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Hyaluronic acid, Manuka honey and Acemannan gel: Wound-specific applications for skin lesions

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ABSTRACT

Healing of open wounds is of great medical importance. Wound healing is a complex process that aims to restore the function and structure of damaged tissue. This study was conducted to compare secondary intention healing of wounds treated daily with a topical application of commercially available hyaluronic acid (HA), Manuka honey (MH), Acemannan gel (AG), or a placebo. Bilateral wounds were surgically created on the backs of six sheep. At two and six weeks post-wound creation, biopsies were obtained to perform histological, immunohistochemical, and molecular analyses of the wound site. Daily clinical evaluations were performed and weekly photographs were taken of the wounds. HA treatment promoted a physiological progression of the healing process in all wound healing phases, while stimulating an abundant cutaneous adnexa and promoting rapid healing, representing the most compelling treatment. MH-treated wounds were slightly dry. However, the main effect of MH was to promote cell proliferation and neovascularization, with an overall pro-inflammatory effect. Results suggest that MH treatment enhances the healing process. AG treatment dehydrated the wounds and stimulated late granulation tissue and cell proliferation. Moreover, AG-treated wounds produced a mild late pro-inflammatory and neovascularization effect. Our data indicate that AG treatment can have a positive influence on moist wounds with abundant granulation tissue and exudate.

1. Introduction

The main function of skin is to protect against external agents. The loss of a large portion of the skin can lead to disability and functional and cosmetic recovery can take a long time. For these reasons, healing of open wounds has always been of great human and veterinary medical interest and importance.

Wound healing is a dynamic and complex multi-stage process that results in the restoration of tissue integrity and function. It is composed of three phases: i) haemostasis and inflammation, which involve the formation of a blood clot and the release of growth factor from platelets; vascular permeability, chemotaxis, migration of neutrophils, monocytes, macrophages and lymphocytes to the wound site that release cytokines and prevent wound infection; ii) a proliferative phase, in which granulation tissue formation occurs; this provisional extracellular environment consists of vessels, fibroblasts, and inflammatory cells (at the same time neovascularization, re-epithelialization, and wound contraction take place); iii) maturation and remodeling, when the granulation tissue is replaced by mature collagen (Fossum, 2012). At times, the normal wound healing process fails to adequately restore skin integrity, leading to potentially severe complications such as chronic wounds or scarring (Han and Ceilley, 2017). Many studies have investigated the efficacy of different topical treatments on all phases of wound healing. However, there have been no studies to compare their effectiveness from the clinical, histological, and immunohistochemical perspective. In this study, three different commercially available topical treatments were evaluated and compared: hyaluronic acid (HA), Manuka honey (MH), and Acemannan gel (AG).

The glycosaminoglycan HA, also known as hyaluronan, can be found in many mammalian tissues and body fluids, with the highest concentrations in connective tissue and skin (Weindl et al., 2004). In human and animal models, it has been observed that the topical application of HA accelerates the wound healing process (Taylor et al., 2004). However, the efficacy of topical HA has not been evaluated in sheep.

Due to its intrinsic proprieties, HA can stimulate and accelerate healing mechanisms during all phases of the wound healing process. During the haemostasis phase, HA combines with fibrin to generate a

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scaffolding that promotes the migration of cells involved in repair mechanisms (West et al., 1985). During the inflammatory phase, HA stimulates the migration of macrophages and granulocytes to the damaged tissues, with a significant impact on phagocytosis. It also induces pro-inflammatory cytokines and enhances cell infiltration. In addition, the antioxidant properties of HA appear to moderate inflammation and prevent oxygen free radical damage of granulation tissue (Lundin et al., 1985). Finally, HA accelerates the granulation phase (proliferative phase) through its effects on repair processes such as fibroblast migration and proliferation, collagen synthesis, and endothelial cell proliferation (Liguori et al., 1997; Weindl et al., 2004). It has been demonstrated that HA fragments, which are released following injury, increase the expression of chemokine IL-8 in endothelial cells, thereby stimulating the endothelium to recognize injury and initiate wound repair (Taylor et al., 2004).

