Histomorphometric evaluation of MMP-9 and CD31 expression during healing under Negative Pressure Wound Therapy in dogs

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Abstract

The aim of the study was to evaluate the molecular and histological effects of negative pressure wound therapy (NPWT) on vascularisation in clinical cases of open wound treatment in dogs. Open wounds (n=10) were randomly assigned to one of two groups: NPWT treatment (n=5) or foam treatment (polyurethane-foam dressing, n=5). Wounds were matched based on age and underlying cause and analysed with respect to neovascularisation (CD31) and matrix proteinase changes (MMP-9). Histological slides were blinded and analysis was performed using automated histomorphometric software. Values determined at day zero after debridement were used as a reference and wound development at day six was evaluated using linear mixed models. Signalment, pre-treatment time and underlying cause were similar between groups. NPWT resulted in a highly significant increase of vascularisation (p<0.001) compared to controls, in which mean vascularization indices decreased from day one to day six. MMP-9 activity decreased from day 0 to 6 with no significant differences between groups. This study indicates that NPWT exerts a substantial effect on vascularisation and tissue organization within wounds in dogs.

Keywords: negative Pressure Wound Therapy, open wound treatment, neovascularisation, matrix-metalloproteinase 9, dog

Histomorphometrische Beurteilung der MMP-9 und CD31 Expression während der Wundheilung unter Unterdrucktherapie beim Hund

Ziel der Arbeit war es, die molekularen und histologischen Effekte der Vakuumtherapie (NPWT) auf die Vaskularisation während der offenen Wundtherapie in klinischen Fällen bei Hunden zu erfassen. Offene Wunden (n=10) wurden randomisiert in zwei Gruppen unterteilt: NPWT Therapie (n=5) oder Abdeckung mittels Polyurethan Wundauflage (n=5). Die Wunden wurden in Hinblick auf das Wundalter sowie die Ursache gepaart und hinsichtlich Neovaskularisation (CD31) und Veränderung der Matrix-Metalloproteinase-Aktivität (MMP-9) untersucht. Die Beurteilung der einzelnen Histologiepräperate erfolgte geblindet mittels automatisierter Histomorphometrie Software. Werte die an Tag 0 nach Debridement erfasst wurden dienten hierbei als Referenzwert für die Entwicklung der Wunden an Tag sechs, welche mittels eines gemischten Modells analysiert wurde. Signalement, Dauer der Vortherapie sowie Ursache waren zwischen den Gruppen vergleichbar. NPWT führte in Vergleich zur Kontrolle zu einer signifikanten Steigerung der Vaskularisation (p<0,001). In der Kontrollgruppe verringerten sich die Vaskularisations Indices an Tag sechs verglichen zu Tag null. Die MMP-9 Aktivität nahm zwischen Tag null und sechs ab, ohne signifikante Unterschiede zwischen den Gruppen. Diese Arbeit zeigt das NPWT Therapie einen signifikanten Effekt auf die Vaskularisation und Gewebeorganisation von Wunden beim Hund hat.

Schlüsselwörter: Vakuumtherapie, offene Wundtherapie, Neovaskularisation, Matrix-Metalloproteinate 9, Hund

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Introduction

The effectiveness of Negative Pressure Wound Therapy (NPWT) for the enhancement of wound healing has been known for some time (Argenta et al.,2006). Studies in humans, pigs and mice have shown that NPWT results in a uniform reduction of interstitial oedema due to active fluid drainage, causing a reduction of interstitial pressure and an increase of blood flow and enhanced granulation (Argenta et al., 2006; Wackenfors et al., 2005; Morykwas et al., 2001; Saxena et al., 2004). Although these primary effects have been consistently documented in clinical cases in human and veterinary medicine, the underlying mechanisms are still under investigation (Glass et al.,2014; Derrick and Lessing,2014).

