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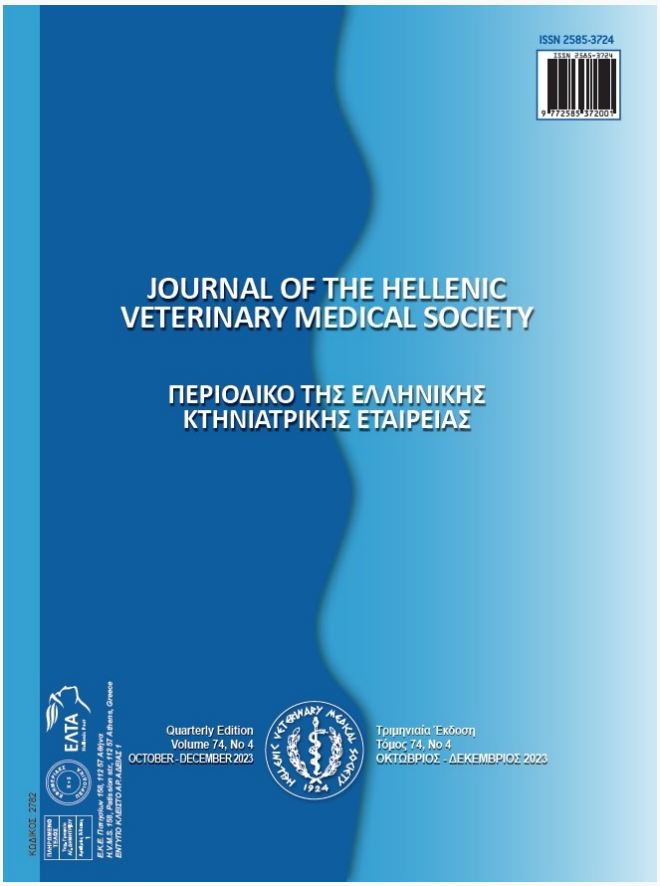

The effect of medical-grade honey or hypericum on matrix metalloproteinases expression in feline wound healing

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Research article
Ερευνητικό άρθρο

The effect of medical-grade honey or hypericum on matrix metalloproteinases expression in feline wound healing

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ABSTRACT: The aim of the present study was to evaluate the relation between MMP expression and the local application of medical-grade honey or Hypericum in second-intention cutaneous wound healing in healthy cats. Our hypothesis was that MMP expression may be affected by medical-grade honey or Hypericum application in cutaneous wounds in cats. Eight female spayed purpose-bred healthy DSH cats were included in this blind study. Under general anesthesia eight 2 X 2 cm square wounds, four on each side of the dorsal midline were created including the subcutaneous fat until the thoracolumbar fascia was apparent. The four wounds on each side were randomized to receive treatment or serve as untreated controls using computer software (random number generator). Two of the wounds were treated daily with medical-grade honey ointment [LMS] two were treated daily with Hypericum-based ointment (HYP) two were used as untreated controls for medical-grade honey (LMSC), and two were used as untreated controls for Hypericum-based ointment (HYPC). The LMS and HYP treatments were applied aseptically once daily for 25 days. Biopsies were obtained from the two cranial wounds for MMPs measurements on day 0 and from the two caudal wounds for MMPs measurements on days 14 and 25. An evaluation of the expression of MMP-2, MMP-9, and TIMP-1 by real-time PCR was performed to investigate their association with the different measurement days. MMP-2, MMP-9, and TIMP-1 expressions did not differ between the LMS and HYP-treated wounds. No significant differences between sampling days for MMP-2 MMP-9 and TIMP-1 expressions were noted in all treated and control wounds (P= 0.188, 0.580, and 0.407 respectively). In conclusion, MMP-1, MMP-9, and TIMP-1 expressions were not affected by medical-grade honey or Hypericum applied locally in cutaneous wounds in cats.

Keywords: Cat; honey; hypericum; matrixmetalloproteinases; wound healing

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INTRODUCTION

Cutaneous deficits in cats may be the result of traumatic injuries, surgical removal of masses, or infectious lesions. These cutaneous deficits can be healed primarily or by second intention through contraction and epithelialization. Second-intention healing in cats is slower than in dogs and wounds denuded of subcutaneous tissue may lead to delayed granulation tissue formation and often result in pseudohealing, pocket wounds, or chronic wound formation (Bohling et al., 2004; Bohlingand Henderson, 2006; Bohling et al., 2006) Therefore, the need to enhance the healing mechanism has led to the manufacturing of local products in companion animals including ointments and dressings(Volk et al., 2013).

