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Research paper

Development of a sprayable hydrogel formulation for the skin application of therapeutic antibodies

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ABSTRACT

A formulation of an antibody with antibacterial properties for topical use on Staphylococcal skin infections was developed and characterized. The best formulation was obtained with 1.5% (w/v) sodium carboxymethylcellulose containing 10 mg/ml immunoglobulin.

Spraying forces and rheological behavior were measured in order to characterize the hydrogel formulation. The percentage of antibody aggregates in gel as well as the antibody release, folding and target binding properties of the released antibody were analyzed to proof an acceptable shelf life and no significant changes in the activity of the antibody over time. No microbial contamination was observed in the chosen non-airless application container. Functional testing of the topical skin formulation was performed with an *ex vivo* biopsy culture model of dog skin. Histological analysis indicated efficacy in protection from *Staphylococcus* mediated skin damage and antibody delivery restricted to the epidermal surface.

The results demonstrate that this hydrogel is suitable for cutaneous antibody applications in the medical field.

1. Introduction

The increasing appearance of multi-resistant bacterial strains makes the treatment of skin infections a growing challenge. The problem exists likewise for human beings and companion animals and there is good evidence that transmission of multi-resistant strains between pets and human beings can occur in both directions adding a new dimension of public health implication [1,2]. *Staphylococcus pseudintermedius (S. pseudintermedius)* is the most common skin pathogen in dogs [3], which is associated with a broader drug-resistance than Methicillin resistant *Staphylococcus aureus* (MRSA) in human beings [4]. Methicillin resistant *S. pseudintermedius* (MRSP) was first reported from dogs in North America in the late 1990s and accounted for > 30% of staphylococcal isolates from American dogs within 10 years [5–7]. MRSP is now identified worldwide and high prevalences have been reported from China (~50%) and Japan (~70%) [8,9]. In Europe, where MRSP had been identified in 2006, the prevalence is ~30% [4,10].

For dogs restrictions on antimicrobial prescribing apply and last resort antibiotics are strictly reserved for use in human beings [11], which raises the need of alternatives to antibiotics. Currently, 92% of dogs with pyoderma receive systemic antibacterial therapies [12]. In the future, it would be desirable to make use of the unique accessibility of the skin for topical therapy [13]. At present, topical antimicrobial treatments of cutaneous infections are gaining interest as adjunctive therapy to systemic treatment or even as therapy instead of standard antibiotics. Recent studies have provided good evidence that topical therapy can be effective in superficial pyoderma, including cases with multi-resistant strains [14–16]. Local delivery of polyclonal IgG to infected areas have been shown to lead to a synergistic therapeutic efficacy in combination with systemically administered antibiotics in a murine wound infection model [17]. Aqueous CMC (carboxymethylcellulose) gel containing polyclonal human IgG has been shown to reduce the severity of an infection with MRSA in a murine surgical implant model when applied locally at the site of infection [18]. The formulation of polyclonal antibody in a sprayable hydrogel appears to be appropriate when haired skin is affected. Gels are greaseless, easy to spread and remain as thin layer on the skin [19]. A sprayable gel allows a cutaneous application at the site of infection with a minimal risk of contamination. Furthermore, a non-airless application container was chosen to keep the antibody gel sterile without the use of preservatives.

We describe the formulation of a polyclonal antibody directed against alpha-toxin of *Staphylococcus* which plays a critical role in skin

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infections [20] derived from colostral whey of vaccinated cows as sprayable hydrogel.

2. Material and methods

2.1. Preparation of hydrogel

Placebo hydrogels were prepared by the addition of the required amount of Sodium Carboxymethylcellulose (CMC) (Ashland, Kentucky, USA) to the respective dispersant (highly purified water or phosphate buffered saline (PBS) pH 7.4) under vigorous stirring. The mixture was heated to 40 °C and stirred until the CMC was dissolved. Afterwards, the hydrogel was steam sterilized (121 °C, 2 bar, 15 min).

Antibody containing hydrogels were prepared according to the before mentioned procedure with some modifications. A stock gel with a higher CMC concentration was prepared and after steam sterilization, the hydrogel was diluted to the appropriate concentration with a sterile filtered (0.22 μm) solution of the antibody.

2.2. Sprayability of hydrogel

Sprayablity of the hydrogel was evaluated by spraying the gel through a 3 K® Horizontal Spray Nozzle (Ursatec, St. Wendel, Germany) and weighing the sprayed amount (AB304-S, Mettler Toledo, Gießen, Germany).

2.3. Spraying forces

Spraying forces were tested using a TA.XT Plus Texture Analyser (Stable Micro Systems). A stainless steel plate was attached to the machine and used to compress the spray pump and determine their force needed to release the gel.

2.4. Rheological behaviour

Gel characteristics were determined with a MCR 100 rheometer (Anton Paar, Graz, Austria) and a stainless steel plate/plate system (diameter 25 mm).

Viscosity measurements were performed in oscillation mode with a constant deformation of 0.5% and a constant angular frequency of $10\,s^{-1}$. The samples were equilibrated to a temperature of 8 °C (storage temperature) and 32 °C (skin temperature), respectively.

The rheological properties of hydrogels were measured in rotation mode with a logarithmic increase in shear rate from 1 to $900\,s^{-1}$ followed by a logarithmic decrease in shear rate from 900 to $10\,s^{-1}$. The rheological properties were investigated at a room temperature of 20 °C as well as at storage temperature of 8 °C.

2.5. In vitro release of antibody from gel

Freshly prepared polyclonal IgG antibody containing hydrogel was exactly weighed into the donor compartment of Teflon® flow through cells and separated by a cellulose acetate membrane (0.45 μm). The flow through cells were closed, connected to silica tubings and placed on a heating plate (32 °C). PBS buffer pH 7.4 was used as release medium and was pumped with a speed of 0.5 ml/h through the cells with a multi-syringe pump. The release fractions were collected and analysed by size exclusion chromatography.

2.6. Size exclusion chromatography (SEC)

SEC was used to analyze the formation of soluble aggregates of the polyclonal IgG antibodies. SEC analytics was conducted on a Waters 2695 system (Waters GmbH, Eschborn, Germany). The flow rate of the running buffer (50 mM phosphate, 300 mM NaCl, pH 7.0) was 0.5 ml/min. Hydrogel samples were diluted with running buffer (ratio 1:10) and 25 μl of each sample was injected onto an YMC Pack-diol 300 column and detected with UV-detection at 280 nm. Protein concentrations were determined by SEC using a standard curve.

2.7. Circular dichroism (CD)

For far-UV CD, the polyclonal IgG antibody formulations were diluted to concentrations between 0.159 mg/ml and 0.209 mg/ml with phosphate buffered saline pH 7.4 and measured with a Jasco J-715 spectropolarimeter (Jasco International, Tokyo, Japan) in a quartz cuvette with a path length of 0.1 cm at 20 °C. Far-UV CD spectra were collected in a continuous scanning method from 190 to 250 nm at a scanning speed of 50 nm/min, a response time of 1 s, a bandwidth of 1 nm, steps of 0.1 nm and an accumulation of 4 scans. Using the Spectra Analysis Software (Jasco International, Tokyo, Japan), the spectra were background corrected for the spectrum of the respective buffer or placebo gel and curves were smoothed. Data is recorded in millidegrees of ellipticity as a function of wavelength.