MH is a honey obtained from bees that pollinate the manuka tree (*Leptospermum scoparium),* a tree native to Australia and New Zealand. MH has been demonstrated to have therapeutic advantages in wound healing over other honeys (Alvarez-Suarez et al., 2014). The main benefits of MH are found in its potent antibacterial, antioxidant, and wound healing properties (Alvarez-Suarez et al., 2014). Moreover, it has a high osmolarity, a high sugar content, and a low pH (3.5–4.5), which inhibits microbial growth. It stimulates the bactericidal action of macrophages and, in chronic wounds, it reduces protease activity and increases fibroblast activity and oxidation (Lusby et al., 2002; Sell et al., 2012; Alvarez-Suarez et al., 2014). The pronounced antibacterial activity of MH depends on a non-peroxide antibacterial compound called methylglyoxal (MGO) (also referred to as “Unique Manuka Factor”) in addition to defensin-1, various polyphenolic compounds, and complex carbohydrates (Alvarez-Suarez et al., 2014). The biological effect of MH on wounds occurs due to the stimulation of macrophage migration, with the acceleration of tissue turnover and the formation of a protective surface barrier thus decreasing healing time (Alvarez-Suarez et al., 2014).

AG is a β(1, 4)-linked mannane acetylated compound, and the primary polysaccharide extracted from the *Aloe vera* plant. It is a commercially available topical wound medication that is commonly used to enhance the healing of wounds, burns, and ulcers, while at the same time speeding the healing process (Chithra et al., 1998a; Dart et al., 2005; Maenthaisong et al., 2007; Hamman, 2008; Sahu et al., 2013). The compound stimulates macrophages to enhance the secretion of interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). These cytokines stimulate the proliferation of fibroblasts, the development and growth of epidermal cells, and collagen deposition. They also promote a prolonged stimulation of granulation tissue (Zhang and Tizard, 1996; Liptak, 1997; Chithra et al., 1998a,b,c; Dart et al., 2005; Sahu et al., 2013). Furthermore, AG induces the expression of vascular endothelial growth factor (VEGF) and other factors related to wound healing (e.g., keratinocyte growth factor-1 and type 1 collagen), giving it a pro-angiogenic effect (Liptak, 1997; Dart et al., 2005; Jettanacheawchankit et al., 2009; Majewska and Gendaszewska-Darmach, 2011; Boonyagul et al., 2014). It may also have a stabilizing effect on growth factors, prolonging their effects on fibroplasia (Dart et al., 2005). This study explores the efficacy of different topical treatments on experimentally created wounds on sheep.

2. Materials and methods

2.1. Animal model

Ethical approval for the experiment was obtained from the Body for the Protection of Animals (OPBA), ministerial decree n° 51/2015-PR, released by the Health Department of Italy.

Six female Bergamasca sheep of similar age and size were chosen for inclusion in the study. During two weeks of acclimation at Padova University (MAPS Department), parasitological and biochemistry examinations were conducted. Sheep represent a better experimental model than equines and carnivores because they are less neurologically developed, while still being of sufficient size for the creation of experimental lesions. Sheep have also been identified as a possible research animal model for human medicine (Music et al., 2018; Lankadeva et al., 2018). Considering the “3Rs” principle (replacement-reduction refinement) (Russel and Burch, 1959), sample size was based on previous studies and, at the end of the project, the animals were relocated to a teaching farm.

2.2. Experimental design

The effects of three topical treatments were analyzed using the following: HA (Connettivina®, Fidia 2 mg/g), MH (Medihoney® Wound gel, Comvita), AG (Carra vet® Acemannan Wound gel) and compared to a placebo (phosphate saline buffer, PBS). The protocol was established by Broeckx et al. (2015). Six equidistant and symmetrical (with each other) lesions were created on the backs of six sheep, for five different therapeutic treatments and one lesion treated with the placebo (Martinello et al., 2018). In this study, the effects of three conventional topical treatment (Acemannan gel, Manuka honey and Hyaluronic acid) were analyzed and compared to a control group (phosphate saline buffer, PBS). After application of the treatments, the lesions were bandaged with sterile gauze using the “wet to-dry” method. Once per day, the wounds were cleaned with sterile saline solution, the therapeutic treatments (except for the placebo) were reapplied, and the bandages were changed.

At two time points (15 and 42 days post-lesion creation), two 6 mm punch biopsies were collected from each lesion using appropriate sedation and analgesic drug administration. Of the two biopsy samples collected for each time point, one was used for histopathological and immunohistochemistry testing and the other was used for molecular analysis.

2.3. Clinical evaluation

Wounds were photographed and measured every day to evaluate the progress of the lesions. A clinical evaluation was conducted every week by the same blinded researcher. Presence and character of the exudate, hydration status of the wound, and presence of granulation tissue were recorded as part of each evaluation (Hadley et al., 2013) (Table 1).