Medical-grade honey promotes wound healing by accelerating granulation tissue and epithelialization development, collagen maturation, and by exerting antimicrobial effects (Molan 1999; Pleeging et al., 2020). Medical-grade honey accelerated wound healing in equines by local or subcutaneous application in contaminated wounds compared to untreated controls (Bischofberger et al., 2011; Bischofberger et al., 2013; Davidson, 2015; Mandel et al., 2019). Medical-grade honey showed promising healing effects for cutaneous defects in cats but was not effective on acute cutaneous defects in healthy dogs (Lukanc et al., 2018; Lukanc et al., 2020; Repellin et al., 2021). *Hypericum Perforatum* was reported to promote wound healing by accelerating granulation tissue formation in humans, cattle, rats, and cats (Yadollah et al., 2015; Tresch et al., 2019; Vatnikov et al., 2019; Samadi et al., 2020; Marino et al., 2021).

Matrix metalloproteinases (MMPs) are metalloendopeptidases that utilize Zn^{2+} or Ca^{2+} for activation (Sabino and Keller, 2015; Pavletic, 2018; Kandhwal et al., 2022). Twenty-eight different MMPs were discovered to date (Kandhwal et al., 2022). MMPs are divided into collagenases (MMP-1, MMP-8, MMP-13, and MMP-8) gelatinases (MMP-2 and MMP-9), stromelysins, membrane-type MMPs, and novel MMPs (Pavletic, 2018, Kandhwal et al., 2022). Their activation is regulated at the gene level, at the molecular level, and by endogenous tissue inhibitors known as TIMPs which bind directly to block MMPs function (Baker et al., 2002; Pavletic, 2018; Kandhwal et al., 2022). The wound healing process requires a balance between MMPs and their inhibitors (Pavletic, 2018). While MMPs expression is low in normal skin, their activity is increased following injury and excessive activity plays a role in chronic wound formation (Zhang et al., 1988; Xue et al., 2006; Sabino and Keller, 2015; Lazaro et al., 2016). MMPs participate in all phases of normal wound healing including the inflammatory phase and repair phase having a positive effect (Kandhwal et al., 2022). During the inflammatory phase, collagenases aid in the cleavage of the extracellular matrix collagen allowing gelatinases to perform collagen degradation into smaller parts (Pavletic, 2018). MMPs also help in the basement membrane and extracellular matrix degradation forming openings that allow the migration of endothelial cells to create new capillaries (Pavletic, 2018).There is a limited number of feline studies investigating the contribution of ocular surface components to MMPs in relation to corneal epithelial wounds and the relation between MMP expression in cutaneous healing following locally injected platelet-rich plasma (Petznick et al., 2013; Angelou et al., 2022).However, there are no studies investigating MMP expression in cutaneous healing in cats following the application of medical-grade honey or hypericum.

The purpose of the present study was to evaluate the relation between MMP expression and the local application of medical-grade honey or Hypericum in second-intention cutaneous wound healing in healthy cats. Our hypothesis was that MMP expression may be affected by medical-grade honey or Hypericum application in cutaneous wounds in cats.

MATERIALS AND METHODS

The study was approved by the State Veterinary Services (certificate # 598/2019) and the Bioethics committee of the School of Veterinary Medicine (certificate # 633,001/3,372). Eight female spayed purpose-bred healthy DSH cats aged 1-4 years and weighing 3-4 kg, current in vaccination were included in this blind study. Before the study commencement, all cats underwent physical examination, hematologic, biochemical, and fecal parasitology examinations, and were checked for feline immunodeficiency and feline leukemia viruses. The cats were separately kept in indoor kennels of the companion animal clinic fed a dry diet and were offered water freely. One week before the beginning of the study each cat had a padded bandage applied from the cranial thorax to the lumbar region caudally in order to get accustomed. After the end of the study, all cats were adopted.