2.8. ELISA to measure alpha-toxin binding

96-wells plates were coated with 0.3 μg/ml recombinant alpha toxin. After blocking with BSA and washing with PBS containing 0.05% Tween 20 the polyclonal IgG antibody was added to the plate starting with 100 μg/ml and diluted in 1:2 dilution steps. Detection was done with a peroxidase conjugate goat-anti-bovine Ig antibody (Jackson ImmunoResearch). TMB was used as substrate and the reaction was stopped with H_2SO_4 (1 N). Absorbance was measured at 450 nm.

2.9. Alpha-toxin neutralization assay

Anti–alpha-toxin neutralizing activity of the antibody was measured with a red blood cell–based neutralization assay. The ability of the antibody before and after formulation to lyse rabbit erythrocytes (RBC) was tested in a 96-well format. Specifically, 140 μl from each antibody sample was loaded into the first well and then serially diluted 2-fold, up to 1: 2048. After the dilution of each sample, 140 μl of alpha-toxin (10 ng/ml) was added to each well incubated at RT for 2 h. 5×10^6 rabbit RBC in PBS was added to each well and following incubation at RT for 2 h, plates were centrifuged for 5 min, 100 μl of the supernatant was removed gently to a new microtiter plate, and absorbance was read at 415 nm.

2.10. Bacterial colonization model of dog skin

Healthy dog skin was obtained from dogs that were euthanatized after the end of a terminal animal experiment, that was approved by the Committee for Experiments on Animals of the Utrecht University, The Netherlands (AVD115002016531).

Samples of 8 mm were taken with a stiefel biopsy punch and cultivated in a trans-well system (0.4 μm pore size, Greiner Bio-One, Germany) with air-liquid interface for 30 h. Cultivation medium was Williams E supplemented with growth factors as previously described by Abramo et al [21]. Prior to cultivation either 1×10^8 CFU bacteria, bacteria and hydrogel or bacteria and hydrogel containing 10 mg/ml antibody were applied on the epidermal surface of the skin samples by pipetting. After incubation samples were either stored at −150 °C or at RT in 4% Formalin.

2.11. Immunofluorescence microscopy

A combination of bacteria labeled with FITC (Fluorescein isothiocyanate) and 10 mg/ml antibody in hydrogel was applied on the epidermal surface of skin samples by pipetting. Immunofluorescence staining was performed using 3 μm cryosections of the samples after 30 h cultivation in the transwell-system. After incubating with Hank's

Balanced Salt Solution HBSS (Thermo Fisher Scientific) with 1% BSA for 10 min, sections were incubated with an Alexa Fluor® 647-conjugated goat anti-bovine IgG (Jackson ImmunoResearch Inc.) for 1 h at room temperature in the dark. The skin cells were counterstained with DAPI.

After washing with buffer, coverslips were placed on the slides and fixed with ProLong Gold antifade reagent (Thermo Fisher Scientific). Images were taken with a Leica confocal Microscope CTR6500 using Leica Application Suite Advanced Fluorescence (LAS AF) software.

2.12. Histology

H&E and PAS staining was performed on slides from paraffin-embedded skin samples stored in formalin. Slide preparation was done as an automated routine paraffin histology service by the department of pathology of the University Medical Centre Utrecht.

Scans were made with Nano Zoomer Digital Pathology (Hamamatsu) for semiquantitative evaluation. Viable cells, cells with pyknotic nuclei and dead cells (ghost cells) were counted on ten randomly chosen epidermal areas of each section (under 30-fold magnification)

3. Results and discussion

Our aim was the development of a formulation for a therapeutic antibody directed against Staphylococcal alpha-toxin for topical application on dogs with pyoderma.

Alpha toxin as pore-forming cytolytic toxin is a key virulence factor of *S. aureus* that has been strongly associated with skin and soft tissue infections in humans. It plays a dual role in skin infections by contributing to disease severity and evasion of host immune defenses. Neutralization of alpha toxin has shown a therapeutic effect in various models of infected wounds [22,23].

S. pseudintermedius is comparably with *S. aureus* responsible for severe and necrotizing infections in humans and dogs. In contrary to *S. aureus*, the pathophysiological mechanisms involved in the virulence of *S. pseudintermedius* are not completely understood. Recently a role of pore forming toxins has been described [24].

In culture supernatants of various clinical *S. pseudintermedius* isolates we detected a toxic activity that could be neutralized by our anti-alpha toxin antibody. Based on our findings *in vitro* we aimed to develop a formulation of the antibody for further preclinical and clinical testing.

Sodium carboxymethylcellulose (CMC) was identified as a possibly suitable gelling agent since it is physiologically inert and generally recognized as safe (GRAS) by the FDA (Code of Federal Regulations Title 21, Chapter I, Subchapter B, Part 182, Subpart B, §182.1745 Sodium carboxymethylcellulose). Furthermore, it is currently widely used in cosmetics, food and pharmaceuticals and can be provided in sufficient quality for a pharmaceutical product.

3.1. Hydrogel preparation

Requirements that the hydrogel in our antibody product has to fulfil are sprayability in a reproducible manner for standardized dosage as well as a degree of viscosity that allows adhesion to the skin.

3.1.1. Rheological properties

CMC was investigated as thixotropic gelling agent in order to allow spraying of the hydrogel formulation through a horizontal spray nozzle. This rheological behavior is dependent on the degree of substitution and the viscosity of the polymer. For this study, we have chosen a high viscosity type CMC with a degree of substitution of 0.65–0.90 [25].

The first aim of this study was to find the appropriate CMC concentration with thixotropic behavior, which enables constant and reproducible spraying of the hydrogel. The rheological properties of the hydrogels were investigated. Thixotropic behavior of the CMC formulations can be seen in Fig. 1. By an increasing shear rate the shear stress also increases, whereas with a decreasing shear rate the process is reversed. The hysteresis loop, the area enclosed by the up and down curve, is a typical characteristic for thixotropic behavior [26]. This behavior of the hydrogel is desired as it allows shear thinning during the spraying process and afterwards a rearrangement of the hydrogel structure and its viscous properties on top of the skin. Because the gel returns to its viscous state after spraying, it adheres better to the skin.