More experimental and clinical studies have been published on NPWT than on any other wound dressing in small animal medicine so far with increasing interest (Demaria et al., 2011; Pitt et al., 2014; Nolff et al., 2015; Nolff et al., 2016). Unfortunately, so far there is only one study available that specifically investigated the cellular effect of NPWT compared to a control in beagle dogs (Demaria et al., 2011). Despite documenting superior granulation in the NPWT group, they were not able to detect any histological effect based on a semi-quantitative score (Demaria et al.,2011). Given the favourable effect of NPWT in clinical canine cases (Pitt et al.,2014; Nolff et al.,2015; Nolff et al.,2016), and the documented effects on cells and growth factors in other species this finding seems unlikely (Jacobs et al.,2009; Labler et al., 2009; McNulty et al., 2009; Derrick and Lessing 2014; Glass et al.,2014; Liu et al.,2014; Wang et al.,2014; Xia et al., 2014; Ma et al., 2016; Tanaka et al., 2016). The aim of this study was to evaluate the effect of NPWT treatment on neovascularisation (microvessel density) and matrix-metalloproteinasis-9 (MMP-9) excretion in clinical cases of open wound therapy in dogs, and to compare it to a standard treatment protocol.

Animals, Material and Methods

The current study was performed as part of a prospective study conducted between July 2014 and September 2016 after approval of the ethic commission of the veterinary faculty (22-27-02-2014). Dogs were included if they were presented with comparable wounds (age, underlying cause, infection status, location) requiring open wound treatment and written owner consent was available.

Treatment protocols

All patients (n=7) underwent complete surgical debridement with en bloc resection of the initial wound bed at initiation of therapy in order to create a fresh surgical wound (n=10 wounds). In Group A, NPWT was performed at a continuous pressure of -125 mmHg (ActiVac, KCI Medical, Germany). In Group B, the wounds were initially covered using a foil coated polyurethane-foam dressing (Allevyin, Smith and Nephews, Germany) sutured to the surrounding skin. Dressing changes were performed at day 3 and 6. Tissue biopsies were obtained at day 0 (after debridement) and at day 6 in all patients, and then they were used for further immunohistological investigation. Further patient treatment was adjusted to the condition of the individual patient.

Histology and Immunhistochemistry

Standard haematoxylin and eosin (HE) stained slides were available from all patients. For the detection of vascular endothelial cells, tissue sections were incubated with a polyclonal rabbit anti-murine CD31 antibody (AP15436PU-M, Acris Antibody GmbH, Germany ; dilution 1: 100). Immunostaining for MMP-9 was performed using a polyclonal rabbit anti-murine gelatinase-B (MMP-9) (RM105MMP9, Triple Point Biologics, United States; dilution 1: 300) that is directed against the whole molecule, as described previously for canine specimens (Miao et al., 2003). For CD31, tissue sections were boiled in 10 mM citrate buffer at a pH of 6 for ten minutes followed by cooling at room temperature for another 20 minutes for antigen retrieval. Non-specific binding was blocked using inactivated goat serum, diluted 1:5 in phosphate-buffered saline (pH 7.1). This was then replaced by the primary antibody diluted in PBS before incubation in a moist chamber at 37 °C for 30 minutes. As a detection system, the avidin–biotin–peroxidase complex was applied as described previously. As positive controls, a cell pellet of a canine macrophage/monocytic tumour cell line originating from a disseminated histiocytic sarcoma (DH82 cells) was used for MMP-9 and normal canine skin for CD31. Sera from non-immunized Balb/cJ mice for the monoclonal antibody and from non-immunized rabbits for the polyclonal antibodies diluted to a protein concentration (globulin fraction) identical to the concentration of the antibodies were used as negative controls.

Histomorphometry

Sections were blinded before further evaluation. For each parameter, five sections per patient and time-point were evaluated. Ten high power fields (magnification 400×) per section were randomly chosen along the wound surface at the area of most intense staining (hot spots). Digital images were obtained and analyzed using automated histomorphometric software in order to mi-

nimize intra-observer bias (Aperio Image Scope Software v11.2.0.780T, Leika, Germany).

Microvessel Index

The microvessel index was determined as number of vessels per high power field as previously described. However, since vessel diameter or architecture can bias manual counting, we additionally performed an automated histomorphometric evaluation. Based on the description of Low and Di Pietro (2010) the percentage of positively stained pixels per high power field was used to determine the vascularity index (Low et al. 2010).

$$Vascularity\ index = \frac{positive\ stained\ pixels\ per\ HPF}{all\ stained\ pixels\ per\ HPF} \times 100$$

Positive pixels were assessed using the positive pixel count Makro of Aperio Image Scope Software at the following setting: Hue value 0.1, Hue width 0.5, color saturation threshold 0.04, Iwp (High) 220, IWP (Low) = IP (High) 90, Ip(low) = Isp(high) 90, Isp(low) 0, inp(high)-1 (Fig.1A and B).