Procedures

The cats received premedication with acepromazine maleate (0.05 mg/kg IM) and buprenorphine (0.1 mg/kg IM), induction of anesthesia was performed by propofol (2mg/kg IV), and maintenance was by isoflurane in oxygen (Angelou et al., 2022). Each cat was put on ventral recumbency, and the dorsolateral region extended from the thorax to the lumbar area was clipped and prepared for aseptic surgery. Eight 2 X 2 cm square wounds, four on each side of the dorsal midline were created being 3 cm apart from each other and 3 cm from the dorsal midline. A # 15 blade was used for the creation of the full-thickness wounds including the subcutaneous fat until the thoracolumbar fascia was apparent. Four simple interrupted 3/0 polyamide sutures were put through the skin and fascia at the corners of the squares to prevent slippage from the neighboring tissues. The four wounds on each side were randomized to receive treatment or serve as untreated controls using computer software (random number generator). Two of the wounds were treated with medical-grade honey ointment [LMS] (L-Mesitran soft, Triticum, Netherlands) two were treated with Hypericum-based ointment (HYP)[Hypermix-vet gel, RI.MOS, Italy]two were used as untreated controls for medical-grade honey (LMSC), and two were used as untreated controls for Hypericum-based ointment (HYPC).One side was randomized, andthe opposite side was adapted to always accommodate a pair of treatments and control. The LMS and HYP treatments were applied aseptically once daily for 25 days. Postoperatively, the wounds were dressed in three layers including a nonadherent dressing as a contact layer, a cotton pad bandage, and an adhesive bandage around the trunk. All cats had an Elizabethan collar placed. The cats recovered from anesthesia and were placed in their kennels and received buprenorphine (0.02 mg/kg IM) and tramadol (1 mg/kg SC, BID for 5 days). The CSU feline pain scale was used for pain assessment before and after bandage changes. In case of pain of more than five days duration, the cat received tramadol until the resolution of clinical signs. Pethidine (3 mg/kg IM) was administered as a rescue analgesic. Bandage changes were performed daily until the end of the study for 25 days using dexmedetomidine (0.04mg/kg IM). Atipamezole was used for reverse. Bandage changes were performed aseptically. Before ointment applications wounds and surrounding areas were cautiously cleaned with sterile saline-soaked gauze to prevent wound healing disturbance. Biopsies were obtained from the two cranial wounds for MMPs measurements on day 0 and from the two caudal wounds for MMPs measurements on days 14 and 25. As a result, a minimum of one of each treated and one control wounds on days 0,14, and 25 were obtained.

Evaluation of Matrix metalloproteinase expressions

An evaluation of the expression of MMP-2, MMP-9, and TIMP-1 by real-time PCR was performed to investigate their association with the different measurement days (Angelou et al., 2022). From the biopsies obtained from the wounds on days 0, 14, and 25 the specimens were frozen in liquid nitrogen and stored at -80^{0}C to be assessed at a later time. To generate accurate results, the appropriate housekeeping gene (YWHAZ) was used for data normalization as reported by others (Penning et al., 2007; Kessler et al., 2009;Jursza et al., 2014).RNA extraction from biopsies was done as reported by other authors (Konstantinidis et al., 2021), and reverse transcription was performed with the SuperScript III first-strand synthesis system (Invitrogen), according to the manufacturer's guidelines. Specific primers were depicted, related to the feline GenBank sequences, and for each respective target were: TIMP1 (sense: 5′-TGGCTGCGAAGAATGCACCGTAT-3′andantisense:5′-CTGGAAACCCTTGTCAGTGCCTGT-3′); MMP9 (sense: 5′- CGCACGACATCTTTCAGTTCCA-3′and antisense: 5′- CCGAGAACTCACACGCCAATA-3′); MMP2 (sense: 5′- GGGTGACCTTGACCAGAGCACGAT-3′ and antisense: 5′- GGTCCAGATCAGGCGTGTAGCCAAT-3′). For YWHAZ were (sense: 5′- ACAAAGACAGCACGCTAATAATGC-3′ and antisense: 5′- CTTCAGCTTCATCTCCTTGGGTAT-3′) [Kessler et al., 2009]. Primer pair specificities were confirmed by arranging each respective amplicon. The qPCR reaction (20 μl) comprised of 1× PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.2 μM of each primer, 0.2 mM of each dNTP, 3 mM MgCl2, 3 U of Platinum™ Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1× EvaGreen™ dye (Biotium, Hayward, CA, USA) and 2 μl of cDNA. Thermal cycling conditions were done as follows: 94°C for 3 min, and thereafter by 47 cycles into 2 steps: denaturation at 94°C / 20 s and annealing-extension at 60°C / 45 s for MMP2 and TIMP1 primers or at 57°C for MMP9 and YWHAZ primers, respectively. Fluorescence was documented following each cycle completion and a melting curve was created, by heating from 75°C to 90°C, in increments of 0.2°C/6