2.4. Histopathological analysis

All biopsy samples (12 AG-treated, 12 MH-treated, 12 HA-treated, and 12 placebo-treated) were evaluated by histopathology. The samples were embedded in optimal cutting temperature (OCT) compound (Kaltek) and frozen in isopentane and liquid nitrogen. They were then

Table 1
Score system used in the clinical examination.

Parameters	Score	
Hydration	-2	Desiccation + +
	-1	Desication +
	0	Normal
	1	Maceration +
	2	Maceration + +
Presence of exudate	1	None
	2	Mild
	3	Moderate
	4	Abundant
Granulation tissue	1	Absent
	2	Mild
	3	Moderate
	4	Abundant

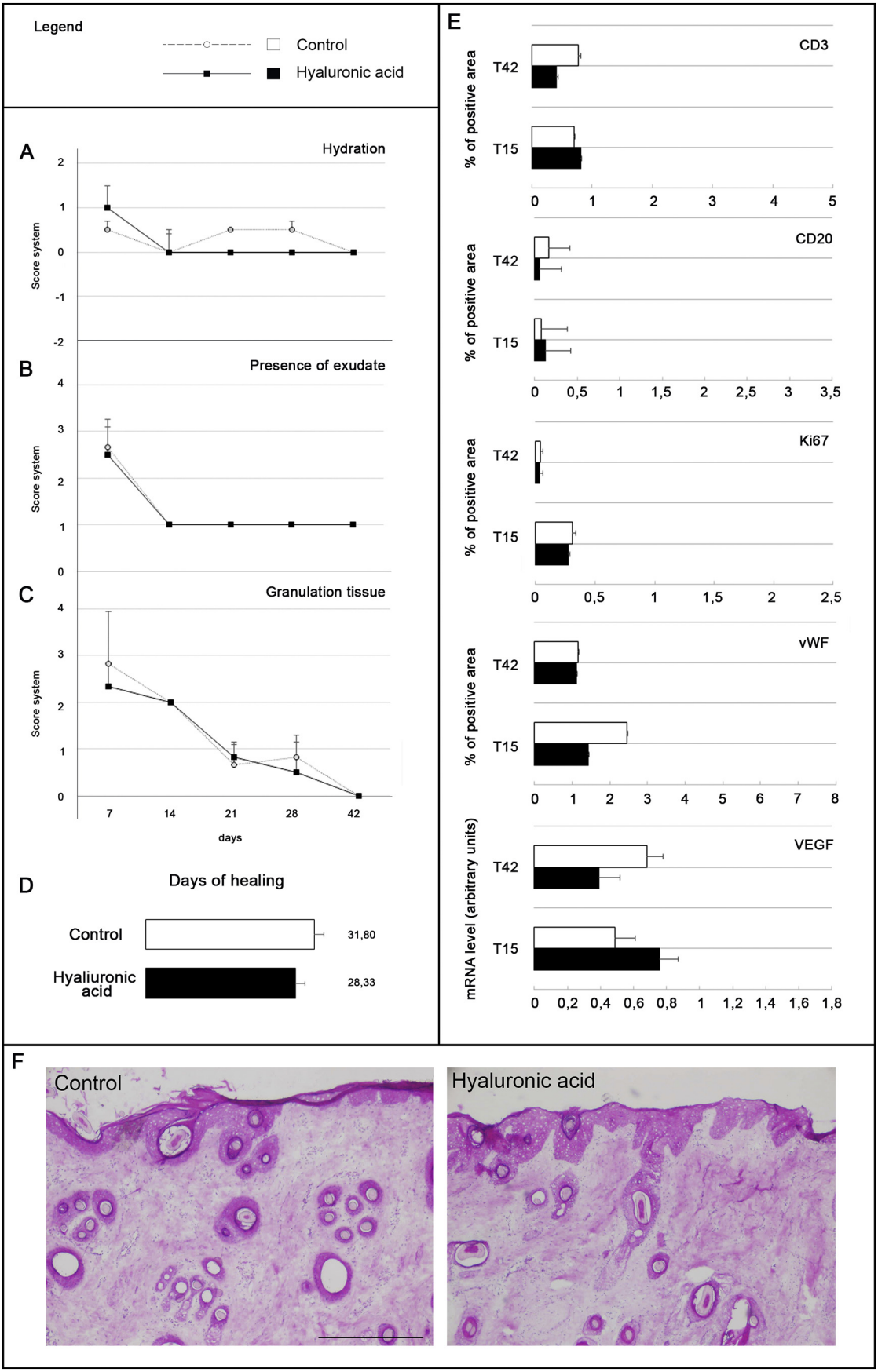

Fig. 1. Analysis of Hyaluronic acid treatment (black lines and black bars) vs control group (dashed lines and white bars). **A, B, C**: clinical evaluation, the score system is indicated in Table 1. **D**: wound closure time. **E**: immunohistochemistry and mRNA gene expression analyses; percentage of positive staining for CD3, CD20, KI67, vWF and mRNA level of VEGF; each graph represents the main ± SD and asterisk indicates significant differences between each treated group and control group ($p < .05$). **F**: representative Hematoxylin-Eosin photomicrographs of treated wound at 42 days from lesion; scale bar: 500 μm.