Matrix metalloproteinate 9

MMP-9 Intensity was also quantified using the positive pixel count Makro of Aperio Image Scope Software [g] at the following setting: Hue value 0.1, Hue width 0.5, colour saturation threshold 0.04, Iwp (High) 220, IWP (Low)= IP (High) 160, Ip(low) = Isp(high) 100, Isp(low) 0, Inp (high)-1. Total MMP-9 stain was determined by addition of areas of medium and high intensity staining per slide divided through all detected pixels (Fig. 1 C and D).

Statistical methods

Data were coded in Excel and analyzed using SPSS® 20.0 Statistics. Descriptive statistics such as mean and range were computed. In order to address individual
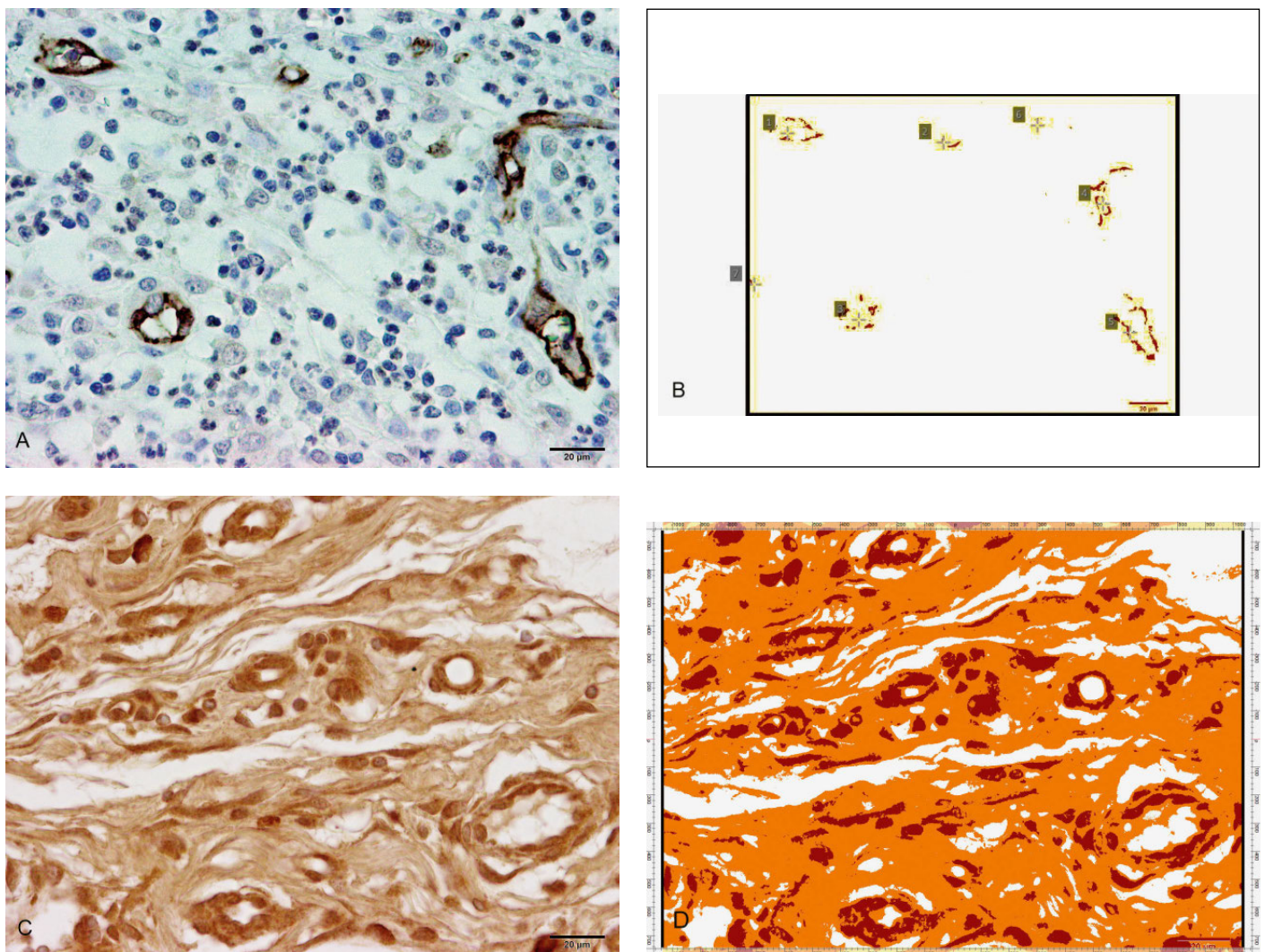
Figure 1: Automated histomorphometric evaluation of CD31 (A and B) and MMP-9 (C and D) percentage using the positive pixel count algorithm of Aperio Image Scope.