sec. Reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). Reaction effectiveness was determined for each primer set, using 10-fold serial dilutions (2*107–2*101 copies/reaction) of plasmids ligated with each amplicon, and was found close to 100%. Melting curve analysis did not display nonspecific amplicons in any of the reactions (Angelou et al., 2022). All RNA extracts were evaluated in triple. Target gene expression levels were established by the comparative threshold cycle (ΔCT) method (Schmittgenand Livak, 2008). Data were demonstrated as fold changes over untreated controls given by $2^{-\Delta\Delta CT}$. All bands were normalized to the expression of the housekeeping gene that was transcribed at a constant level. The band intensity values were expressed based on the absorbance.

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were conducted using computer software (SPSS Statistical Package for the Social Sciences, version 25.0). G-Power analysis was used to calculate the sample size. Based on previous studies, a group size of 8 cats could yield at least 80% power to determine differences at P≤0.05 (Bohling et al., 2004; Bohling et al., 2006; Angelou et al., 2022). For MMP-2, MMP-9, and TIMP-1 expression double repeated measures analysis of variance was performed, with these variables as dependent, cats (eight cats) as the sampling unit, and wounds (4 wounds) and the sampling day (0, 14, 25 days) as dependent variables. The observed significance levels of statistical tests were predetermined at α=0.05 (P≤0.05) and were estimated by the Monte-Carlo simulation method (Mehta and Patel, 2013).

RESULTS

MMP-2, MMP-9, and TIMP-1 expressions did not differ between the LMS and HYP-treated wounds. No significant differences between sampling days for MMP-2 expression were noted in all treated and control wounds (P= 0.188). There were no statistically significant differences in terms of time-wound interaction for MMP-2 (P = 0.094). On day 14 the mean MMP-2 expression in the LMS-treated wounds was 3.000 ± 2.8350 times higher than these on day 0 and the mean MMP-2 expression in the HYP-treated wounds was 18.563 ± 23.4914 times higher than theseon day 0.On day 14 the mean MMP-2 expression of LMSC or HYPC wounds was 9.938 ± 17.4417 times higher than those on day 0 LMSC or HYPC wounds. On day 25 the mean MMP-2 expression in the LMS-treated wounds was 0.850± 0.5806 times less than these on day 0 and the mean MMP-2 expression in the HYP-treated wounds was 1.325± 2.2372 times higher than these on day 0. On day 25 the mean MMP-2 expression of LMSC or HYPC wounds was 1.100± 0.8912 times higher than those on day 0 LMSC or HYPC wounds (Fig. 1).