Table 2
Values of positive area evaluated for each immunohistological parameter (CD3, CD20, Ki67, vWF); values of arbitrary units of mRNA levels, evaluated for VEGF.

	CD3		CD20		Ki67		vWF		VEGF	
	T15	T42	T15	T42	T15	T42	T15	T42	T15	T42
Control	0,7 ± 0,02	0,77 ± 0,03	0,08 ± 0,02	0,16 ± 0,06	0,31 ± 0,02	0,04 ± 0,03	2,46 ± 0,02	1,15 ± 0,02	0,49 ± 0,11	0,68 ± 0,13
Hyaluronic acid	0,81 ± 0,02	0,4 ± 0,04	0,12 ± 0,05	0,06 ± 0,05	0,27 ± 0,03	0,03 ± 0,02	1,43 ± 0,01	1,12 ± 0,03	0,76 ± 0,12	0,39 ± 0,10
Manuka honey	4,07 ± 0,12	3,27 ± 0,13	0,89 ± 0,07	2,62 ± 0,06	0,99 ± 0,04	1,79 ± 0,03	2,36 ± 0,04	7,30 ± 0,11	0,13 ± 0,11	1,45 ± 0,12
Acemann gel	1,19 ± 0,03	1,28 ± 0,05	0,32 ± 0,04	1,10 ± 0,05	0,42 ± 0,04	2,09 ± 0,04	1,29 ± 0,05	1,34 ± 0,04	0,93 ± 0,12	0,76 ± 0,13

cut with a cryostat into 5 μm slices before being mounted on slides and stained with Hematoxylin and Eosin (H&E). In order to perform a full thickness examination, all samples were examined at six different pre-selected depths. The following characteristics were evaluated for all samples: the presence of dermal and subcutaneous infiltrates, (immature) granulation tissue, undifferentiated mesenchymal tissue, and the development of adnexa. These characteristics were scored using a 0 to 4 scale (0 = absence, 1 = presence, 2 = small amount, 3 = moderate amount, 4 = abundant amount). Data were presented as the relative frequencies of the assigned values and calculated for each sheep and for each parameter.

2.5. Immunohistological evaluation

Serial slices were immunostained with polyclonal rabbit anti-human CD3 (cluster of differentiation 3, T-cell co-receptor) (Dako, 1:100), polyclonal rabbit anti-human CD20 (cluster of.

differentiation 20, B-lymphocyte antigen) (Thermo Fisher, 1:100), monoclonal mouse anti-human.

Ki67 (Dako, 1:10), and monoclonal rabbit anti-human vWF (von Willebrand factor) (Dako; 1:3200). Immunolabeling was achieved with a highly sensitive horseradish peroxidase (PO) mouse or rabbit diaminobenzidine kit, with blocking of endogenous PO (Envision DAB + kit; Dako) in an.

autoimmunostainer (Cytomation S/N S38–7410-01; Dako). An antibody diluent (Dako) with.

background-reducing components was used to block hydrophobic interactions. The average of three.

fields from each slice was quantitatively evaluated for each immunohistological parameter and all measurements were performed with a computer-based program (Leica microscope DM LB2 with Leica Application Suite LAS V4.0) using 20× magnification.

2.6. Real-time PCR analysis

All biopsy samples were used to analyze the mRNA expression of VEGF. Total RNA extraction was performed using TRIzol reagent (Life Technologies) and quantified with a NanoDrop spectrophotometer (Thermo Scientific). The complementary cDNA was synthetized to perform real-time PCR using the AB 7500 Real Time PCR system (Applied Biosystems). All samples were tested in triplicate, and untreated skin was used as a calibrator sample. The 2-ΔΔCt method was used to analyze and normalize the RNA expression of the target genes with respect to the endogenous housekeeping genes RPS24 and 18S. PCR primers were designed using Primer Express 3.0 software (Applied Biosystems).