differences at initiation of therapy, values for this time-point were taken as reference zero and development from zero to day six was determined. To account for repeated measurements per animal, a mixed linear regression model was used to assess differences between treatment groups. Significance was set at *p < 0.05.*

Results

A total of 7 dogs (10 wounds) were enrolled within the study. Two dogs presented with identical wounds at dif-

Figure 2: NPWT treated tissue (A) already exhibited a high grade of tissue organisation. Inflammatory cells (red arrowheads) were mainly arranged along the NPWT sponge borders (*). Fibroblasts were oriented paralell to the wound surface (black arrows) while vessels were aligned tangential to the surface (black arrowheads). Control wounds (B) displayed an amorpous arrangement of multinucleated cells (arrowheads) and fibrin. . Haematoxilin and eosin stained overview (100x magnification).

ferent locations (Dog No. 5: bilateral flank injection site abscess and dog No. 6: bilateral inguinal wounds after surgical site infection) representing perfect pairs, another dog developed open wounds after serious infection of two toes at the same hindlimb at different time-points (dog No. 7). Finally, the remaining partners were different patients with comparable disease (two pairs of dogs with surgical site infections (No. 1 and 2 as well as No. 3 and 4)). Mean age of patients treated with NPWT was 76 months (range 16-153 months), and mean body weight was 38.4 kg (range 9-93 kg). Patients with foam-treated wounds had a mean age of 59 months (range 16-103 months), and a mean body weight of 32.5 kg (range 9-93 kg). Mean duration of previous treatment in wounds treated by NPWT was 54.8 days (range 2-240) and 53.8 days for foam-treated wounds (range 3-240 days). The mean time to closure in NPWT treated wounds was 9 days (range 6-11) compared to 33.25 days (range 16-43) in foam treated wounds.

General histological findings

HE stained overviews indicated a higher grade of granulation tissue maturation and in NPWT treated specimens (Fig. 2A). Vessels were frequently aligned parallel towards each other and tangential with respect to the surface. Different layers of highly organized fibroblastic cells were detectable beneath the foam aligned parallel to the wound surface. The NPWT foam residues were covered by multinucleated cells. In contrast, foam-treated wounds displayed an amorphous array of numerous multinucleated cells and individual clusters of fibrocytes, fibrin and vessels (Fig. 2B).

Neovascularisation

Mean vessel density per HPF was 4.9 in NPWT (range 0-15) and 6.9 in foam-treated wounds (range 0-25) at day 0. At day 6, NPWT wounds displayed a mean microvessel density of 10 (range 2-22) compared to foam-treated wounds, which had a mean microvessel density of 6 (range 0-18). The development from day 0 to day 6 showed a highly significant ($p < 0.001$) increase in microvessel density in NPWT-treated wounds (Fig. 3A).