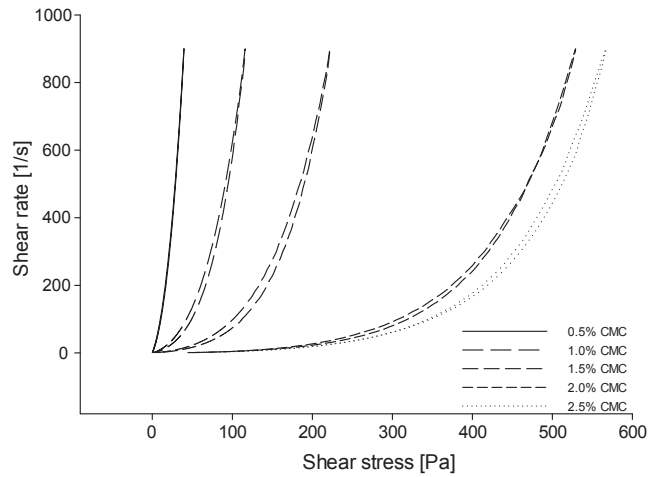

Fig. 1. Rheogram of different CMC concentrations. Data represent one of three measurements showing comparable results.

3.1.2. Sprayability

Besides the rheological properties, the handling related characteristics of the CMC formulations were investigated. Fig. 2 shows the sprayed amount of different CMC concentrations with an Ursatec 3 K® horizontal spray system. It can be seen that the released amount of hydrogel up to a concentration of 2.0% CMC is equal to the released amount of water. A constant amount of about 150 mg is released from the nozzle after the fifth to sixth spray (combined SD of spray 6–12 form three independent spray nozzels (= 21 sprays): 1.9 mg for 0.5% CMC, 2.9 mg for 1.0% CMC, 3.5 mg for 1.5% CMC, 4.8 mg for 2.0% CMC). In comparison to pure water, the hydrogel shows a delayed release from the nozzle due to its higher viscosity. In addition, this figure shows a clear margin of sprayability at a concentration of 2.5%. The viscosity of this formulation reached a range, where the energy produced by the nozzle is no longer high enough to liquify the hydrogel to such an extent that a constant amount is released.

3.1.3. Spraying force

Based on the findings above the required pressing force to activate the spray process was measured (Fig. 3). The figure depicts an increasing spray force with increasing CMC concentration whereas hardly any differences between the concentrations 0.5%, 1.0% and 1.5% could be found. With regard to a preferably high viscosity of the hydrogel in order to achieve maximal adhesiveness on the skin and the possibility to allow constant dosing with moderate effort, a concentration of 1.5% CMC was evaluated to be optimal for our purposes.

3.2. Antibody containing hydrogels

Topical application of a therapeutic anti-bacterial antibody is a novel way to treat skin diseases and to our knowledge a corresponding formulation has not been described so far. Requirements that the antibody containing hydrogel has to fulfil are stable viscosity and structure of the hydrogel during storage as well as stability and functionality of the antibody in hydrogel.

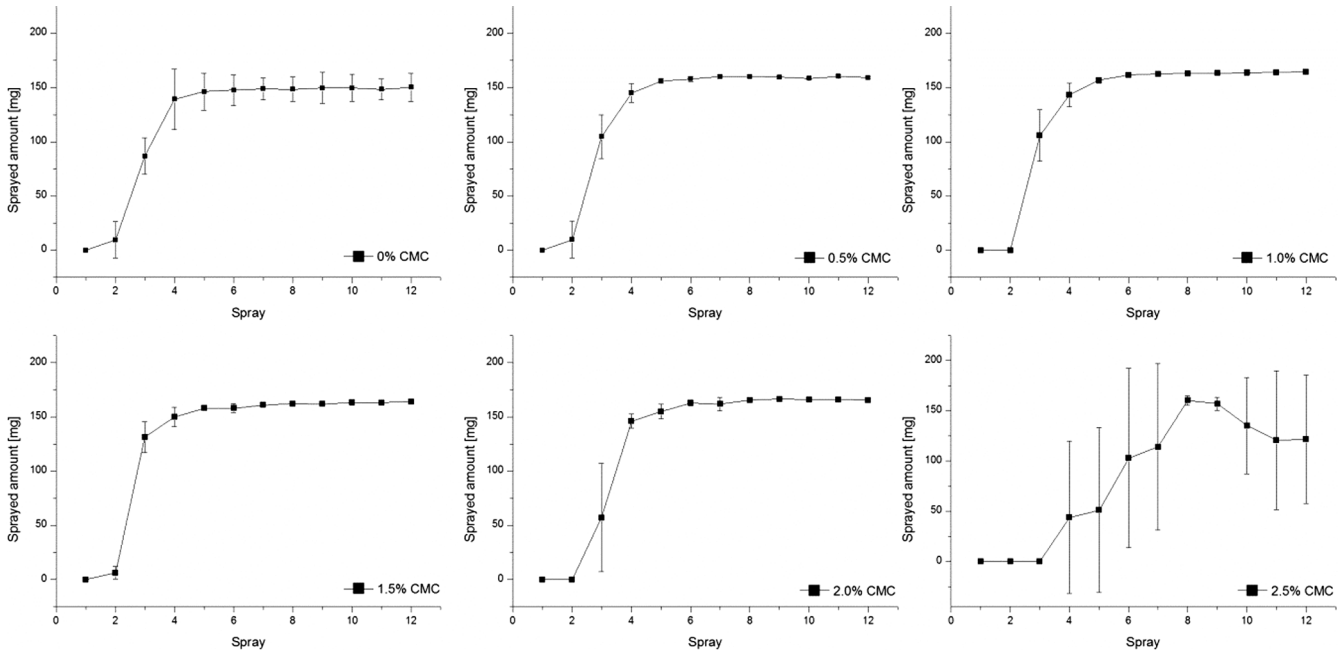

Fig. 2. Sprayability of different CMC concentrations with an Ursatec 3 K® horizontal spray system. CMC concentration of 0% represents pure water. Data is presented as mean ± SD (n = 3).

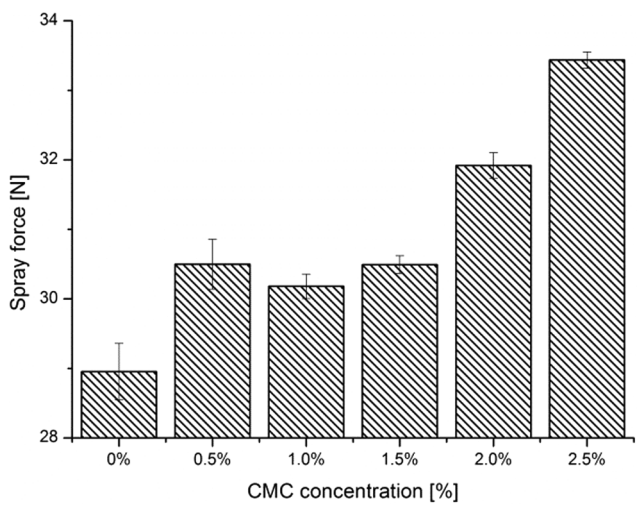

Fig. 3. Spray forces required to activate the spray process of different CMC concentration from an Ursatec 3 K® horizontal spray system. Data is presented as mean ± SD (n = 3).

