



Effects of amitraz plus *Parapoxvirus ovis* on EGF, VEGF, IGF-1 and IGF-2 in canine generalized demodicosis

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ABSTRACT

Objective. The purpose of the study is to investigate the effect of treatment with amitraz plus-*Parapoxvirus ovis* (IPPVO) on serum concentrations and skin expressions of insulin-like growth factor (IGF)-1 and -2, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), in dogs suffering from generalised demodicosis (GD). **Materials and Methods.** GD affected dogs were injected 1 ml IPPVO on days 0, 2 and 9 subcutaneously in addition to amitraz (0.025%) treatment twice weekly for 80 days. IGF-1, IGF-2, EGF and VEGF concentrations in blood serum were measured by canine-specific enzyme-linked immunosorbent assay kit. EGF, VEGF, IGF-1, and IGF-2 expressions in skin biopsy specimens were examined immunohistochemically. **Results.** After the treatment of the dogs with amitraz plus-IPPVO in GD, we demonstrated a significant reduction in both circulating concentrations and skin expressions of EGF, VEGF, IGF-1, and IGF-2, which have a role in preserving skin integrity and wound healing. **Conclusions.** Results of this study suggest that IGF-1, IGF-2 EGF, and VEGF have a crucial role in the progression of GD in dogs. It is believed that the findings from this study will contribute to the development of new strategies for the treatment of GD, which is an important health problem for dogs.

Keywords: *Demodex canis*; dog; generalized demodicosis; growth factor; skin (Source: MeSH).

RESUMEN

Objetivo. El propósito del estudio es investigar el efecto del tratamiento con amitraz más-*Parapoxvirus ovis* (IPPVO) sobre las concentraciones séricas y las expresiones cutáneas del factor de crecimiento insulínico (IGF) -1 y -2, factor de crecimiento epidérmico (EGF), vascular factor de crecimiento endotelial (VEGF), en perros que padecen demodicosis generalizada (GD). **Materiales**

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y métodos. A los perros afectados por GD se les inyectó 1 mL de *IPPVO* los días 0, 2 y 9 por vía subcutánea además del tratamiento con amitraz (0.025%) dos veces por semana durante 80 días. Las concentraciones de IGF-1, IGF-2, EGF y VEGF en suero sanguíneo se midieron mediante un kit de ensayo inmunoabsorbente ligado a enzimas específico para caninos. Las expresiones de EGF, VEGF, IGF-1 e IGF-2 en muestras de biopsia de piel se examinaron inmunohistoquímicamente.

Resultados. Después del tratamiento de los perros con amitraz más-*IPPVO* en GD, demostramos una reducción significativa tanto en las concentraciones circulantes como en las expresiones cutáneas de EGF, VEGF, IGF-1 e IGF-2, que tienen un papel en la preservación de la integridad de la piel y la herida. curación. **Conclusiones.** Los resultados de este estudio sugieren que IGF-1, IGF-2 EGF y VEGF tienen un papel crucial en la progresión de la GD en perros. Se cree que los hallazgos de este estudio contribuirán al desarrollo de nuevas estrategias para el tratamiento de la GD, que es un problema de salud importante para los perros.

Palabras clave: *Demodex canis*; perro; demodicosis generalizada; factor de crecimiento; piel (Fuente: MeSH).

INTRODUCTION

Demodicosis is an inflammatory skin disease caused by *Demodex* spp. agents. *Demodex canis* is one of the three mites causing demodicosis in dogs (1). The disease is characterized by alopecia, erythema, pustule, scaling and crusting in many areas of the skin and secondary deep or superficial pyoderma may occur. There are two clinical forms of demodicosis: young and adult, in both two forms, localized or generalized form can be seen. Localized demodicosis is characterized by disease-related lesions in 1 to 5 different regions of the body, mainly in the facial region and forelimb. Mostly in dogs with weak immune system, localized form progresses to generalize form (2). Generalized demodicosis causes immunologic reactions with the inflammation of hair follicles and sebaceous glands in dogs, furunculosis and nodular dermatitis can be observed, and the integrity of the skin is impaired (3).

The loss of the integrity of large parts of the skin in dogs suffering from GD leads to a clinical picture that can last until death (2,4). Treating the disease requires a very difficult process. While, having the toxicity potential within the application difficulties, a monoamine oxidase inhibitor and α 2-agonist molecule amitraz is still effectively used in the treatment of demodicosis in dogs plus macrocyclic lactones and metaflumizones (5,6). The use of *Parapoxvirus ovis* (*IPPVO*) to stimulate non-specific immune system in combination with amitraz in the treatment of GD in dogs has been found to provide a better success in treatment (7).

