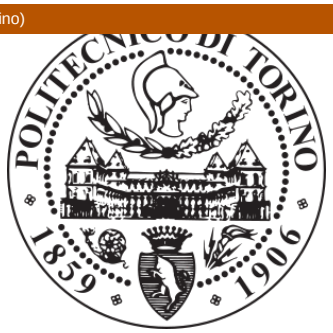

An in vitro skin wound infection model and bioevaluation of antimicrobial wound dressings

An *In-vitro* Skin Wound Infection Model and Bioevaluation of Antimicrobial Wound Dressings

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Introduction: Chronic wounds and associated delayed healing has become a great challenge in wound care and management. In this respect, development of human-based three-dimensional *in-vitro* systems with bacterial infection and biofilm formation is highly demanding to study biological properties of novel antimicrobial polymeric biomaterials (AMBs) as well as to understand underlying pathophysiology of chronic wounds for successful healing. An *in-vitro* wound infection model will serve as an advanced and complex system to perform more reliable preclinical studies for the bioevaluation of cytotoxicity, antimicrobial as well as wound healing properties of AMBs in a biomimetic system of the *in vivo* state of chronic wounds.

Methods: The 3D skin equivalent was obtained having both a dermal and an epidermal compartment, by embedding human primary fibroblasts in rat tail tendon collagen type I hydrogel (mimicking skin extracellular matrix) and then seeding human primary keratinocytes on it to generate the epidermal layer. The model was characterized for morphological characteristics and dermal/epidermal markers through histological and immunohistochemical analysis. To generate wound infection model, this system was inoculated with clinically challenging bacteria e.g. *Staphylococcus aureus* (ATCC 29213) into full thickness wounds. Commercially available wound dressings like "Atrauman® Ag" were evaluated for antibacterial as well as cytotoxic properties in this 3D system. To assess the viability of the system quantitatively, cytotoxicity assays e.g. CytoTox-ONE™ (Promega) were used.

Fig 1: Development of *In-vitro* Skin Model

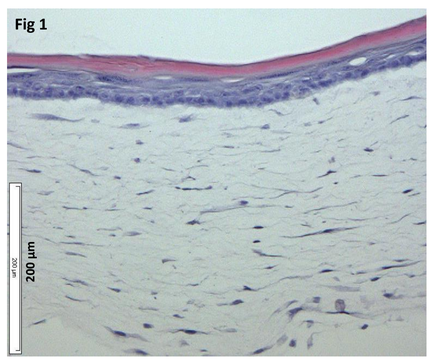

Fig 1: Hematoxylin/Eosin (H & E) stained histological images of *in vitro* 3D human skin model. The results show the two structurally distinct layers of skin: the outer epidermis layer and the underlying thicker dermis layer that consists of connective tissue. The epidermal part shows well differentiated layers of keratinocytes namely stratum corneum, granulosum, spinosum and basale. Furthermore, the contact between dermal and epidermal surface is not straight rather it is undulating in a way that it intertwines with dermal layer; mimicking the *in vivo* situation.

Fig 2: Characterization of *In-vitro* Skin Model

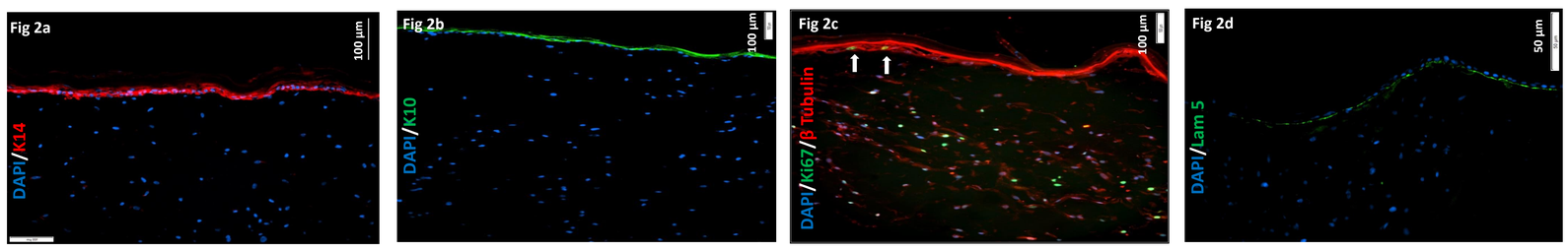

Fig 2: *In-vitro* human skin model recapitulates the epithelial differentiation found in healthy human skin; and thus can be termed as skin equivalent. The epidermis is stratified squamous epithelium that makes the protective covering of the skin. The innermost cells of the epidermis have the capacity for DNA synthesis and mitosis. Under a trigger of terminal differentiation, the basal cell begins its journey to the surface. In transit, it undergoes a series of morphological and biochemical changes associated with the expression of differential markers at different stages of its differentiation cycle.

The images show immunolabelling for specific epidermal differentiation markers of *in-vitro* 3D human skin: (a) Keratin 14 (K14) red, (b) Keratin 10 (K10) green, (c) Ki67 (a cellular proliferation marker protein) green, (d) Laminin 5 (Lam 5) green. Cell nuclei are shown in blue by using DAPI staining.