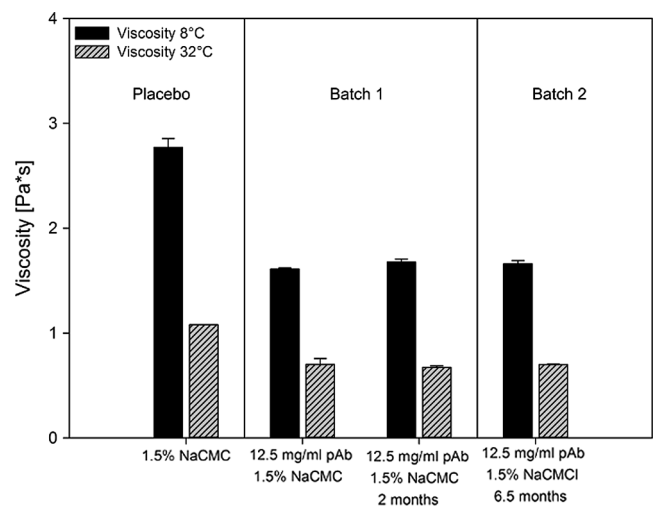

Fig. 4. Viscosities of placebo and antibody containing hydrogels at storage temperature (8 °C) and skin temperature (32 °C) directly after preparation and two or six months after storage. Data is presented as mean ± SD (n = 3).

3.2.1. *Viscosity and structure of hydrogel during storage*

After determination of an appropriate CMC concentration, hydrogels containing antibody were prepared. By addition of the antibody solution, the viscosity of the hydrogel decreases (Fig. 4). This decrease of viscosity may be caused by an intercalation of the antibody molecules between the hydrogel structures. Higher viscosity is related to strong intermolecular interaction between CMC molecules. Intercalation of the antibody molecules disturbs and reduces these interactions. Consequently, hydrogel viscosity decreases. Furthermore, the viscosity at low storage temperature is higher than at skin temperature. Temperature dependence of the viscosity of CMC is a well-known phenomenon [27].

These data revealed stability of the structure and viscosity of the hydrogel for over six months, when the hydrogel was kept under sterile conditions (Fig. 4).

Under non-sterile conditions a complete loss of thixotropic behavior as well as strong decrease of viscosity could be observed due to microbial contamination and degradation. This phenomenon can be seen in Fig. 5. The viscogram of the non-sterile hydrogel changed from the curve representing a thixotropic system to a straight line at very low viscosity representing a Newtonian system. This leads to the conclusion that the preservation of sterility in the application container is a main requirement not only for preventing degradation of the antibody and possible infection of the patient, but also regarding formulation integrity. Consequently, the use of an Ursatec 3 K® system is appropriate due to sprayability and preservation of sterility.

3.2.2. *Stability of antibody in hydrogel*

Besides stability of the hydrogel, stability of antibody in the hydrogel was investigated. These included three objectives: Does the antibody tend to aggregation in the hydrogel? Is the secondary structure of the antibody stable? Is the antibody in the hydrogel still functionally

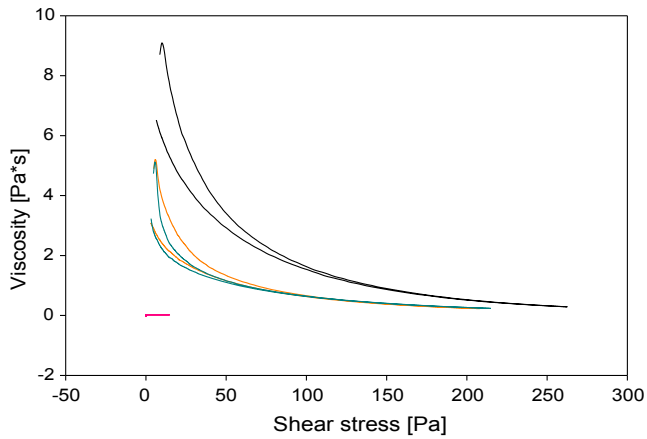

Fig. 5. Viscogram of placebo hydrogel (black), freshly prepared antibody containing hydrogel (orange), antibody containing hydrogel after six months storage (green) and antibody containing hydrogel with microbial contamination (pink straight line in the left lower part of the diagram without thixotropic behavior). Data represent one of three measurements showing comparable results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

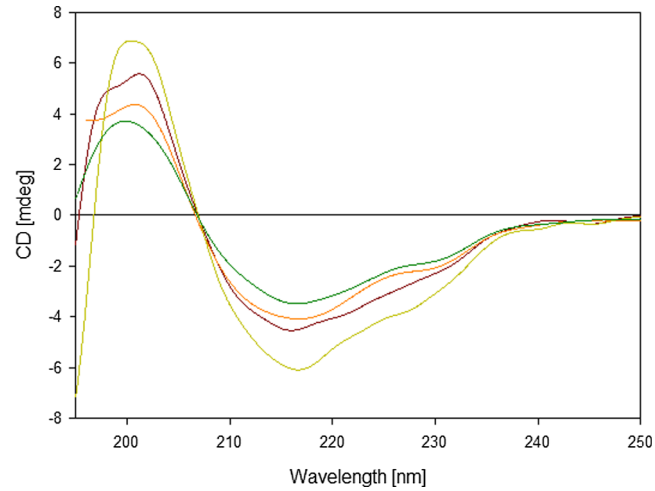

Fig. 7. Far-UV Circular dichroism spectra of IgG antibodies in PBS (red), in hydrogel (orange), in PBS after 7 weeks of storage (yellow) and in hydrogel after 7 weeks of storage (green). Data represent mean of three measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

active?

The potential aggregation was evaluated by size exclusion chromatography (Fig. 6). These data show aggregation of the antibodies in hydrogel upon storage at 2–8 °C for batch one. The relative amount of dimers and larger soluble aggregates increases and recovery strongly decreases by 20% within eleven weeks. This loss in recovery may be caused by insoluble aggregates, which are not detected in SEC. A second batch shows the same tendency but less marked even after > 6 months of storage.

The secondary structure is also an important hallmark for the activity of an antibody. Prevention of secondary structure in the hydrogel compared to PBS and upon storage was analyzed (Fig. 7). The far-UV CD region (180–240 nm) corresponds to the peptide bond absorption and gives information on the secondary structure of a protein. These data show the far-UV spectra of IgG antibodies in PBS directly after preparation and after seven weeks of storage at 2–8 °C and the respective samples formulated in 1.5% CMC gel. The spectra show typical curves of immunoglobulins representing high beta-sheet content. This can be concluded from the negative maximum ~217 nm, a zero ellipticity at ~210 nm and a positive maximum ~200 nm [28–30]. Differences in the amplitudes are caused by variation in the concentrations and the heterogeneity of antibody batches. Concluding, there are no changes in secondary structure of the IgG antibody upon storage in PBS pH 7.4 or 1.5% CMC gel.

From this data, it can be concluded that the secondary structure of the antibody is stable in the gel formulation at the concentration desired for *in vivo* studies (10 mg/g).