No significant differences between sampling days

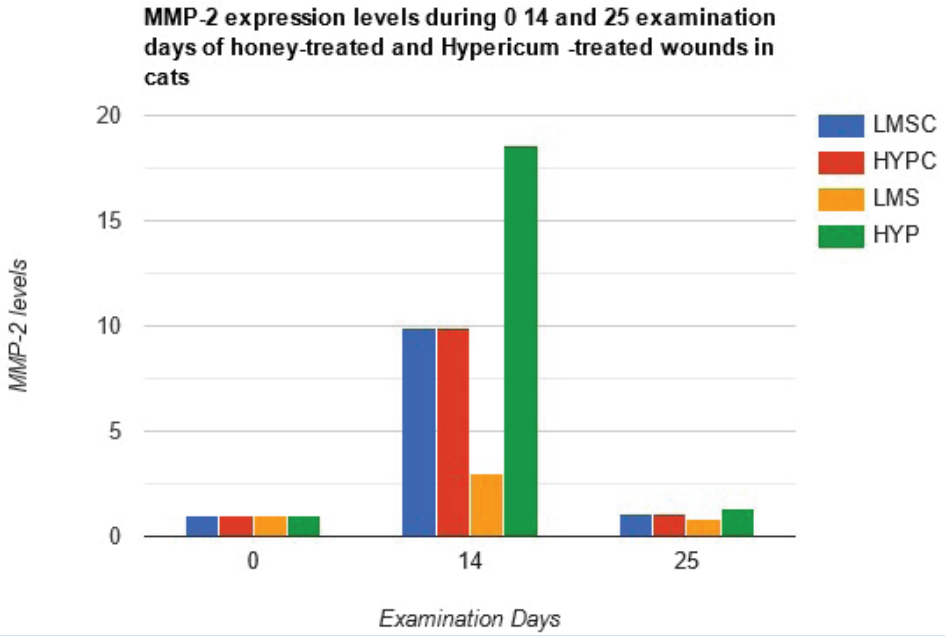

Figure 1. MMP-2 expression levels during examination days 0 14 and 25 of LMS and HYP–treated cutaneous wounds in cats.

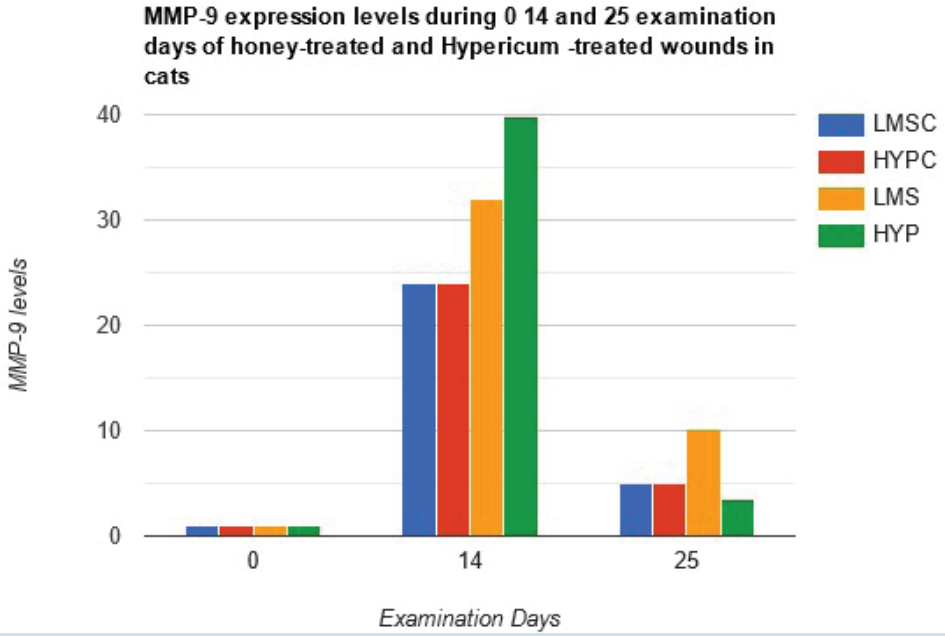

Figure 2. MMP-9 expression levels during examination days 0 14 and 25 of LMS and HYP–treated cutaneous wounds in cats.

for MMP-9 expression were noted in all treated and control wounds (P= 0.580).There were no statistically significant differences in terms of time-wound interaction for MMP-9 expression (P = 0.496).On day 14 the mean MMP-9 expression in the LMS-treated wounds was 31.950 ± 37.2330 times higher than these on day 0 and the mean MMP-9 expression in the HYP-treated wounds was 39.888 ± 27.2045 times higher than these on day 0.On day 14 mean MMP-9 expression of LMSC or HYPC wounds was 23.950± 33.0045 times higher than those on day 0 LMSC or HYPC wounds. On day 25 the mean MMP-9 expression in the LMS-treated wounds was 10.100 ± 17.6394 times higher than these on day 0 and the mean MMP-9 expression in the HYP-treated wounds was 3.463 ± 5.1141 times higher than these on day 0. On day 25 the mean MMP-2 expression of LMSC or HYPC wounds was 5.075± 10.1238 times higher than those on day 0 LMSC or HYPC wounds (Fig. 2).