In particular, basal and suprabasal layers show Keratin 14 and Keratin 10 respectively, while presence of Ki67 cells (arrows) shows the proliferative state of basal epidermal cells. Laminin 5 is used as a marker of dermo-epidermal junction (DEJ) and appeared as a thin line.

Fig 3: Development & Characterization of *In-vitro* Skin Infection Model

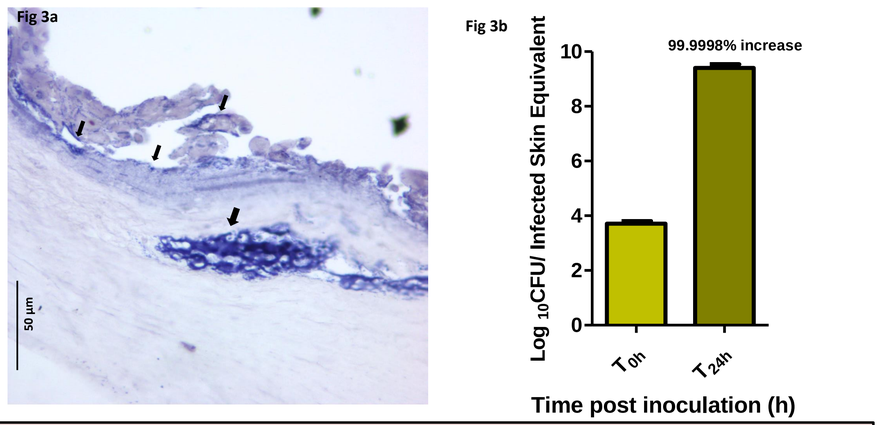

Fig 3: a) Microscopic analysis of bacterial replication and biofilm formation on *in-vitro* skin model. Skin equivalents were exposed to *S. aureus* for 24 h. Thereafter the skin sections were stained with H & E staining and analyzed by light microscopy. The results reveal that inoculated *S. aureus* is able to adhere to the dermal surface, colonize, and replicate to make large structures of biofilm. Also, the bacteria did not persist on the surface, rather invaded through the whole dermal depth. Big arrows indicate bacteria within a biofilm matrix inside dermis. Small arrows indicate the bacteria surrounding keratinocytes in epidermis. How do these bacteria affect skin cells, needs further investigation. **b)** The graph shows replication of *S. aureus* on *in-vitro* skin model over 24 h. This graph shows the bacterial growth rate on this biotic surface, indicating that bacteria are able to increase their population on an *in-vitro* skin to 99.99% in 24 h. The ability of *S. aureus* to form a biofilm on human skin plays an important role in its persistence on the skin infection.

Fig 4: Cytotoxicity Measurement of Materials on *In-vitro* 3D Skin Infection Model

Fig 4: Cell viability of the 3D skin infection model is characterized by measuring the amount of dead cells based on LDH measurements of: 1) *In-vitro* Skin Wound Model after wounding called "Wound Control" 2) *S. aureus* colonization of Wound Control served as "Wound Infection Model" 3) Treatment of Wound Infection Control with the antimicrobial wound dressing Atrauman® Ag named as "Treated (Atrauman® Ag) Group". The results indicate an affected cell viability after wound infection that decreases over time. This effect can be a result of cytotoxic effect of bacteria on cells and/or utilization of the nutrients by bacteria to form biofilm.

Surprisingly, the cytotoxicity values are higher in treated group than in Wound Infection Control. Inability of Atrauman® Ag to eradicate bacteria can be a reason for this increasing detrimental effect on cells in treated group. However, evaluation of cytotoxic effects of Atrauman® Ag on cells alone without bacteria (i.e. Skin Wound Model) has to be investigated to answer this question.

Fig 5: Treatment of *S. aureus* Colonized Skin Infection Model

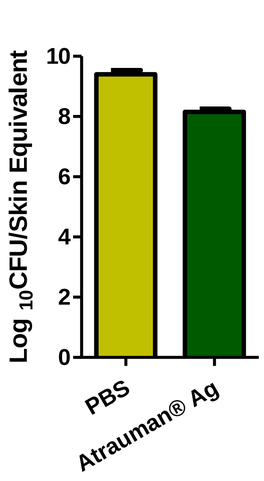

Fig 5: The graph shows the treatment of *S. aureus* colonized skin infection model**.** Skin equivalents were infected with *S. aureus* and treated with Atrauman® Ag versus PBS. Twenty-four hours after treatment the number of bacteria was determined. The results show that Atrauman® Ag was able to reduce bacterial burden to some extent but not to eradicate the bacteria completely.

Summary: With an increasing need for novel strategies to combat persistent infectious wounds, there is an urgent need to understand basic aspects of the host-pathogen relationship; as well as a more *in-vivo* like screening tool to study the efficacy of antimicrobials. In this study, we intend to successfully create a full thickness infected skin equivalent, to serve as a risk assessment platform, representing healthy and infected skin.

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