RPS24- forward 5' TTTGCCAGCACCAACGTTG 3′,
reverse 5'AAGGAACGCAAGAACAGAATGAA 3′,
18S - forward 5'AAACGGCTACCACATCCAAG 3′,
reverse 5'TCCTGTATTGTTATTTTTCGTCAC 3′,
VEGF- forward 5' GCTCTCTTGGGTGCATTGGA 3′,
reverse 5' TGCAGCCTGGGACCACTT 3′.

2.7. Statistical analysis

Data on clinical, histological, immunohistochemical, and molecular parameters were analyzed using.

PROC MIXED, with animal as both a random and repeated effect. The linear model included the fixed effect of treatment (AG – MH – HA vs placebo), time (week 1, 2, 3, 4, 5, and 6 for clinical evaluation and days 15 and 42 for histological, immunohistochemical, and molecular analysis), and their interaction. The assumptions of the linear model were graphically inspected using residual plots. For data that were not normally distributed (Shapiro-Wilks test < 0.90), the Mann-Whitney test was used (wound closure time, % re-epithelialization, presence of exudate). The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of hyaluronic acid

The healing progress of wounds treated with HA appeared similar to the progress of wounds treated with the placebo. HA-treated wounds presented the same moist environment as placebo-treated wounds (Fig. 1A) and, in the first week of the healing process, the volume of exudate, which was always serous-sanguineous and pink-red in colour, gradually diminished (Fig. 1B). Moreover, the granulation tissue modification showed a physiological trend (Fig. 1C). Weekly photographs indicated an acceleration of the healing process using HA, as compared to the control group (Fig. 1D). Complete re-epithelization was detected at 42 days post-wound creation for both the HA and placebo-treated lesions, which was not the case for the other treatment groups.

Expression of CD3 and CD20 did not indicate a significant difference in immune response between the HA and control groups (Fig. 1E, Table 2). Furthermore, similar levels of proliferative cells (evaluated by Ki67 immunostaining, vWF protein expression, and VEGF mRNA expression) were observed, indicating that HA treatment did not influence neovascularization (Fig. 1E, Table 2).

Histopathological examination (Fig. 1F) showed no significant differences between the HA treatment group and the control group at 15 (data not shown) and 42 days post-wound creation. At 42 days, subcutaneous inflammation and granulation tissue were completely absent in all wounds and a small amount of dermal inflammation was observed in only 17% of wounds from both groups.

3.2. Effect of Manuka honey

Clinical evaluation indicated differences in the healing process between wounds treated with MH and wounds treated with the placebo. The level of hydration (Fig. 2A) and the volume of exudate (Fig. 2B) was lower in the MH-treated wounds compared to control wounds, while the maturation of granulation tissue (Fig. 2C) appeared physiological.

Treatment with MH significantly stimulated the expression of CD3 at 15 and 42 days post-wound creation and the expression of CD20 after 42 days. Throughout the healing process, MH activated Ki67 and by 42 days it had enhanced the neovascularization process. Expression of vWF and VEGF mRNA increased significantly compared to the control