There are a number of scientific studies showing that changes in serum protein levels and skin expressions of growth factors have a role in the wound recovery with the deterioration of the skin integrity (8,9,10,11,12,13). Epidermal growth factor (EGF) accelerates wound recovery by stimulating fibroblast proliferation, accelerating the formation of granulation tissue, increasing epithelization and stimulating new vessel formation (14). EGF administration has a curative effect to diabetic foot wounds (15,16). Vascular endothelial growth factor (VEGF), a subtype of the platelet-derived growth factors family, is produced by keratinocytes (17), renal mesangial cells (18) and tumor cells (19). VEGF is involved in normal physiological functions such as bone formation, wound recovery and growth (20,21). It is suggested that VEGF plays a role in vascular formation, which has an important role in wound recovery (22). IGF-2 is associated with many adulthood pathological conditions such as skin diseases (23), wound healing process (24) and tumoral diseases (25). High IGF-2 expression in the skin recovery process is associated with differentiation and function of fibroblasts (8). Scientific studies investigating the expression of growth factors and protein levels in canine demodicosis with impaired skin integrity are quite limited (26,27,28). The objective of the current study, therefore, was to demonstrate effects of amitraz plus-*IPPVO* on serum protein levels and skin expressions of EGF, VEGF, IGF-1 and IGF-2 in dogs suffering from GD.

MATERIALS AND METHODS

Animal material. Ten client-owned dogs between 6 to 10 months of age, different breeds and either sex, which presented to the Veterinary Teaching Hospital, were enrolled in the study. Generalized demodicosis was diagnosed by clinical examination and was confirmed by parasitological examinations in deep scraping samples. Furthermore, histopathological examination of tissue biopsy specimens collected for the determination of growth factors by immunohistochemistry was also confirmed parasitological diagnosis.

Ethics. The owners of the dogs were informed about the treatment procedure and signed consent forms were provided. All experiments were performed after approval of Ondokuz Mayıs University Local Ethics Committee for animal experiments (2015/09).

Parasitological investigation. For parasitological examinations, on the day before treatment (day 0), deep scraping samples (~4 cm²) were taken from the upper part of the affected areas of legs from each dog. Before scraping to push the mites out from the depths of the hair follicles deep skin scraping was done by squeezing the skin. Subsequently, the indicated area was scraped with a scalpel until capillary bleeding occurred (29). The material was transferred to a microscope slide, mineral oil was dripped onto it, and it was covered with a glass coverslip and examined under a light microscope at 100X magnification to count the number of eggs, larvae, nymphs and adults *D. canis* forms (2). On the 80th day, the same areas were scraped again, and parasitological examinations were performed.

Treatment protocol and sampling. Venous blood of 5 ml was taken from the cephalic vein into a vacutainer without anticoagulant for biochemical analysis. The centrifugation of blood samples at 1550 g for 10 min at 4°C was performed to separate serum. Sera were stored at -80°C for the analyses. Six-millimeter skin punch biopsy specimens were used for sampling (30). After clipping and cleaning, 2 milliliters of 2% injectable lidocaine was subcutaneously applied to the targeted area. Only one lesioned site was selected for sampling of the dogs with GD before the treatment and afterwards, biopsy samples from the skin was fixed in 10% formalin for routine histological and immunohistochemical

examination. Biopsy sites were recorded in each animal. After the whole recovery (80 days after treatment) different sites were chosen for control sampling. The previous protocol was performed for collecting the control samples. Dogs diagnosed as GD were treated according to a standard protocol reported by Pekmezci et al (7).

Histopathological examinations. After fixation with 10% formalin and paraffin embedding, 5-6 µm thick sections were cut. Sections of the tissues were examined using microscope (Nikon Eclipse E600, Nikon Instruments Inc., Tokyo, Japan) after staining with Hematoxylin and Eosin. Additional sections were prepared for the following immunohistochemical examinations.

Immunohistochemical examinations. Streptavidin-biotin peroxidase complex kit was used to determine the immunopositivity (SABC; Zymed Laboratories, Inc.; San Francisco, CA, USA). Paraffin sections were plated on 3-aminopropyltriethoxysilane (APES) coated slides. Sections deparaffinized twice in xylene were passed through absolute, 96% and 70% ethanol series for rehydration. All steps were carried out in a humid environment and at room temperature. Phosphate buffer (pH 7.4) was used in the washings. Sections were kept in methanol with 3% H₂O₂ for 10 min. for inhibition of endogenous peroxidase activity and then were heated in a microwave oven at 600 watts with citrate buffered (pH 6) antigen retrieval solution to reveal the antigenic structure which bounded by formaldehyde solution for 10 min. and cooled. Sections were exposed to 5% goat serum for 10 min. and with primary antibodies overnight at 4 °C as follows to prevent non-specific antigenic binding as follows: Rabbit polyclonal EGF antibody (1:100; MBS2003642, My BioSource, Inc. San Diego, CA, USA), rabbit polyclonal VEGF antibody (1:50; orb191500, Biorbyt, Cambridge, UK), rabbit polyclonal IGF-1 antibody (1:500; orb312277, Biorbyt, Cambridge, UK), rabbit polyclonal IGF-2 antibody (1:500; orb10887, Biorbyt, Biorbyt, Cambridge, UK). Reactivity of all the antibodies used against canine species was verified by the companies that antibodies were purchased. Sections were incubated with secondary antibody for 20 min. and incubated with HRP for 20 min. The AEC chromogen was applied for 5 min. (by controlling the reaction of the chromogen under a microscope). Staining procedure was done using Mayer's hematoxylin solution, then washed by