No significant differences between sampling days for TIMP-1 expression were noted in all treated and control wounds (P= 0.407). There were no statistically significant differences in terms of time-wound in-

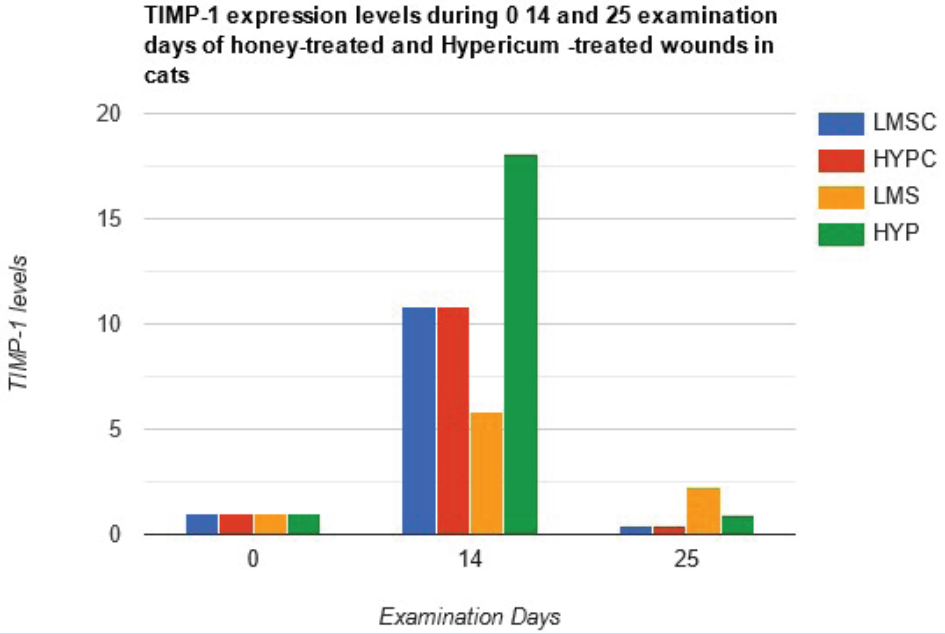

Figure 3. TIMP-1 expression levels during examination days 0 14 and 25 of LMS and HYP–treated cutaneous wounds in cats.

teraction for TIMP-1 expression (P = 0.352). On day 14 the mean TIMP-1 expression in the LMS-treated wounds was 5.8± 5.79 times higher than these on day 0 and the mean TIMP-1 expression in the HYP-treated wounds was 18.113± 22.6478 times higher than these on day 0. On day 14 the mean TIMP-1 expression of LMSC or HYPC wounds was 10.850± 19.5542 times higher than those on day 0 LMSC or HYPC wounds. On day 25 the mean TIMP-1 expression in the LMS-treated wounds was 2.288± 4.1834 times higher than these on day 0 and the mean TIMP-1 expression in the HYP-treated wounds was 0.8875 ± 1.1307 times less than these on day 0.On day 25 the mean TIMP-1 expression of LMSC or HYPC wounds was 0.400± 0.28 times less than those on day 0 LMSC or HYPC wounds.MMP-2, MMP-9, and TIMP-1 expression levels were decreased reaching baseline as the healing process reached day 25 (Fig.3).

DISCUSSION

In the study presented here MMP-2, MMP-9 and TIMP-1 expressions failed to show any differences during measurement times (days 0, 14, and 25) in both treatment and control wounds. Thus, our hypothesis was rejected. This is the first time in the literature investigating the effect of medical-grade honey and Hypericum ointments applied daily in cutaneous wounds on the activity of MMP-2, MMP-9, and TIMP-1 in cats.