This observation was substantiated by applying the automated vascularity score as second independent method (Fig. 3B). NPWT wounds reached a vascularity count of 0.4% (range 0.08-2.3%) at day 0, which showed a 2.9 fold increase to 1.27% (range 0.03-3.9%) at day six. In contrast, foam-treated wounds began with a mean vascularity index of 0.8% (range 0.02-4.8%) that showed a 0.3 fold decrease to 0.2% (range 0.009-1.7%) at day 6. As with the conventional microvessel density, this development was highly significant ($p < 0.001$).
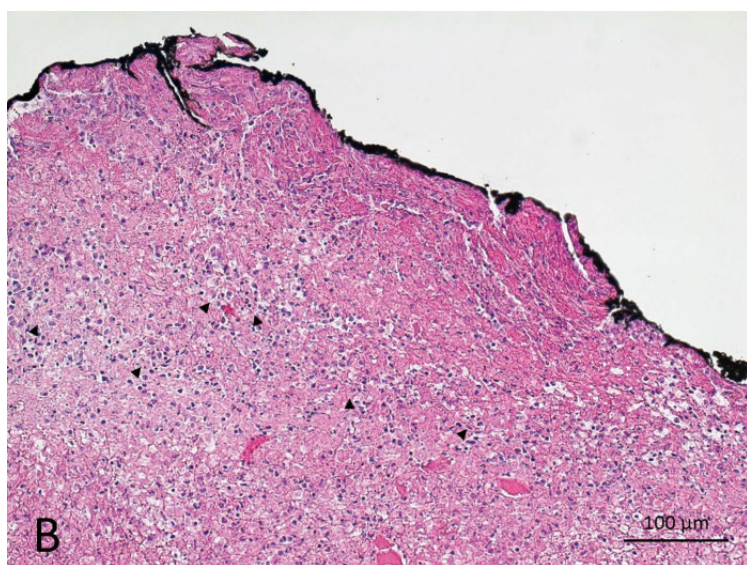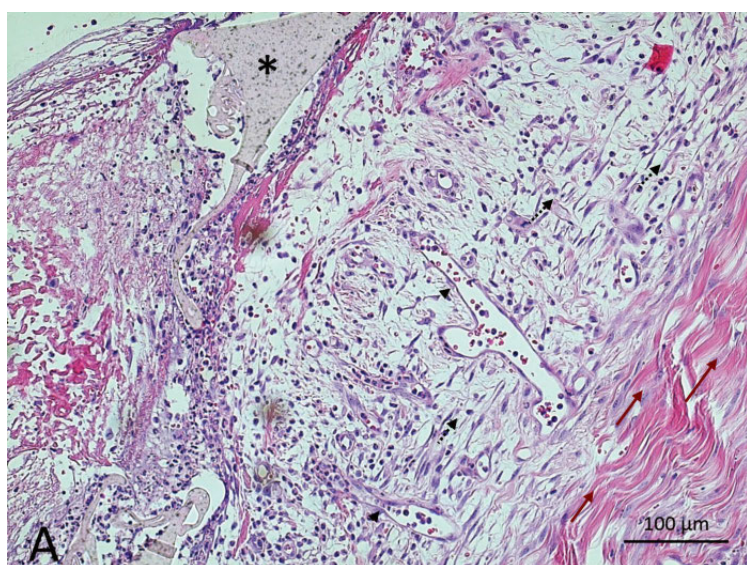
MMP-9

NPWT wounds had a total of 71.2% MMP positive stained area (range 14.6-96.5%) compared to 75.1% for the foam-treated wounds (range 38.4-97.8%) at day 0. The amount of positively stained area decreased by day 6 in both groups (NPWT 23.9% (range 0.2-74.2%); Foam 29.9% (range 0.09-98.5%) MMP-9 positively stained area (Fig. 3C)). No statistical significant differences were detected between treatments.

Discussion

The main finding of this study is that we were able to detect a higher neovascularisation rate as well as an increase in tissue organisation under NPWT treatment that was accompanied by a faster closure compared with foam-treated controls. Comparable findings have been previously documented in experimental studies in mice and swine were they could be linked to effects on growth factor and cytokine expression as well as faster maturation of myofibroblasts resulting in earlier organisation of collagen compared to control treatment (Jacobs et al., 2009; Labler et al., 2009; McNulty et al., 2009; Erba et al., 2011; Derrick and Lessing, 2014; Glass et al., 2014; Liu et al., 2014; Wang et al., 2014; Xia et al., 2014; Ma et al., 2016; Tanaka et al., 2016). Neovascularisation is a prerequisite for granulation tissue formation, as increased numbers of cells can only be viable with increased vascular supply (Wackenfors et al., 2005; Scherer et al., 2008; Ma et al., 2016). Moryckwas et al. (2001 found that vascularisation and increased granulation were closely correlated in NPWT treated wounds in swine. Ma et al. (2016) even reported that the amount of neovascularisation and vessel maturation was closely correlated with the overall prognosis of successful wound healing.