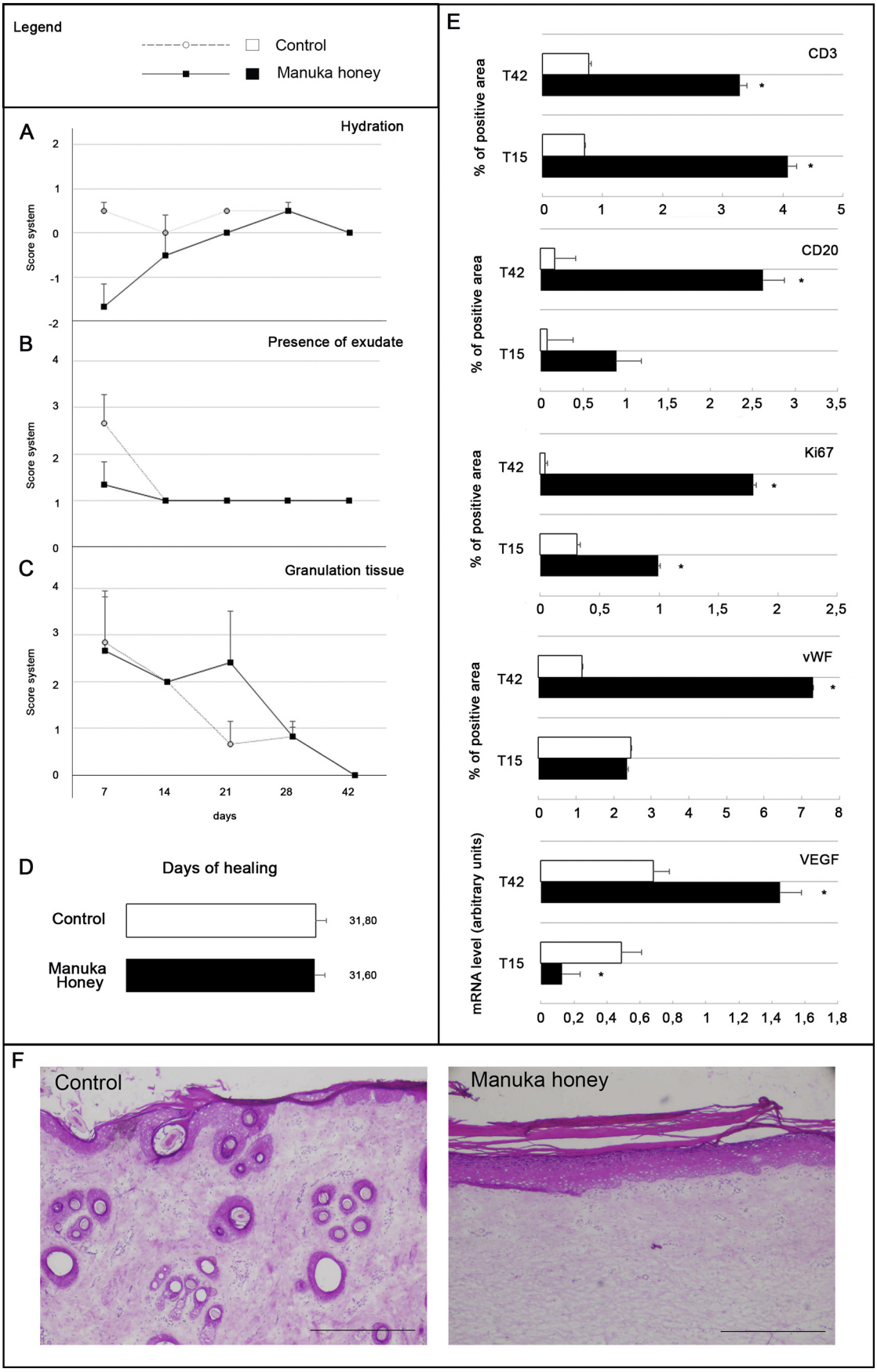

Fig. 2. Analysis of Manuka honey treatment (black lines and black bars) vs control group (dashed lines and white bars). **A, B, C**: clinical evaluation, the score system is indicated in Table 1. **D**: wound closure time. **E**: immunohistochemistry and mRNA gene expression analyses; percentage of positive staining for CD3, CD20, KI67, vWF and mRNA level of VEGF; each graph represents the main ± SD and asterisk indicates significant differences between each treated group and control group ($p < .05$). **F**: representative Hematoxylin-Eosin photomicrographs of treated wound at 42 days from lesion; scale bar: 500 μm.