Taken together, the secondary structure of the antibody is not affected by the formulation in hydrogel and the antibody shows stability over four weeks of storage at 2–8 °C.

The pH of the antibody containing hydrogel is pH 7.08 and is therefore highly suitable for dogs with a healthy skin pH as well as dogs with atopic dermatitis or pyoderma with an elevated skin pH slightly above pH 7 [31].

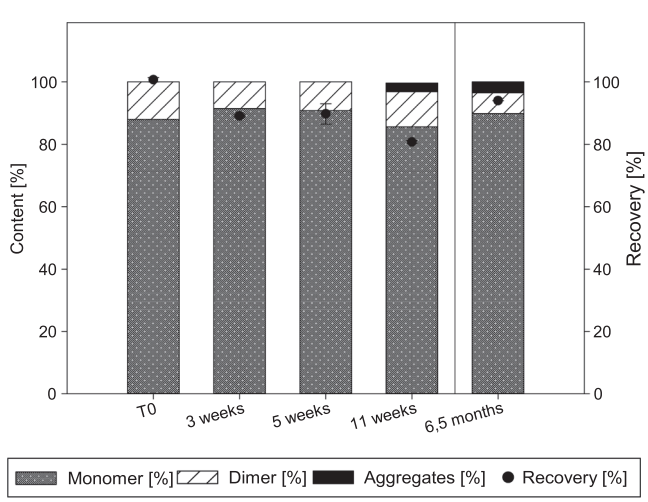

Fig. 6. Relative content of monomer, dimer and larger aggregates as well as recovery of antibodies in CMCs hydrogels of two different batches (separated by black line) directly after preparation and after storage. Data is presented as mean ± SD (n = 3).

3.2.3. Functionality of the antibody in hydrogel

The aim of the following test was to analyze if besides the structural characteristics also the functional properties of the antibodies are preserved in hydrogel.

For this purpose, we made use of the knowledge that the used antibody pool contains a specific activity against alpha-toxin of *S. aureus*. To measure the antigen binding of the IgG antibody a microtiter plate was coated with recombinant alpha-toxin and antibody in PBS as well as after release from hydrogel were titrated on the plate.

The overlay of the binding curves (Fig. 8) shows that the full binding activity on alpha-toxin is preserved and formulation in hydrogel has no influence on the target binding of the antibody.

Besides the ability to bind alpha toxin the neutralization capability of the antibody is a meaningful measure for the quality of the antibody. The neutralization of alpha-toxin by the antibody was analyzed in a hemolysis assay based on rabbit erythrocytes (RBC assay). The antibody was titrated to a fixed amount of alpha-toxin and rabbit red blood cells. Hemoglobin in the supernatant of lysed red blood cells was quantified by a colorimetric measurement.

Antibody in PBS as well as after release from hydrogel of concentrations of 4 µg/ml and higher showed the ability to neutralize 10 ng/ml alpha-toxin and therefore protect red blood cells from toxic lysis (Fig. 9).

Overlay of the neutralization curves from antibody in PBS and hydrogel demonstrate that the full alpha-toxin neutralization capability is preserved in the formulation. A further characteristic to evaluate the

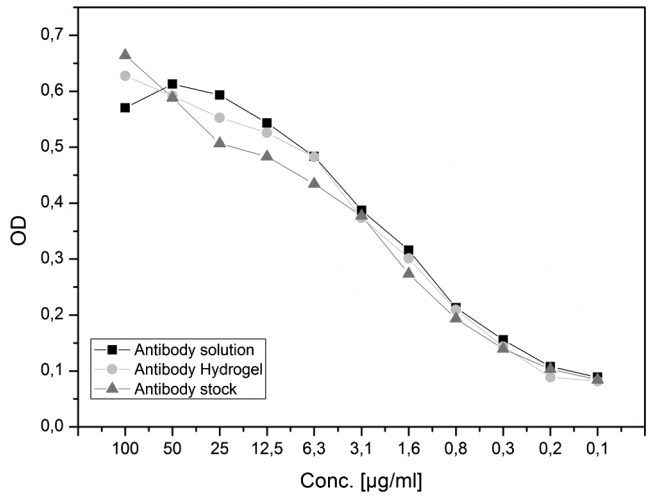

Fig. 8. Comparison of the capability of antibody in PBS and hydrogel to bind alpha-toxin in a direct ELISA. Data represent one of three measurements showing comparable results.

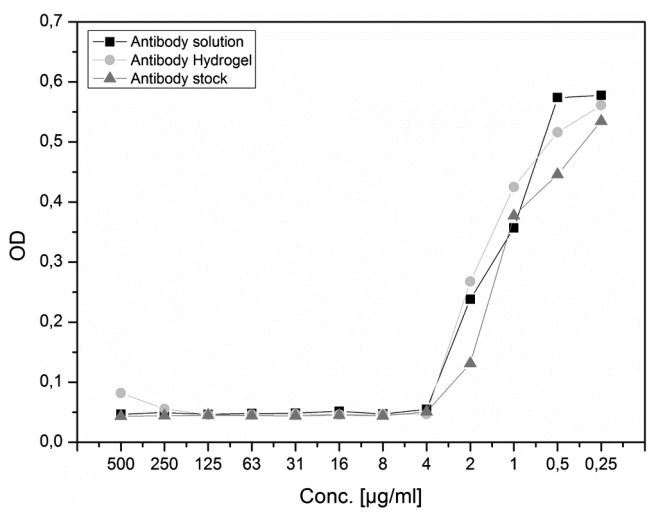

Fig. 9. Comparison of the capability of antibody in PBS and hydrogel to neutralize alpha-toxin in a rabbit red blood hemolysis assay. Data represent one of three measurements showing comparable results.

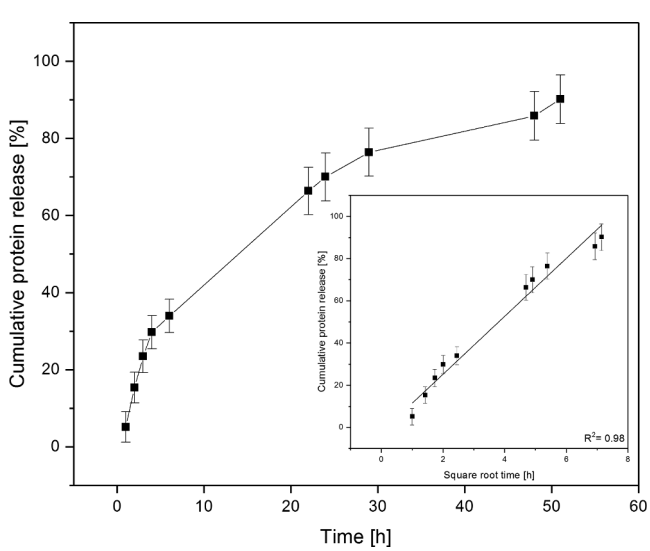

Fig. 10. Release profile of fresh hydrogel containing Ab. Data is presented as mean ± SD (n = 3).

quality of the hydrogel was the antibody release profile. From Fig. 10 it can be seen that nearly 40% of the antibody is released after six hours. After 50 h an almost complete release could be realized. Additionally, antibody release follows Higuchi kinetics (square root of time, $R^2 = 0.98$), as expected for the liberation from hydrogel matrices [32].