Interestingly, the only available study evaluating the effect of NPWT on granulation tissue quality in dogs documented faster, smoother granulation of wounds under NPWT therapy when compared to the controls; however they failed to detect a histological difference between treatment groups (DeMaria et al., 2011). Given the fact that improved wound healing, in terms of faster closure and rapid build-up of macroscopically healthy well vascularised granulation tissue, has been constantly documented in various experimental and clinical studies in dogs so far (De Maria et al. 2011, Pitt et al. 2014, Nolff et al 2015, Nolff et al. 2016), it seemed unlikely that this macroscopic effect would not be reflected histologically as seen in other species, and we were now able to document that the clinical effects are indeed accompanied by an improved histological tissue organisation and vascularisation. The fact that this was not documented by DeMaria et al. (2011) is most likely accountable to major differences in the way the histologi-
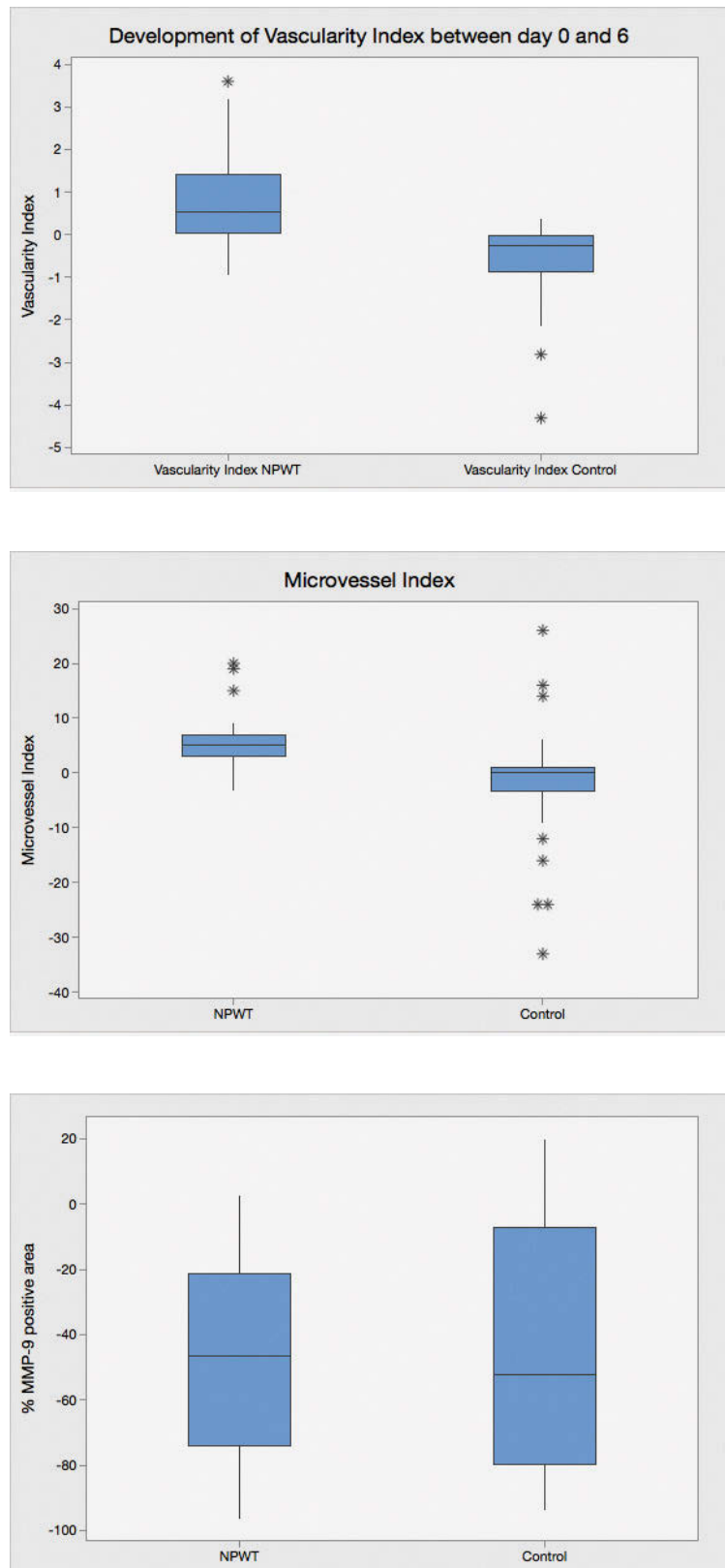