The role of MMPs during all phases of cutaneous wound healing is considered to be critical (Xue et al., 2006; Gill and Parks, 2008; Sabino and Keller, 2015; Kandhwal et al, 2022). MMP-2 and MMP-9 expression is reported to be in low levels in normal skin but increases significantly during the acute wound healing process (Sabino and Keller, 2015). During the inflammatory phase of healing MMPs regulate the inflammatory process by possibly mediating chemokine activity and promoting extravasation and epithelial migration of leucocytes to the wound (Kandhwal et al, 2022). Migrating keratinocytes secrete MMP-2 and MMP-9 that mediate keratinocyte motility favoring epithelialization (Xue et al., 2006; Gill and Parks, 2008; Sabino and Keller, 2015). During the remodeling phase of healing, MMPs secreted by fibroblasts promote irregular scar degradation and aid in scar tissue contraction (Kandhwal et al, 2022). However, it is believed that MMP-2 and MMP-9 may also inhibit cell proliferation (Gill and Parks, 2008). A recent feline study investigated the role of MMP-2, MMP-9, and TIMP-1 on cutaneous wound healing following platelet-rich plasma application; significant differences in MMP-2 and TIMP-1 expressions in both treated and untreated control wounds on days 14 and 25 compared to day 0 were reported (Angelou et al., 2022). In this study, MMP-2, expressions were significantly higher on day 14 and significantly decreased on day 25 in the treated and untreated control wounds compared to day 0 of healing. In contrast, no significant differences in MMP-9 expressions were recorded on days 14 and 25 of healing in the treated and untreated control wounds compared to day 0 of healing (Angelou et al., 2022). TIMP-1 expressions showed significantly higher levels on day 14 of healing which significantly decreased on day 25 in the treated and untreated control wounds compared to day 0 of healing (Angelou et al., 2022). In this study, all wounds in both platelet-rich plasma-treated wounds and their untreated controls healed over 25% on examination day 25.

Medical-grade honey is thought to improve the wound healing process because of hydrogen peroxide production following contact with wound exudate, flavonoids, and sugars that result in hyperosmotic along with antibacterial effects (Molan, 1999). This high osmotic effect and activation of proteases due to hydrogen peroxide production may be the reason for the quick debridement activity of honey (Pleeging et al., 2020). It has been reported that medical-grade honey acting as an immunomodulator exhibits both pro-inflammatory and anti-inflammatory effects(Majtan,2014).In low levels of inflammatory/stimulatory mediators, honey induces the production of inflammatory cytokines and MMP-9 from keratinocytes; however, the maximal level of MMP-9 is observed at the edges of the wound 24 hours after an injury and decreases significantly by 48 hours (Tarlton et al., 1997). In infected wounds or when inflammation is in progress, honey inhibits the production of inflammatory cytokines and MMP-9 (Majtan, 2014). Medical-grade honey and Hypericum seem to improve cutaneous second-intention healing in cats (Lukanc et al., 2018; Lukanc et al., 2020; Marino et al., 2021). In contrast, medical-grade honey showed minimal effects on cutaneous wound healing in dogs (Repellin et al., 2021). TIMPS reported that their concentration remains at high levels in chronic wounds and in wounds of diabetic patients (Krejner et al., 2016).

In contrast to Angelou et al (2022) study, we found that during similar examination days there were no significant alterations in MMPs and TIMP-1 expres-

sions in both LMS and HYP and their respective untreated controls. In our study, MMPs and TIMP-1 expressions were increased on day 14 compared to their controls and decreased thereafter on day 25 but no significant differences were detected. In our study, both MMPs and TIMP-1 expression were decreased reaching baseline as the healing process reached day 25 when all wounds were almost healed as reported in Angelou et al. (2022) study. Even the MMP-2 expression of HYP wounds doubled on day 14 compared to untreated control failed to show any significant difference. The reason for the difference between our findings and those of others (Majtan, 2014; Angelou et al. 2022) in relation to MMP-2 and TIMP-1 expressions may be not easily determined. These differences may be related to the different animals used, the different methodologies applied, the different measurement times utilized, or the small sample size of our study. The large standard deviation observed in the MMPs and TIMP-1 expression values in the present study may be due to the fact of outlier presence in our data or to the small number of the cats included in the study.

The limitations of the present study include the small number of cats examined and the use of LMS or HYP in surgically created cutaneous wounds in normal cats. The results might be different if the study was performed in a clinical setting, in infected wounds.

In conclusion, MMP-1, MMP-9, and TIMP-1 expressions were not affected by medical-grade honey or Hypericum ointments applied locally in cutaneous wounds in cats. Further investigations are needed to verify the MMPs involvement and function in cutaneous wound healing progression in cats.

CONFLICT OF INTEREST

None declared.

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