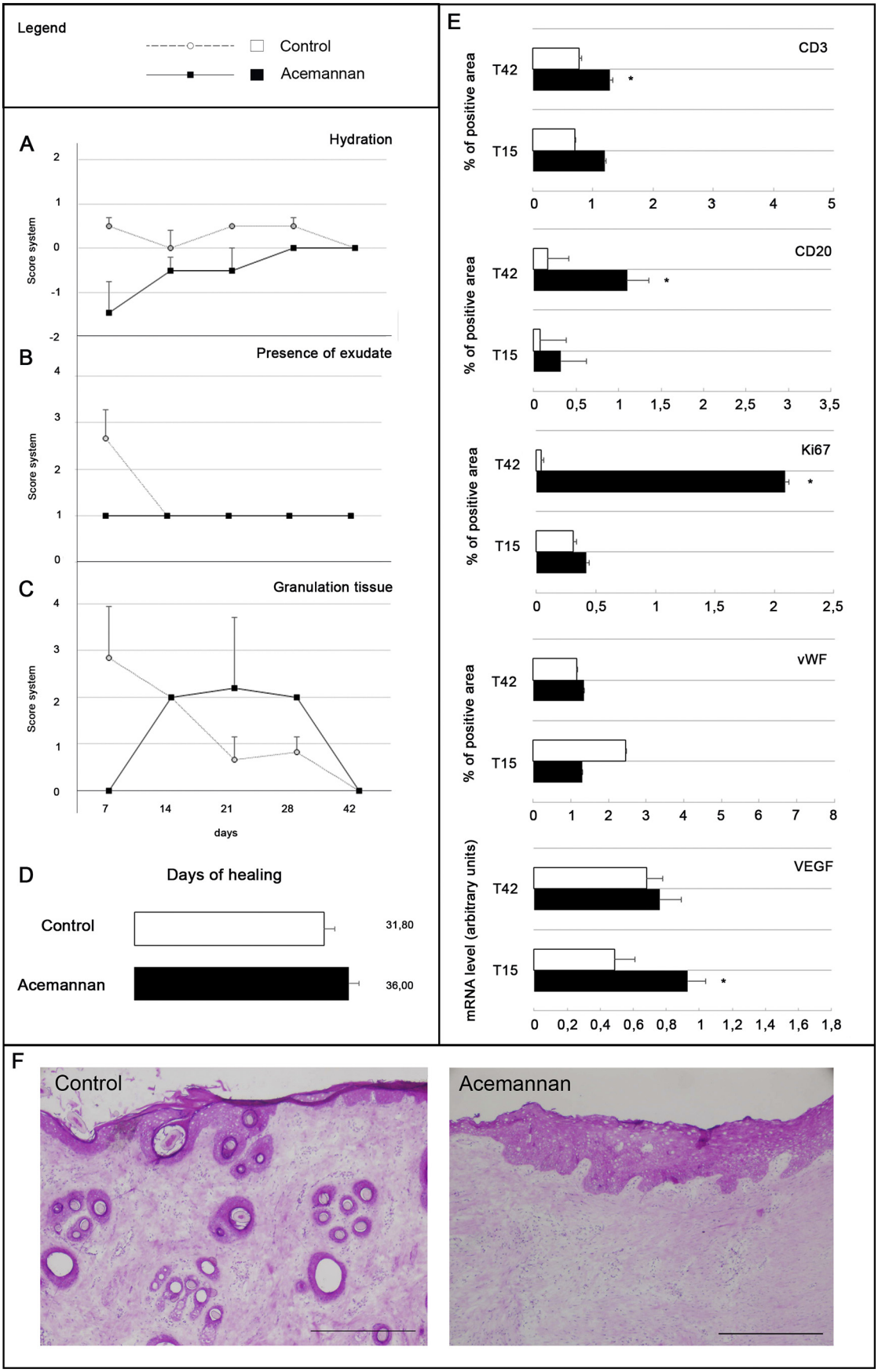

Fig. 3. Analysis of Acemannan gel treatment (black lines and black bars) vs control group (dashed lines and white bars). **A, B, C**: clinical evaluation, the score system is indicated in Table 1. **D**: wound closure time. **E**: immunohistochemistry and mRNA gene expression analyses; percentage of positive staining for CD3, CD20, KI67, vWF and mRNA level of VEGF; each graph represents the main ± SD and asterisk indicates significant differences between each treated group and control group ($p < .05$). **F**: representative Hematoxylin-Eosin photomicrographs of treated wound at 42 days from lesion; scale bar: 500 μm.

group (Fig. 2E, Table 2). Histopathology showed no remarkable differences between the MH-treated wounds and the control wounds at 15 days post-wound creation (data not shown). At 42 days, 40% of wounds treated with MH had a small amount of dermal inflammation, but no subcutaneous inflammation. Granulation tissue was observed in 17% of MH-treated cases. In addition, the epidermis appeared immature and was characterized by hyperkeratosis associated with deposition of keratin in the upper layers of the epidermis (Fig. 2F).

3.3. Effect of Acemannan gel

Treatment with AG resulted in drying of the wounds through day 28 post-wound creation (Fig. 3A) and there was almost no exudate produced throughout the healing process (Fig. 3B). In addition, the granulation tissue appeared later than in the control group, as it was only observed after 14 days (Fig. 3C). AG appeared to activate the immune response late in the healing process, with the levels of CD3 and CD20 significantly higher at day 42 compared to the control group. By day 42, cell proliferation was significantly stimulated by AG treatment, as indicated by Ki67 protein expression. AG did not appear to activate neovascularization and there was only a slight increase of VEGF mRNA expression at 15 days post-wound creation (Fig. 3E, Table 2). Histopathology showed a moderate amount of subcutaneous inflammation in 50% of AG-treated wounds. Immature granulation tissue was seen in 87% of AG-treated wounds by day 42 (Fig. 3F).

