Modulation of immune response in canine chronic wound stimulated with Class C CpG oligonucleotide

Olla A. Khalifa 1, A.M.Alakraa 2 and A.H.Elkasapy 2

1 Genetics and Genetic engineering. of Animal Wealth Development Dept., Faculty of veterinary medicine, Benha Univ., Benha, Egypt,
2 Dept. of Surgery, Anesthesiology and Radiology, Faculty of veterinary medicine, Benha Univ., Benha, Egypt
E-mail: olla.khalifa@fvtm.bu.edu.eg

Abstract
Chronic wound healing is a severe problem in veterinary practice, therefore, a new agent for wound healing is recommended. Synthetic oligodeoxynucleotides containing one or more CpG motifs (CpG-ODN) are used to stimulate the immune system and improve skin wound healing. Ten clinically healthy animals were used to estimate and compare the rate of wound healing. The animals were arranged into two groups (five animals each). The estimation of wound healing was carried out by clinical observation of the signs of healing in 21 days, the observation was based on the presence of sepsis and unhealthy granulation tissue formation. Histopathological findings depend on the rate of new vascularization, amount of collagen bundles, epithelial thickness and cellular component. Molecular assessment based on the expression of IL10 & TGF-β. This study aimed to evaluate the role of Class C CpG oligonucleotide as new modality to overcome the delay of chronic wound healing in canine.

Keywords: Chronic wound, CpG-ODN, Canine, Immune response.

1. Introduction
Wounds sustained in one or more of phases of wound healing (mainly in inflammatory stage) and not healed as other wounds do are called chronic wound[1]; balance between production and degradation of collagen missed in chronic wounds while its persistent in acute wound [2]. Acute and chronic wounds unlike each other being healed in a different manner [3]. Alterations of the keratinocyte activation cycle with persistent inflammation lead to a non-healing state, and wound ulcer chronicity with different complications like infections [4]. Some factors move wounds forward to chronicity such as age, repeated trauma, systemic illnesses like diabetes, immunosuppression, and diseases make ischemia [5]. Treatment of chronic wound aims to overcome main causes of it like bacterial load, ischemia and imbalance of proteases [6] using different methods including suitable antibiotic, debridement, irrigation, vacuum-assisted closure, warming, oxygenation, moist wound healing, removing mechanical stress, and addition of materials secrete or enhance levels of healing factors [7].

There were some of growth factors and cytokines promote and controlling wound healing [8]. Transforming growth factor-β (TGF-β) considered a potent chemo attractant for endothelial cells, fibroblasts and innate immune cells, such as neutrophils and monocytes [9]. Persistent inflammation state observed in chronic wounds, referred to high leukocyte counts present in TGF-β1 transgenic mice skin [10-12], on the other hand absence of inflammatory processes in TGF-β1 knockouts one [13, 14].

Interleukin-10 (IL-10) is homodimeric cytokine produced by different cell types as T cells, monocytes, and macrophages. It’s also produced from keratinocytes after injury [15]. IL-10 have different properties activating macrophage/ monocyte functions and decrease production of pro-inflammatory cytokine [16], regulating fibrogenic cytokines, like transforming growth factor-b (TGF-b) as it has important role in the tissue remodeling regulation [17].

ODN D-SL03 (double-stem loop ODN) is a synthetic oligonucleotide class C that contains unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs) involve double stem loops, a phosphorothioate backbone and two palindromes with AACGTT motif and TTCGAA motif in each loop. It have immunostimulatory effects activates B cells and NK cells and act as a TLR9 agonist in different vertebrate species (human, mice and dogs) [18].

In mice and humans unmethylated CpG motifs activates innate immune system via TLR9 [19], stimulates inflammatory response and production of growth factor like VEGF [20]. Some studies revealed relation between activation of the innate immune system and wound healing [21]. It was proven that treatment with CpG ODN enhance skin repair and wound healing in primates and mice [22].

Before surgical intervention systemic pretreatment with CpG-ODN improves dermal regeneration, especially in case of some chronic wound healing conditions, such as diabetes, immunosuppression, and infection. This proven by [23] who showed that mice treated with CpG Fp 6 days prior to skin wounding showed increased epithelialization, reduced numbers of leukocytes, increased numbers of macrophages, improving wound healing than control one, support late tissue-remodeling processes that lead to decrease of inflammation and solid wounds during skin regeneration.

Previous studies indicated that antibiotic treatment decrease bacteria and wound inflammation from the skin, on the other hand delays wound healing. This adverse effect is overcome by CpG-ODN treatment [24]. For this reason we establish our study to evaluate effect of local CpG-ODN on acceleration of wound healing in chronic wound in canine.
2. Materials and Methods

2.1. Experimental Animals

Ten mongrel dogs aged 3-4 years and weighting 13-15 Kg were used in this study, kept in separate kennel under standard environmental situations. The animals were given free water and feed was given twice daily. Under the influence of general anesthesia [25], in the right chest area of the vertebral column 3 cm diameter acute full-thickness skin wound were made.

2.2. Assessment of wound contraction rates:

Wounds were digital photographed for clinical assessment using ruler for wound healing rate calculation at days 0, 7, 14, and 21 after injury.

