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The occurrence of biofilm in an equine experimental wound model of healing by secondary intention

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ABSTRACT

In humans, biofilm is a well-known cause of delayed healing and low-grade inflammation of chronic wounds. In horses, biofilm formation in wounds has been studied to a very limited degree.

The objective of this study was thus to investigate the occurrence of biofilm in equine experimental wounds healing by secondary intention.

Tissue biopsies from non-contaminated, experimental excisional shoulder and limb wounds were obtained on day 1–2, day 7–10 and day 14–15 post-wounding. Limb wounds were either un-bandaged or bandaged to induce exuberant granulation tissue (EGT) formation and thereby impaired healing. Presence of biofilm in tissue biopsies was assessed by peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) and confocal laser scanning microscopy (CLSM).

Bandaged limb wounds developed EGT and displayed delayed healing, while shoulder and un-bandaged limb wounds healed normally. Biofilm was detected in limb wounds only. At day 14–15 biofilm was significantly more prevalent in bandaged limb wounds than in un-bandaged limb wounds ($P = 0.003$). Further, bandaged limb wounds had a statistically significant increase in biofilm burden from day 7–10 to day 14–15 ($P = 0.009$).

The finding that biofilm was most prevalent in bandaged limb wounds with EGT formation suggests that biofilm may be linked to delayed wound healing in horses, as has been observed in humans. The inability to clear bacteria could be related to hypoxia and low-grade inflammation in the EGT, but the interaction between biofilm forming bacteria and wound healing in horses needs further elucidation.

1. Introduction

Biofilms are defined as aggregates of bacteria embedded in a biopolymer matrix that show increased tolerance towards host defenses and antimicrobials (Burmølle et al., 2010). Biofilm infection is a well-known cause of chronicity in wounds in humans (Bjarnsholt et al., 2008; James et al., 2008). In horses, chronic wound healing is common; and particularly wounds on the distal limb fail to heal. Often these wounds have a propensity for developing exuberant granulation tissue (EGT) (Adam and Southwood, 2006). Hypoxia (Sørensen et al., 2014) and low-grade chronic inflammation (Bundgaard et al., 2016; Wilmink et al., 1999b) has been implicated in the pathogenesis of EGT formation in equine limb wounds, similar to findings in human chronic wounds with biofilm involvement (Fazli et al., 2011; James et al., 2016). Two recent studies have described biofilm in equine surgical and traumatic chronic wounds (Freeman et al., 2009; Westgate et al., 2011). Similar to human studies, these studies have suggested that biofilm might be implicated in impaired wound healing in horses.

To assess whether biofilm could be linked to impaired wound healing in horses, the presence of biofilm was assessed by peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) and confocal laser scanning microscopy (CLSM) in an equine experimental wound model. Wounds healing by secondary intention under three different scenarios were examined: limb wounds

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bandaged with non-adhesive gauze (a dressing known to provoke EGT formation and delayed healing), un-bandaged limb wounds, and un-bandaged shoulder wounds.

2. Material and methods

2.1. Horses

In this study, a convenience sample was obtained from two previous experimental studies: study A, where seven horses were included, and study B, where two horses were included (Bundgaard et al., 2016; Sørensen et al., 2014). Overall nine mature mix-breed geldings, 4–8 years old, weighing 384–537 kg (average 459 kg), standing at 149–171 cm were included in this study. After thorough clinical and hematological examination, the horses were found to be healthy. The horses underwent deworming with ivermectin (Study A: Eqvalan Merial Nordic, Hørsholm, Denmark; study B: Noromectin Vet, Biovet, Fredensborg, Denmark), vaccination against equine influenza and tetanus (Study A: EquipFT, Orion Pharma Animal Health, Copenhagen S, Denmark; study B: ProteqFlu-Te, Merial Nordic, Hørsholm, Denmark), teeth floating, and hoof trimming as necessary prior to the study. The horses were housed in box stalls and had access to pen/paddock 2–3 h/day. The horses were fed concentrates according to their needs, and ad libitum grass hay and water. The horses were on a daily basis examined for any signs of lameness, discomfort, illness, and bandage slippage. The experimental protocols were approved by the Danish Animal Experiments Inspectorate (Study A: license no. 2005/561-1027; study B: license no. 2010/561–1882), and procedures were carried out per the Danish Animal Testing Act.

2.2. Experimental procedure

The horses had wounds created either under general anesthesia (study A) or standing after sedation and nerve blocks using 2% lidocaine hydrochloride (Lidokain, SAD, Copenhagen, Denmark) and 0.5% bupivacaine (Marcaine, AstraZeneca, Albertslund, Denmark) (study B). Horses received ampicillin 10 mg/kg (Pentrexyl, Bristol-Myers Squibb, Virum, Denmark) 30 min prior to wounding. On the day of surgery and the day after, horses received flunixin meglumine 1.1 mg/kg (Finadyne, Intervet, Skovlunde, Denmark) to minimize discomfort associated with the surgical procedure.