Poelstra et al describe for 10 mg/ml human IgG from 2% CMC gel a more rapid release. Nearly 90% of the human IgG is released after 9 h and a complete antibody release is observed after about 30 h [18]. This difference in liberation velocity may be due to different parameters of the *in vitro* release procedure, such as temperature (37 °C vs. 32 °C) and release model (beaker vs. flow through cell). Furthermore, the authors do not specify the investigated CMC type or the viscosity of the resulting hydrogel. However, based on the given information, one can assume a notably reduced viscosity of the hydrogel compared to our 1.5% CMC hydrogel. This lower viscosity of the matrix may lead to the accelerated release rate observed by Poelstra et al. However, the release kinetics (Higuchi square root of time) of both types of antibody containing CMC gels are comparable, indicating a diffusion controlled liberation of the antibody from the hydrogel.

Our work shows the ability of the preparation of a sterile sprayable hydrogel formulation of antibodies for easy use on skin of dog patients.

3.3. Efficacy of the antibody in hydrogel on skin of dogs

To evaluate the efficacy of the antibody containing hydrogel after topical application we designed a new test model that features the toxic damage of bacterial skin infections. This enables a proof-of-concept study that illustrates the potency of the antibody gel *ex vivo*.

3.3.1. Bioavailability of the antibody in hydrogel on infected ex vivo canine skin

To analyze co-localization of the anti-alpha toxin antibody in hydrogel with the dog skin pathogen *S. pseudintermedius* after a time window of 30 h the *S. pseudintermedius* strain 69687, labeled with the fluorescence marker FITC and hydrogel containing antibody were applied by pipetting on a sample of çanine skin and cultivated in a transwell air-liquid interface system for 30 h. Transversal cryosections of the biopsy were stained with a secondary Alexa Fluor 647 labeled antibody recognizing bovine IgG (red) and counterstained with DAPI (blue). Bacteria (in green) as well as antibody (in red) and a co-localization of both (overlay in yellow) were detected on the surface of the epidermis and in the *stratum corneum* (Fig. 11D). Single color channel measurements show the staining of skin only by DAPI (Fig. 11A), bacteria only by FITC (Fig. 11B) and antibody gel only by Alexa Fluor-647 (Fig. 11C) and make the spectral overlap of the two fluorochromes DAPI and FITC visible. The antibody is co-localized with *S. pseudintermedius* indicating bioavailability.

The co-localization study was also used to measure the penetration using intact canine skin after 30 h incubation on an air-liquid interface. Bacteria as well as antibody remain on the skin surface and no antibody was detectable in the recipient compartment. Neither bacteria nor the antibody penetrated the epidermis indicating that the antibody does not enter deeper skin compartments or the systemic circulation.

3.3.2. In vitro efficacy of the antibody

The chosen *S. pseudintermedius* strain 69687 was isolated from skin of a dog in the UK in 2012. It belongs to the phenotypic group of multidrug resistant MRSP and shows resistance to seven antibiotic classes [1].

Various *S. pseudintermedius* isolates have been shown to secrete toxins, which can damage keratinocytes [24]. To verify the alpha-toxin producing capability of 69687 and neutralization of this toxin by the antibody *in vitro* the hemolysis of rabbit erythrocytes by culture supernatant of 69687 as well as neutralization of alpha-toxin in the supernatant by the antibody was analyzed in a hemolysis assay.

The supernatant was titrated to a fixed amount of antibody and

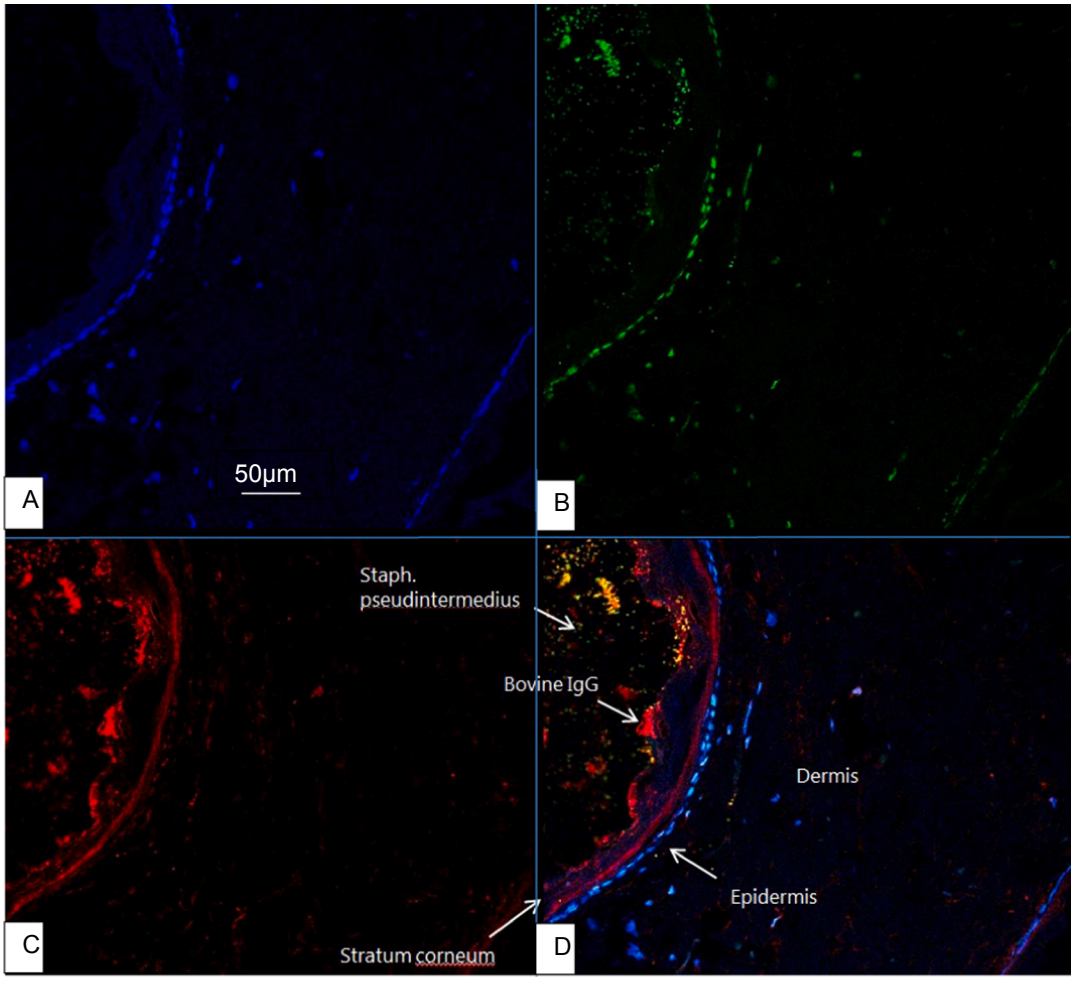

Fig. 11. Localization of antibody in hydrogel and Staphylococci on transversal sections of canine skin after 30 h incubation. Skin cells are labeled in blue, bacteria in green and antibodies in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rabbit red blood cells. Hemoglobin in the supernatant of lysed red blood cells was quantified by a colorimetric measurement.