2.3. CpG-ODN Phosphorothioated (ODN D-SL03):

ODN D-SL03 is a type C CpG ODN and a TLR9 agonist in dogs, 5′ TCG CGA ACG TTC GCC GCG TTC GAA CGC GG 3′ (Trilink Biotechnologies, San Diego, CA, USA).

2.4. Study Groups

Two study groups were conducted; five dogs in each group. Group I (control group): the wounds were treated with normal saline only twice per week. Group II (CpG-ODN treatment) wounds were injected with 75 ug CpG-ODN/dog subcutaneously twice weekly [26]. In all groups wounds left to heal with the second intention for three weeks.

2.5. Analysis of gene expression by qPCR

Specimen from wound tissue were collected for total RNA extraction using Easy Red TM kit (Intron Biotechnology, Korea). 2X Reverse Transcriptase Master Mix (Applied Biosystem, USA) were used for the synthesis of complementary DNA (cDNA) from purified RNA. In Real-time PCR System (Applied Biosystem 7500 Fast, USA) with SYBR Green Master Mix (TOPreal TM qPCR 2X PreMIX), mRNA levels of genes were detected under the subsequent cycle conditions: 95°C for 10 min, 95°C for 15 sec for 40 cycles then 60°C for 1 min. The formula: 2^(-ΔΔCT) were used to calculate fold-change for each gene [27].

Primer sequence: IL-10, forward, 5′-ccgggctgagaaccaagc -3′ and reverse, 5′-aatgctgctcttcacctgccac -3′ [28]; transforming growth factor β (TGF-β), forward, 5′- caagatctggctggaattgta -3′ and reverse, 5′- cagagacctgctctcgtc -3′ [29]; RPS-5, forward, 5′- tctactggtgagaaccccct -3′ and reverse, 5′- cctcgtctcagctc -3′ [30] all manufactured by Invitrogen, Thermo Fisher Scientific, USA . The gene expression levels of IL-10 and TGF-β were normalized to RPS-5 as housekeeping gene in dogs.

2.6. Statistical analysis

Data were analyzed using independent sample t test. Results represented as the means ± SEM. A value of P < 0.05 was defined as statistically significant. All statistical tests were achieved using SPSS software [31].

2.7. Histopathological evaluation

Samples of skin biopsy at 21 days postoperative were exposed to routine histological procedures, first fixed in 10% formalin, then suspended in a paraffin solution, and cutted by a microtome transversely into 4 μm thin sections, then stained with hematoxylin-eosin and examined for histopathological changes. The histopathological scores were established by [32].

3. Result

3.1. Clinical findings:

The control group which composed of five animals subjected to sharp wound measured by 3 cm. The wound showed a good signs of healing during the first week and the wound measures 2.1±34 cm. The center of the wound lies under 3mm from the edges of the wound and showed healthy appearance without flakes and sepsis. The edges of the wound showed sufficient tendency to healing. The wound edges showed turning in and sings of sepsis appeared in the space between the edges and wounded area. Estimation of the wound after two weeks showed that the wounded area became 1.5±22 cm, the center of the wound elevated and covered by unhealthy granulation tissue formation and become over the level of the edges. Purulent discharges accumulated under the edges and within the unhealthy granulation tissue. After 3 weeks the wound area calculated 0.8±32 cm. the granulation tissue formation shed out, the abraded area appeared bled and return under the level of the wound edges. The scar tissue formation began from the edges toward the center and the purulent exudation subsided.

The five animals of the second group which received CpG-ODN one time weekly explain the ideal and good phenomena of the wound healing. The edges of the wound illustrate the ideal factors of the role of the edges in the wound healing (no sepsis, fissuring, swelling or turning in). The central of the wound not elevated over the level of the edges all over the period of the healing without unhealthy granulation tissue formation. The measures demonstrate the continuous and equal healing rate in accordance to the period as shown in Table (1).

3.2. Histopathological examination

The specimen from the animals after 21 days exhibited re-epithelization process extended from the edges to the wound surface and covered by scab tissue or keratine. The proliferative epidermal epithelium showed focal areas of incomplete re-epithelization, composed of granulation tissue admixed with changeable numbers of lymphocytes, a small number of macrophages surrounded by few layers of keratinocytes. Fig. (1)

The CPG-ODN group showed after 21 days complete re-epithelization with uniformly thickness and highest level of collagen deposition. The specimen presented highest number of new blood vessels before granulation tissue. The dermis was infiltrated by high aggregate lymphocytes and macrophages Fig. (2)

3.3. Expression of IL10 & TGF-β in canine treated with Cpg-ODN:

Wound healing process depend on inflammatory procedure. Cytokines production in wound skin were improved by Cpg-ODN. For identification of such inflammatory cytokines in skin wounds quantitative RT-PCR were used to measure mRNA expression of IL10 & TGF-β in dogs with second intention chronic wound followed by Cpg-ODN treatment to assess healing.

As shown in Fig. (3), the cytokines including IL-10 and TGF-β were considerably increased in the Cpg-ODN treated group as compared to control group. Both IL 10& TGF-β expression were up regulated in Cpg-ODN treated group with a substantial enlarge in expression of IL10 than TGF-β (P<0.05).

