fibroblasts may be not the ideal cell type to assess ICAM-1 expression changes. Moreover, other integrins like integrin $\alpha_v\beta_3$, by which cells adhere to VN (Czekay and Loskutoff, 2009) will bear more importance.

As bacterial contamination exerts its deleterious effects, among others, by LPS generation we added LPS to mimic bacterial contamination. Surprisingly, such treatment did not cause any measurable gene expression alteration. This was quite an unexpected result, since the applied LPS concentration (10 µg/ml) was high. One reason for this may be, that the investigated gene expression alterations are not directly influenced by LPS and incubation time (2 hours) was too short to detect secondary effects.

As a second cell culture model, gene expression experiments in lacerated MCF-7 cells were performed and yielded similar result what added significance to the fibroblast

results reported above. Incubation with 7 mg/L O or O+ neither enhanced nor decreased gene transcription of the assessed genes. The glucose concentration delivered with 7 mg/L of O+ (i.e. 1,85 mM glucose) was tested on lacerated MCF-7 cells in addition. This treatment slightly decreased VEGF-A1 and VEGF-A2 and ICAM-1 levels, but without high significance. A study conducted by Qian et al. demonstrated that VEGF mRNA is inducible in response to high glucose exposure (30mM), whereas lower amounts (3mM) showed no effect (Qian et al., 2011). The here applied glucose concentrations are too low to cause significant changes in gene expression. This suggests that the observed differences between O and O+ in the cell proliferation and cell adhesion experiments are not a consequence of severe changes in gene expression in response to the presence of glucose in O+. It can be speculated that glucose rather interacts with octenidine that changes its cytotoxic properties.

From these experiments, it can be stated that treatment with O and O+ alters gene expression profile of fibroblasts and MCF-7 cells only very subtle. In concentrations close to the stock solutions it caused cellular stress and levels of certain genes rose but with high variation which was more profound after O+ exposure (Figure 19). These primary results purport that O and especially O+ are potent to induce genes involved in ECM degradation (MMPs and PAI-1), cell-cell interaction (ICAM-1) and angiogenesis (VEGF-A). Therefore, it is worth to substantiate these results by repeating these experiments.

In long term application of O+ (24 hours, 96 well microtiter plate with a coat of various O/O+ concentrations up to 1:4096) we observed significantly enhanced proliferation with the lowest O+ concentration over the same O concentration. This allows the speculation that stress response induction is beneficial and possibly occurs by preparing cells for survival.

Antibiotics and antiseptics are suspected to interact with each other upon concomitant application. In clinical practise, the clarification of possible interaction between antibiotics and antiseptics is of great interest. To clarify this, we performed agar diffusion tests with two, a pathogenic and a non-pathogenic bacterial strain. In this

test system, bacteria free zones are generated by diffusion of the antibiotic from the endowment disc on a bacterial lawn and are designated as the zone of inhibition. The diameter of the zone of inhibition is a measure for bactericidal efficiency of the investigated antimicrobials. A positive cooperativity was given when the diameter of the zone of inhibition was greater upon combined application of antibiotics and antiseptics than upon antibiotic application. Simultaneous incubation of O+ and various commonly used antibiotics caused growth inhibition of the non-pathogenic *B. petrii* in all combinations. Remarkably, antibacterial efficiency was enhanced in 13 out of the 20 tested combinations suggesting a synergistic effect between O+ and antibiotics. Similar characteristic could also be ascribed to O and Chlorhexidine which had the most positive and least negative effect on bactericidal properties of the antibiotics. Beta-Isodona® was as potent as O or O+ to increase the efficiency of the antibiotics but on the other hand more negative cooperations were measured than with O or O+. Synergistic effects between Taurolin® and antibiotics were only observed once (with Amoxicillin). In the other cases, bactericidal efficiency of the antibiotics was reduced or no influence was observed. When the experiment was repeated with *S. aureus*, O+ was the best choice among the tested antiseptics. It had the most positive cooperations with the antibiotics and concomitantly, the least negative. Again, Taurolin® was the compound that interfered with the antibacterial efficiency of the antibiotics the most. It is obvious from these results that antibiotics and antiseptics have certain interactions that alter their bactericidal properties and hence, their indication for application. Positively charged octenidine may associate with antibiotic compounds like β-lactame antibiotics via electrostatic interactions and guide it to the bacterial cell wall more efficiently as octenidine has a high affinity for negatively charged cell wall components. Conversely, complex formation may lead to sequestration and thus, reduced availability of the antimicrobial compounds.

Surprisingly, O+ was as potent as O to inhibit bacterial growth. The argument that the presence of glucose may support bacterial proliferation and survival was not effective. Another remarkable observation was that in the course of the growth inhibition experiments, *S. aureus* developed antibiotic resistance. Distinct colonies re-grew

within the zone of inhibition after 48 hours. In combinations with O+ these resistant clones were not detected. This would give a complete new meaning to O+ activity if this result could be further elucidated into detail.

In summary, both O and O+ did not interfere with wound healing processes under certain conditions that were pointed out here.