The presence of a hemolytic activity in the supernatant of *S. pseudintermedius* 69687 could be shown, which could be neutralized by the anti-alpha toxin antibody (Fig. 12). Three independent experiments showed comparable results.

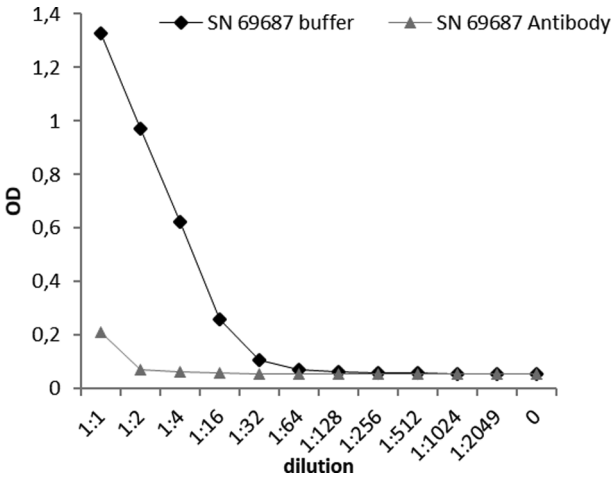

Fig. 12. Capability of antibody in PBS to neutralize alpha-toxin in the supernatant of *S. pseudintermedius* 69687. Data represent one of three independent experiments showing comparable results.

3.3.3. *Efficacy of the antibody in hydrogel in an ex vivo canine skin model*

In a human skin explant wound model to investigate the pathologic effect of alpha toxin on skin, treatment with alpha-toxin from *S. aureus* as well as infection with live bacteria producing alpha toxin resulted in high tissue toxicity and loss of skin epithelial integrity. Alpha toxin-specific antibodies applied in this model were able to protect human skin from structural damage and reduce tissue toxicity due to alpha toxin activity [20].

We established a canine skin explant model for infection with *S. pseudintermedius.* Tissue damage was assessed by H&E (Hematoxylin and Eosin) staining for histopathological changes. Morphological analysis of untreated skin (Fig. 13A) showed a normal skin composed of viable epidermis, dermis and adnexal structures. Epidermis shows normal stratification with clearly visible layers. Infection with live bacteria of the *S. pseudintermedius* strain 69687 producing alpha toxin resulted in high tissue toxicity, loss of skin epithelial integrity and even loss of basal membrane and separation of the epidermal from the dermal layer (Fig. 13B) visible on transversal sections. The alpha toxin-specific antibody in hydrogel applied in this model by pipetting was able to protect canine skin from structural damage and reduce tissue toxicity to a high degree. The epidermis showed normal stratification and adnexal structures between epidermis and dermis were present (Fig. 13D) Hydrogel without antibody as placebo showed no effect. The histologic findings of the infected skin after placebo application were comparable to infected untreated skin (Fig. 13C).

To quantify the toxic damage caused by *S. pseudintermedius* and the efficacy of antibody in hydrogel dead cells appearing as ghost cells,