Fig. (1) Control group viewing a focal area of incomplete re-epithelialization, composed of granulation tissue ad-mixed with inflammatory cells and surrounded by more layers of keratinocytes

Fig. (2) Collagen production in the dermis of wound healing in dog on day 21 after surgery, fibroblasts perpendicular to new blood vessels and high collagen deposition in group two.

Fig. (3) Relative m.RNA expression for (a) IL 10 and (b) TGF-β were normalized against RPS-5 as a house keeping gene. A value of P < 0.05 was defined as statistically significant.
4. Discussion

According to our clinical findings the control group showing a delayed behavior of wound healing where the wounds cannot counter acts the environmental factors which postponed the wound healing [8].

CpG-ODN received group presented uniformly healing pattern over 21 days without sepsis and formation of unhealthy granulation tissue formation. This data get in accordance with [33] who found that CpG ODN increased collagen I production, encouraged cell proliferation in human skin fibroblasts cells and activate immune cells .

CpG-ODN admits many aspects for acceleration of the wound healing, enhancement of new vascularization and wound epithelization. This agree with [22] who recorded that the CpG-ODN accelerate rich new vascularization, dense collagen bundles at the wound junction, the inner edges were united and thicker epithelium.

According to molecular studies we found that CpG-ODN treatment accelerate healing of chronic wound in canine. Synthetic oligodeoxynucleotides (CpG-ODN) represent unmethylated CpG motif has been used to accelerate wound healing and raised innate immunity at the site of injury. It attracts macrophages to the wound site and stimulates them to secrete Vascular endothelial growth factor (VEGF) which plays a critical role in the healing process [34].

We applied CpG-ODN local at the site of wound. It was mentioned that local treatment with CpG-ODN enhances epithelialization of skin wound healing in mice through stimulation of TLR9, increased VEGF production, and promotes cell proliferation of wound healing in the early phases [22].

As shown in Figure (3), the cytokines including IL-10 and TGF-β were considerably increased in the CpG-ODN treated group as compared to control group. [35] revealed that, local but not systemic of CpG-ODN increased IL 6,10, 12 and IFN gamma. It activates immune response especially macrophage through TLR9/MylD88/NF-κB pathway. Also, CpG ODN accelerated wound healing and elevates cytoketes production in injured skin, as it regulates fibroblasts and immune response. Recommending using of CpG ODN as medicament for wound healing treatment [33].

Both IL 10& TGF-β expression were significantly up-regulated in CpG-ODN treated group with a substantial increase in expression of IL10 than TGF-β P<0.05 figure (3). IL-10 have anti-inflammatory, inhibitory, or self-regulating. It regulates damaging effect of inflammatory responses by inhibiting antigen presentation by dendritic cells and prevent macrophage stimulation and infiltration at wound site, decrease expression of proinflammatory cytokine [36]. IL-10 not only indirectly adjust fibrosis through its anti-inflammatory effect, moreover marked increase in expression of IL-10 than proinflammatory cytokines, such as TGF-b1 and -b2, seems to have important role in the regenerative response [37, 38].

TGF-β levels, have major role in chronicity of wounds [39]. It was evidenced that persistent inflammation in chronic ulcers related to level of TGF-β [9, 40]. It cause persistent infiltration of immune and aberrant cell like dermal fibroblast , exaggerate reactive hyperkeratosis and parakeratosis in the epidermis [41].

IL-10 overexpression seems to reduce scar formation in wounds in a dose-dependent manner, in consequence can't distinguish wounded from unwounded skin [42]. Another study considered IL-10 as an anti-scarring agent , have the ability to induce scarless healing, as it promotes regeneration [43]. Over expression of IL-10 make this effect through pleiotropic effects: attenuation of the inflammatory response [37], regulation of the extracellular matrix [44], optimization of fibroblast function and differentiation [45], and increase in endothelial progenitor cell (EPC) [46]. This brief exposure has led to considered CpG-ODN as a potential therapeutic agent reduce scar formation and modulate immune response [34].

5. Conclusion

From our study we concluded that use of CpG-ODN class C in treatment of chronic wound in canine accelerate wound healing. CpG-ODN have been safely administered locally to dogs, suggesting that immunostimulatory oligonucleotides may provide a novel, inexpensive, safe and potent therapy for wound repair with scar less formation.

6. Acknowledgments

This work was supported by research project and authors extended their appreciation to the scientific research fund (SRF) at Benha University, for their fund project no (M5/1/5).

Reference


Eriksson: Autologous cultured keratinocytes
Polis: Total intravenous
Wang: CpG
Klinman: Antibiotics Delay
Schmittgen: Analysis of relative gene
Vollmar: Development, character
Tredget: Out healing in mice.

- EMBO journal.
- Tijdschrift. vol.78, pp.160

BWaelbers, P.Vermoere, I.
- Tijdschrift. vol.78, pp.160

- Biochemical

Olla.A.Khalifa

- Biochemical

Modulation of immune response in canine chronic wound stimulated with Class C CpG oligonucleotide