Concentrations, close to the stock solutions, of O and O+ caused cytotoxic effects in the tissue culture. Despite profound cell cytotoxicity, we found that a lower concentration of O+ better supports cell proliferation than the same concentration of O after 24 hours incubation. This is in accordance with case reports. Both, O and O+, had no harmful effects on cell migration. Molecular mechanisms behind the better biocompatibility of O+ in the 24 hours cell proliferation assays need further elucidation. So far our data strenghtens the speculation that ECM components like CHS could decrease the cytotoxicity of octenidine by sequestering the molecule from the cellular membrane without reducing its microbicidal properties. In addition, here it was demonstrated for the first time, that O+ can enhance bactericidal efficiency of commonly used antibiotics whereby it was superior to O. These results point toward that the dilution of Octenisept® with glucose brings more favorable effects on tissue tolerability and on antibiotic bactericidal potency. The improvement of biocompatibility of Octenisept® would allow to extend the field of application (for instance intra-abdominal) of this antiseptic agent. Furthermore, the development of antibiotic resistance is less likely with O+ than with other antiseptic compounds. This remarkable observation is worth to be further investigated.

6 Abstract

In wound care use of antiseptics is common as is the use of antibiotics. Therefore, it is of great interest to find the optimal mode of antiseptic application in order to support wound healing. Therefore, we tested the impact of commercially available preparations of octenidine-hydrochloride (O: "classical" formulation Octenisept® and O+: glucose in combination with Octenisept®). Additionally, we tried to scout the effects of how octenidine did influence cell proliferation and migration at the molecular level using rt-PCR analysis. We also examined interactions of these antiseptics with various antibiotics on the growth of *B. petrii* and *S. aureus*. As expected high concentrations O and O+ were highly cytotoxic but with 1:4096 dilution of O+ the effect was reverted to enhanced cell proliferation (60 % over control cells). This was not observed so with O. Furthermore, the cytotoxic effects of a high O+ concentration were ameliorated when cells were grown on the ECM component CHS. Thus, this result indicates that cells embedded into ECM components are less sensitive to O+ but not so to O. Furthermore, we could demonstrate that the toxicity of O and O+ can be shifted to be tolerable when the contact time and the concentration of the antiseptics are modulated. From gene expression analysis data we can reason that none of the investigated genes, except TGF-ß, was inhibited. Thus at the gene expression regulatory level these molecules seem provided uninfluenced to the cell and it remains to be determined whether such regulation occurs on the protein synthesis or activation level preferably. In combinations with 20 different antibiotics, O and O+ were able to enhance bactericidal potency of the antibiotics in many cases. Especially, concomitant application of O+ and antibiotics had the most positive cooperativity against *S. aureus* in comparison to other tested antiseptics including Chlorhexidine, Beta-Isodona® and Taurolin®. In conclusion, the dilution of octenidine with glucose has a favorable effect on cell proliferation in vitro and the cytotoxicity of high O+ concentration is substantially decreased in the presence of CHS in comparison to O. Together with the potency to enhance microbicidal efficiency of several antibiotics, the dilution of Octenisept® with glucose could extend the field of application of this agent.

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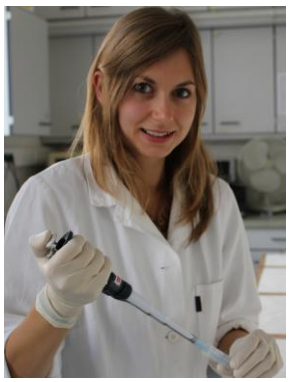
Sabrina Jenull CV



Address	H.v.Dodererstraße 14/1/10, 3100 St. Poelten
Mobile	0676/4132302
Email	sabrinajenull@hotmail.com
Date and place of birth	12/27/1988 in St. Poelten

Education

July 2011 – June 2012	**Master thesis** "The effect of concomitant application of antibiotics and antiseptics on cell proliferation" Supervised by Prof. Manfred Hüttinger Department of pathobiochemistry and genetics – medical university of Vienna
since october 2010	**Master programme of Molecular Biology** University of Vienna Focus on molecular medicine
2010	**Bachelor Thesis** "Signal transduction pathways of lactoferrin" Supervised by Prof. Manfred Hüttinger Department of pathobiochemistry and genetics – medical university of vienna
2007 - 2010	**Bachelor programme of Nutritional Science** University of Vienna
1999- 2007	BG/BRG St.Poelten with focus on science
1995- 1999	Otto-Gloeckel Volksschule in St. Poelten

Practical Experience

August – September 2012	The Wenner-Gren Institute of Stockholm University "The role of the SWI/SNF chromatin remodeling complex in alternative mRNA processing" Supervised by Prof. Ann-Kristin Östlund Farrants **Department of Cell Biology**
April 2011	AKH – Allgemeines Krankenhaus der Stadt Wien

	"DNA methylation in anaplastic large-cell lymphoma" Supervised by Gerda Egger, PhD **Clinical institution of pathology**
October/November 2010	Medical University of Vienna **Department of pathobiochemistry and genetics**
August 2010	"Lebensmittelversuchsanstalt" (LVA) at the microbiological department
August 2004/2005	Niederösterreichische Gebietskrankenkasse (NOEGKK)

Teaching

June 2011, November 2011,	
June 2012	Biochemical practise for nutritionists
October 2011	Block 13 – measurement of lipase activity
January 2012	Block 9 - ELISA

Computer Skills

MS office, Adobe photoshop, Prism3.0

Vienna, November 2012