Figure 3: Boxplot diagrams showing the difference CD31 (A and B) and MMP-9 (C) levels between day 0 and 6 in NPWT and control group. While NPWT showed significant increased microvessel formation, there were no significant differences detected for MMP-9 between groups.

cal evaluation was performed. We chose to use immunohistochemistry to focus on the detection of CD31 expression and MMP-9 development as these were factors were major effects have been previously documented in other species. In contrast, Demaria et al. (2011) used a semiquantitative score which was mainly based on the inflammatory cell population and number of fibroblasts. They did not rate tissue organisation in total and did not include vascularisation in their score. We found that NPWT improved neovascularisation is one of the major effects of the technique, however we did not include a semiquantitative score based on immune cell phenotypes in our comparison. It could be that NPWT actually does not increase the inflammatory response in terms of an increased number of inflammatory cells, but rather by altering their products as has been described previously. However, based on our study design we cannot say anything about this, which makes direct comparison of our study and the DeMaria study difficult.

In contrast to CD31, no significant differences were detected for MMP-9 development between NPWT treated wounds and controls. MMP-9 cleaves the basement membrane and degrades local damaged collagen structures in order to loosen the extracellular matrix up in preparation for neovascularisation and ingrowth of fibroblasts (Lebert et al., 2015; Cho et al., 2016). Beside this important role, recent studies have also reported that MMP-9 itself is an important angiogenic factor (Cho et al. 2016). The synthesis of MMP-9 is controlled by tissue pH, stimulation by other growth factors and exposure to extracellular matrix (Salo et al. 1994). MMP activity needs to be tightly controlled, as a high MMP-9 activity is needed in early phases of healing, followed by a decrease in later stages in order to allow collagen deposition and maturation of the extracellular matrix (Salo et al. 1994). MMP-9 has been widely investigated in terms of its role in wound healing in various species (Lebert et al., 2015; Cho et al., 2016) but unfortunately so far there are no studies that have actually characterised the major MMP class responsible for healing in dog wounds, nor do we have any information regarding the timely events of MMP activation and downregulation during wound healing in this species. This study clearly documents that MMP-9 is upregulated in the early phase of wound healing and shows a significant decline within the first 6 days. However, we were not able to prove our hypothesis that NPWT treatment would result in a faster reduction of MMP-9, as has been previously documented in other species (Glass et al., 2014). The total effect of treatment on MMP-9 expression was low (0.064) resulting in a poor power (0.12) for this parameter. Given the very small effect size, a sample size of almost 1500 individuals per group would be needed to find a statistically significant effect. This implies that rather than our study being under-powered, there is little or no effect to be detected using our methods. We believe one or more of the following three explanations can account for this minimal effect, 1) Day six might have been too late to detect the effect or, 2) MMP-6 might simply not be affected by NPWT as it is in mice and humans or, 3) pathologically increased MMP-9 values might only be a problem in chronic wounds, and usage of NPWT might be beneficial in these cases but might not accelerate to normal development in an undisturbed healing response in an acute wound.