Horses had both metatarsi and one (study A) or two (study B) shoulders clipped and aseptically prepared. Standard surgical preparation was used; 2×4 min scrub with 4% chlorhexidine gluconate followed by multiple applications of 70% isopropyl alcohol. Wounds were created by skin incision in a vertical column on the shoulder(s) and on both metatarsi (study A: 1×1 cm, 2 cm apart, 6 wounds per column; study B: 2.5×2.5 cm, 3 cm apart, 3 wounds per column) using a flexible sterile template made from x-ray film. The wounds were full thickness and included the periosteum at the metatarsi and the fascia at the shoulder in study A, while in study B, periosteum and fascia were left intact. All wounds were left to heal by secondary intention. In study A, the wounds on one randomly chosen limb were bandaged with non-adhesive gauze (Melolin, Smith & Nephew, Hørsholm, Denmark), cotton wrap (Veterinary Gamgee, Robinsons Health Care, Nottinghamshire, United Kingdom), and elastic adhesive wrap (Co-plus and Tensoplast, BSN Medical AB, Billdal, Sweden). The contralateral limb stayed un-bandaged. In study B, all limb wounds were bandaged in a similar manner. All shoulder wounds were left un-bandaged. Bandaged limb wounds, un-bandaged limb wounds, and un-bandaged shoulder wounds are hereafter referred to as wound types.

2.3. Macroscopic wound examinations

Wounds were examined and bandages changed on a regular basis (study A on day 1, 3, 6, 9–10, 12, 14–15; study B on day 1, 2, 4, 7, 10, 14). Quality and quantity of granulation tissue, wound contraction and epithelialization was subjectively assessed. Furthermore, in study A the areas of the wounds were measured using digital photography and the free image analysis software Fiji (https://fiji.sc/). The areas of the wounds (cm^2) were measured three times and the average calculated to produce a single measure for each wound. The area of the granulation tissue, not including the advancing edge of epithelium on each side of the granulation bed, was measured. In study B wounds were measured by planimetry using the Visitrack wound measurement grid (Smith & Nephew, Hørsholm, Denmark) according to the manufacturer's instructions.

2.4. Tissue biopsies

In study A, tissue biopsies were obtained on day 1, 9 (10 in one horse [pilot study]), and 14 (15 in one horse [pilot study]) from a random wound from each location. In study B, tissue biopsies were obtained on day 2, 7, and 14 in a similar random pattern. All wounds were biopsied only once. Horses were sedated and wounds anesthetized by line blocks performed by local infiltration with 2% lidocaine hydrochloride (Lidokain, SAD, Copenhagen, Denmark) (study A) or by nerve blocks obtained with a 1: 1 mixture of 2% lidocaine hydrochloride (Lidokain, SAD, Copenhagen, Denmark) and 0.5% bupivacaine (Marcaine, AstraZeneca, Albertslund, Denmark) (study B). A 6-mm biopsy was obtained from the margins of each wound to include half granulation tissue and half migrating epithelia/skin margin. The tissue biopsies were immediately fixed in 4% formaldehyde and after 72 h transferred to 70% ethanol. After fixation samples were embedded in paraffin, cut into 4-μm sagittal sections, and mounted on glass slides. In total, 68 biopsies were available for this study, 56 from study A and 12 from study B. As biopsies were obtained from different studies they have been pooled, and will be referred to as biopsies from day 1–2, 7–10 and 14–15.

2.5. PNA FISH

PNA FISH was performed as previously described (Fazli et al., 2014; Ring et al., 2016). Briefly, the paraffin-embedded tissue sections were deparaffinized by immersing the tissue sections on glass slides in xylene (VWR International, Fontenay-sous-Bois, France) (2×5 min), 99% ethanol (CCS Healthcare AB, Borlänge, Sweden) (2×3 min), 96% ethanol (CCS Healthcare AB, Borlänge, Sweden) (2×3 min), and in distilled sterile water (3×3 min). A universal bacterial PNA Texas red conjugated probe (AdvanDx, Inc., Woburn, Massachusetts) that hybridizes with 16S rRNA from all types of bacteria was added to the deparaffinized tissue sections and incubated at 55 °C for 90 min. The slides were afterwards washed in a preheated washing solution (AdvanDx, Inc., Woburn, Massachusetts) for 30 min at 55 °C, and additionally counter-stained for 15 min by 4′,6′-diamidino-2-phenylindole (DAPI) to visualize DNA (Life Technologies, Eugene, Oregon). Subsequently, mounting media was added (Prolong Gold, Life Technologies, Eugene, Oregon), and slides were mounted with a coverslip and sealed with clear nail polish ready for imaging.

2.6. Confocal laser scanning microscopy

Microscopic examinations of the tissue sections were performed by CLSM (Axio Imager.Z2, LSM710 CLSM; Zeiss, Oberkochen, Germany) and the accompanying 3D reconstruction software (Zen