4. Discussion

Wound healing is a highly organized and coordinated series of processes that results in the restoration of tissue integrity and function. An interruption in the normal healing process can lead to the development of non-healing chronic wounds. The way wounds are managed affects the speed of healing and the final cosmetic appearance of the wounds.

HA is known for its positive effect on various kinds of wounds (Weindl et al., 2004; Voigt and Driver, 2012) in both veterinary and human medicine. In experimental studies, HA demonstrated superiority compared to other treatments, with respect to shorter wound healing time and histological characteristics (Al Bayaty et al., 2010; Shimizu et al., 2014). The present study confirms that topical application of HA can stimulate healing mechanisms during all three phases of the healing process. HA stimulated the production of granulation tissue, associated with slight maceration of the wound and a limited amount of exudate during the first 7 days of treatment. Although some studies have demonstrated that HA promotes cell proliferation and differentiation, and has anti-inflammatory properties (Nyman et al., 2013; Litwiniuk et al., 2016; Knopf-Marques et al., 2016), this study did not find that HA-treated wounds differed in these qualities compared to placebo-treated wounds. Nevertheless, the present study showed that HA had a greater effect on the stimulation of cutaneous adnexa such as hair follicles, sebaceous, and apocrine glands compared to the other two therapeutic treatments.

The use of honey for treating wound infections is a well-established practice (Alvarez-Suarez et al., 2014). MH is known for its antibacterial activity due to the “Unique Manuka Factor”, which is related to the content of MGO present only in the Manuka tree. Moreover, it seems to possess a pro-inflammatory effect, and increases the release of cytokines from leucocytes regulating angiogenesis and the proliferation of fibroblasts situated along endothelial cells (Bischofberger et al., 2013; Nooh and Nour-Eldien, 2016). The present study confirmed these actions, highlighting an immunomodulatory effect during the healing process, and the expression of CD3 at 15 and 42 days and CD20 at 42 days after the start of treatment. Furthermore, proliferation and neovascularization factors were presented after 15 days and increased their expression at 42 days. There are case studies where MH resulted in the healing of previously non-healing wounds (Sell et al., 2012; Biglari et al., 2013; Bischofberger et al., 2013). For example, MH has been used to treat problematic wounds like leg ulcerations (Gethin and Cowman, 2005).

The beneficial effects of AG have been reported in both human and veterinary medicine (Voigt and Driver, 2012; Aya and Stern, 2014; Neuman et al., 2015). AG has been recommended for full-thickness burns in pigs (Maenthaisong et al., 2007), oral wounds (Sasithanasate et al., 2008; Boonyagul et al., 2014; Boonpaisanseree et al., 2012), lacerations, dermal ulcers, and abrasions in horses and other domestic species (Dart et al., 2005). It enhances contraction and epithelialization of paw wounds in dogs and stimulates granulation tissue formation over exposed bone (Nisbet et al., 2010). Our results showed that AG stimulated development of granulation tissue late in the wound healing process and did not produce much fluid during the healing process compared to the control group. The latter effect was particularly evident in the early healing phases. In fact, the data obtained underline the dryness of wounds and the limited presence of exudate induced by AG treatment. Indeed, other studies have proposed that the greatest effects might be seen during the first 7 days after injury, and that wounds should be treated with AG and bandaged daily (Krahwinkel and Boothe Jr., 2006; Merckoll et al., 2009). Application of AG can continue through the inflammatory phase into the proliferative phase of wound healing (Merckoll et al., 2009). However, we observed a proliferative action, and an immune- and slight neovascularization stimulating effect in the late phase of the healing process. As reported by Hamman (2008), conflicting results obtained from different studies could be due to the use of plants from different locations with variations in chemical composition. Differences in the stability of active ingredients and in isolation techniques used to extract compounds from *Aloe vera* leaf pulp could also be responsible for these discrepancies.

5. Conclusion

In this study, HA, MH, and AG were applied to similar wounds to evaluate the effects of each treatment. Normally, the aims of wound treatment are to accelerate and ameliorate healing to restore the physiological integrity of the skin. HA was the best treatment for acute wounds where the priority is the reduction of healing time together with the progression of the wound healing process. Various wound types such as ulcers, burns and chronic wounds may need different types of interventions. In the present work, MH and AG did not reduce wound closure time, although they may enhance wound healing under certain circumstances. For example, the administration of MH is recommended when the wound appears to be non-healing or chronic because MH has a long-term effect on the healing process. On the other hand, due to its dehydrating effect, AG can be useful during the first week of the wound healing process, in the presence of abundant granulation tissue, elevated moisture, maceration, and abundant exudate.

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Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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