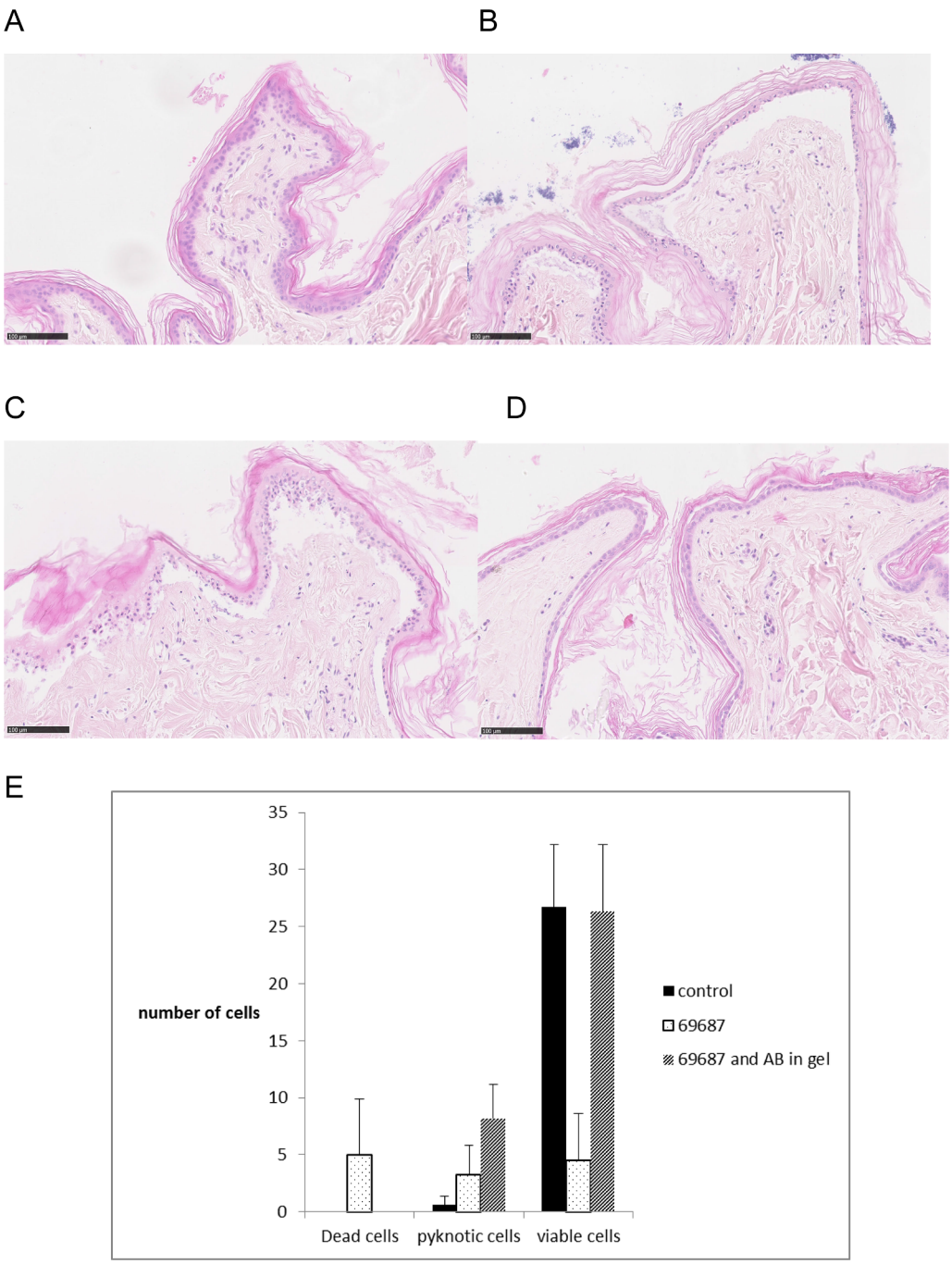

Fig. 13. Hematoxylin and eosin staining of transversal sections of canine skin samples to analyze morphological changes caused by toxic damage (20 fold magnification). A: untreated control skin, The epidermis has normal thickness (1–3 cell layers) and is covered by a basket-weave keratin layer. B: skin infected with *S. pseudintermedius* strain 69687, there is separation of the epidermis from the dermis, resulting in a subepidermal cleft. C: skin infected with *S. pseudintermedius* strain 69687 and treated with placebo gel, the epidermis is separated from the dermis and a subepidermal cleft is visible. D: skin infected with *S. pseudintermedius* strain 69687 and treated with antibody gel resembling non-treated skin without separation of epidermis from dermis. E: Quantification of skin damage by counting of cells classified as viable, pyknotic or dead on H&E slides. Data represent one of three independent experiments using skin of different dogs showing comparable results.

apoptotic cells with pyknotic nuclei and viable cells were counted in a blinded manner at a magnification of 30 fold on ten comparable areas each of H&E stained slides from control samples, samples colonized with *S. pseudintermedius* and treated with hydrogel as placebo or with antibody in hydrogel using Nano Zoomer Digital Pathology (Hamamatsu) (Fig. 13E). The antibody cannot completely prevent skin cells from being damaged shown as morphological changes (pyknotic cells), however it can prevent skin cells from death. In bacterially infected skin about 30% of the cells undergo cell death, 30% show morphological changes (pyknotic) and 30% are viable. In bacterially infected skin treated with antibody however about 75% of the cells are viable, 25% of the cells show morphological changes (pyknotic) and no dead cells can be found. Even though in absolute numbers there are more pyknotic cells in treated versus untreated samples, the relative number of damaged cells is lower.

Periodic acid Schiff (PAS) staining makes the basement membrane zone visible, which maintains skin integrity by anchoring the overlying epidermis to the dermal matrix (Fig. 14A). Infection with alpha-toxin producing *S. pseudintermedius* 69687 leads to damage of the basal membrane resulting in loss of skin integrity (Fig. 14B). Simultaneous application of anti-alpha toxin antibody in hydrogel by pipetting results in partial protection of basal membrane damage (Fig. 14C).

Three independent experiment using skin from different dogs showed comparable results.

In summary topical application of the anti-alpha toxin antibody in hydrogel on *ex vivo* canine skin leads to localization of the antibody on the skin surface without penetration into the epidermis and partial co-localization with colonizing *S. pseudintermedius* producing alpha toxin. The antibody is released from the hydrogel and shows a high efficacy to protect skin from toxic bacterial damage. The data are in accordance

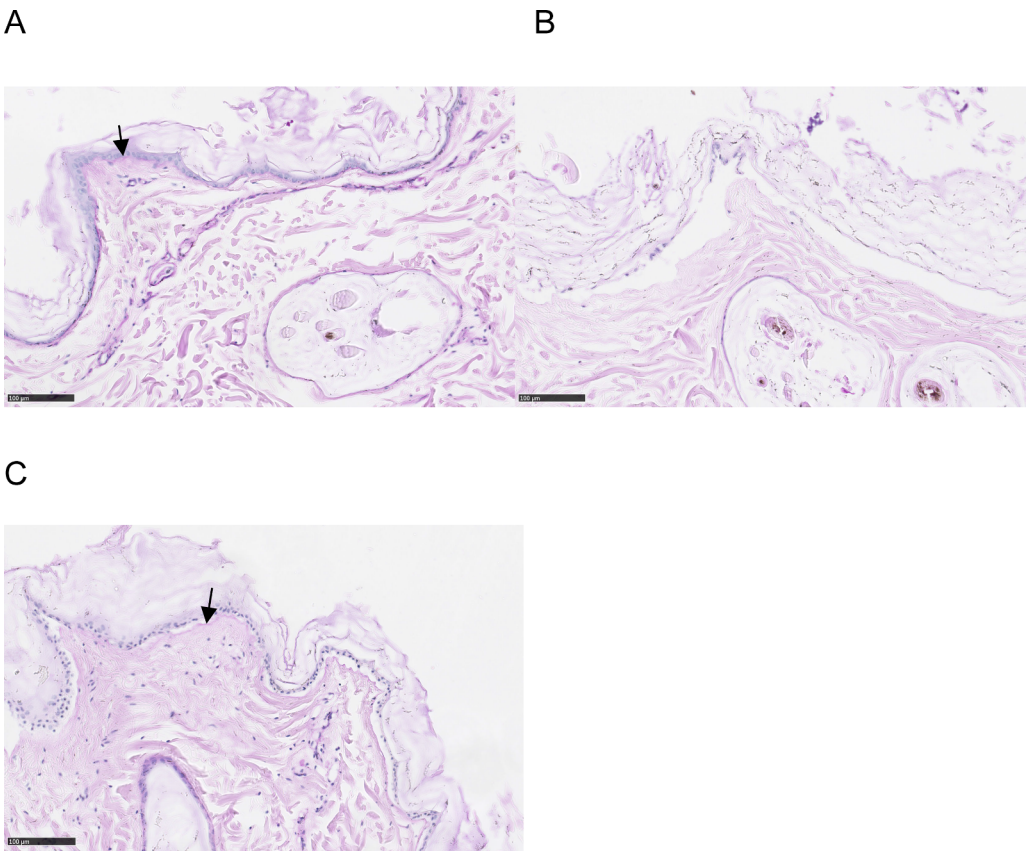

Fig. 14. PAS staining of transversal sections of canine skin biopsies to analyze the integrity of the basal membrane (20 fold magnification). The basal membrane is visible as a line between the epidermal and dermal layer (arrows). A: untreated control skin, B: skin infected with *S. pseudintermedius* strain 69687, C: skin infected with *S. pseudintermedius* strain 69687 and treated with antibody gel. Data represent one of three independent experiments using skin of different dogs showing comparable results.

with the findings of Poelstra et al. showing the local efficacy of antibacterial polyclonal IgG in gel [18].

4. Conclusion

We describe the development of a hydrogel as formulation for immunoglobulins for topical administration on the skin. The formulation fulfills all requested properties by being sprayable and keeping the antibody stable and active as well as releasing functionally active antibody in a therapeutically meaningful timeframe. In an *ex vivo* study on canine skin the antibody containing hydrogel shows efficacy by protecting epidermal cells from damage caused by pathogenic Staphylococci.

Declaration of Competing Interest

The authors claim no conflict of interest.

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