The main limitations of this study are small sample size and limitations on comparability of clinical cases. However, we tried to minimize these limitations by selecting and matching of comparable wounds. Furthermore all wounds were completely debrided and converted into fresh surgical wounds at day 0 and development of all parameters was rated in relation to the situation found at day 0. Nevertheless the small sample size is a major limitation, especially as clinical cases are influenced by numerous factors. However, these factors can only poorly be mirrored in experimental models, which represent another major flaw of purely experimental studies regarding NPWT effects in dogs- as the conditions created there nearly never match the clinical situation were the patient is mostly severely compromised. But it is exactly those situations were NPWT treatment exerts most effects- wounds that have been unsuccessfully treated before, have caused systemic infections and endanger the survival of the patient are the ones that represent the indication of this technique (Pitt et al., 2014; Nolff et al., 2015; Nolff et al., 2016). Our results have to be interpreted under the light of the discussed limitations; however the results indicate the previously detected beneficial effect of NPWT treatment on wound healing in dogs might be due to an increase in tissue organisation and vascularisation, as has been documented in other species before.

Conclusion

From the results of this study, we can conclude that angiogenesis and tissue organisation seems to be supported by NPWT in dogs undergoing open wound therapy.

Conflict of interest statement

The first author occasionally acts as clinical instructor for Acelity. Acelity played no role in this study regarding study design, data acquisition, analysis or interpretation as well as writing of the manuscript.

Acknowledgements

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Evaluation histomorphométrique de l'expression de la MMP-9 et de la CD31 au cours de la guérison des plaies sous thérapie par pression négative chez le chien

Le but de ce travail était de relever les effets moléculaires et histologiques de la thérapie des plaies par pression négative (NPWT) au cours du traitement de plaies ouvertes en clinique chez le chien. Des plaies ouvertes (n=10) ont été répartie de manière randomisée en deux groupes : NPWT (n=5) ou recouvrement au moyen d'un pansement en polyuréthane (n=5). Les plaies ont été appariées en tenant compte de leur âge ainsi que de leur cause et examinées quant à leur néo vascularisation (CD31) et aux variations de l'activité des métalloprotéases matricielles (MMP-9). L'appréciation des divers échantillons histologiques a été faite à l'aveugle, au moyen d'un logiciel d'histomorphométrie automatisé. Les valeurs obtenues au jour 0 après débridement servaient de référence pour l'évolution des plaies au jour 6, évolution analysée avec un modèle mixte. Le signalement, la durée du traitement préalable ainsi que les causes étaient comparables entre les groupes. La NPWT amenait, comparativement au contrôle, à une augmentation significative de la vascularisation (p<0.001). Dans le groupe de contrôle, les indices de vascularisation étaient plus faibles au jour 6 qu'au jour 0. L'activité des MMP-9 diminuait du jour 0 au jour 6 sans différence significative entre les groupes. Ce travail montre que la NPWT a un effet significatif sur la vascularisation et l'organisation des tissus dans les plaies chez le chien.

Stima istomorfometrica dell'espressione di MMP-9 e CD31 per la guarigione della ferita sotto terapia a pressione negativa nel cane

Scopo di questo studio era di rilevare nei cani gli effetti molecolari e istologici della terapia NPWT (Negative Pressure Wound Therapy) sulla vascolarizzazione, durante la terapia con ferita aperta nei casi clinici. Le ferite aperte (=10) sono state randomizzate in due gruppi: con terapia NPWT (n=5) oppure con copertura di poliuretano della ferita (n=5). Le ferite sono state accoppiate per durata e causa della ferita e esaminate sotto l'aspetto della neovascolarizzazione (CD31) e dell'alterazione dell'attività della metalloproteinasi della matrice (MMP-9). La valutazione dei singoli preparati istologici a cieco è stata possibile con l'utilizzo del software di istomorfometria automatizzato. I valori registrati il giorno 0 dopo lo sbrigliamento sono serviti da valori di referenza per lo sviluppo delle ferite al giorno 6, il quale è stato analizzato grazie a un modello misto. Segnalazione, durata del trattamento preliminare e cause sono risultate comparabili tra i gruppi. La NPWT ha condotto a un significativo aumento della vascolarizzazione (p<0.001) rispetto al gruppo di controllo. Nel gruppo di controllo gli indici di vascolarizzazione sono diminuiti al giorno 6 rispetto al giorno 0. L'attività MMP-9 è diminuita tra il giorno 0 e il giorno 6 senza differenze significative tra i gruppi. Con questo studio si dimostra che la terapia NPWT ha un effetto marcante sulla vascolarizzazione e l'organizzazione dei tessuti nelle ferite dei cani.

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