distilled water and mounted with water-based mounting medium. Primary antibodies were omitted from negative control sections, which were incubated with normal serum. Tissues from canine testis, kidney, pancreas, and placenta were served as positive control for the EGF, VEGF, IGF-1, and IGF-2 primary antibodies, immunopositively. Immunopositive area were evaluated semiquantitatively with microscope (Nikon Eclipse E600, Nikon Instruments Inc., Tokyo, Japan).

ELISA analyses. EGF, VEGF, IGF-1, and IGF-2 levels in serum samples were measured by ELISA method using canine-specific commercial ELISA test kits (MyBioSource, Inc. San Diego, CA, USA) as follows: EGF (MBS2605242), VEGF (MBS737581), IGF-1 (MBS041565) and IGF-2 (MBS740813). The tests were carried out according to the manufacturer's procedure. According to the kit procedure, standard diluent for EGF, sample diluent for IGF-1 and phosphate-buffered saline for IGF-2 and VEGF were used as negative controls. Analyzes were done simultaneously in duplicate. The absorbance of color in microplate was recorded using microplate reader (Infinite F50, Tecan Austria GmbH, Grödig, Austria) and serum levels were calculated according to standard concentrations.

Statistical evaluation. Data were analyzed using the statistical package program (SPSS Statistics V21.0, IBM Corporation, Armonk, NY). The results were given as mean±standard deviation. Normality was tested using Shapiro-Wilk normality test. Homogeneity of variances was evaluated using the Levene's test. Group differences for EGF, VEGF, IGF-1 and IGF-2 were determined using Independent samples two-tailed t-tests. A minimum $p < 0.05$ was considered significant for statistical findings.

RESULTS

After completion of treatment, dogs recovered completely without any side effects. Generalized demodicosis lesions were completely recovered after treatment protocol and no mites seen in parasitological investigations of deep scraping samples and in histopathological examinations of biopsy specimens collected for the determination of growth factors by immunohistochemistry.

Serum EGF, VEGF, IGF-1, and IGF-2 concentrations. The serum EGF, VEGF, IGF-1, and IGF-2 concentrations before and after treatment, in dogs with GD are presented in table 1.

Table 1. Serum EGF, VEGF, IGF-1 and IGF-2 concentrations before and after treatment in dogs with generalized demodicosis.

	Before treatment	After treatment
EGF (pg/ml)	136.8±15.2	44.3±7.0*
VEGF (pg/ml)	164.9±29.5	17.1±3.5***
IGF-1 (ng/ml)	206.4±41.1	71.6±7.4**
IGF-2 (ng/ml)	79.7±13.3	20.4±5.2**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Independent samples t-test.

The serum EGF concentration was 136.8±15.2 pg/ml before treatment and 44.3±7.0 pg/ml after treatment. The difference was statistically significant ($p < 0.05$). After treatment, serum EGF was reduced 3.09 fold. Serum VEGF concentration after treatment were significantly lower than before treatment (17.1±3.5 pg/ml vs. 164.9±29.5 pg/ml) ($p < 0.001$). Treatment of dogs suffering from GD caused a significant reduction in serum VEGF concentrations down to 9.64 fold. Serum IGF-1 was significantly decreased in dogs after treatment (71.6±7.4 ng/ml) compared to before treatment (206.4±41.1 ng/ml; $p < 0.01$). Similarly, serum IGF-2 concentration was decreased after the treatment (79.7±13.3 ng/ml vs. 20.4±5.2 ng/ml; $p < 0.01$).

Histopathological findings. The epidermis in dogs with GD was thickened due to proliferation of keratinocytes. Cystic dilatations were observed in hair follicles with multilayer squamous epithelium. *D. canis* mites and keratinous residues were found in most hair follicle lumens (Figure 1a). Lymphocytes, plasma cells, histiocytes, and giant cell infiltrations were observed around the follicular and sebaceous glands in the skin samples of dogs with GD (Figure 1b). After the treatment, it was determined that the thickness of the epidermis regressed to normal and the *D. canis* mites and inflammatory cell infiltrations were not found in the sections.

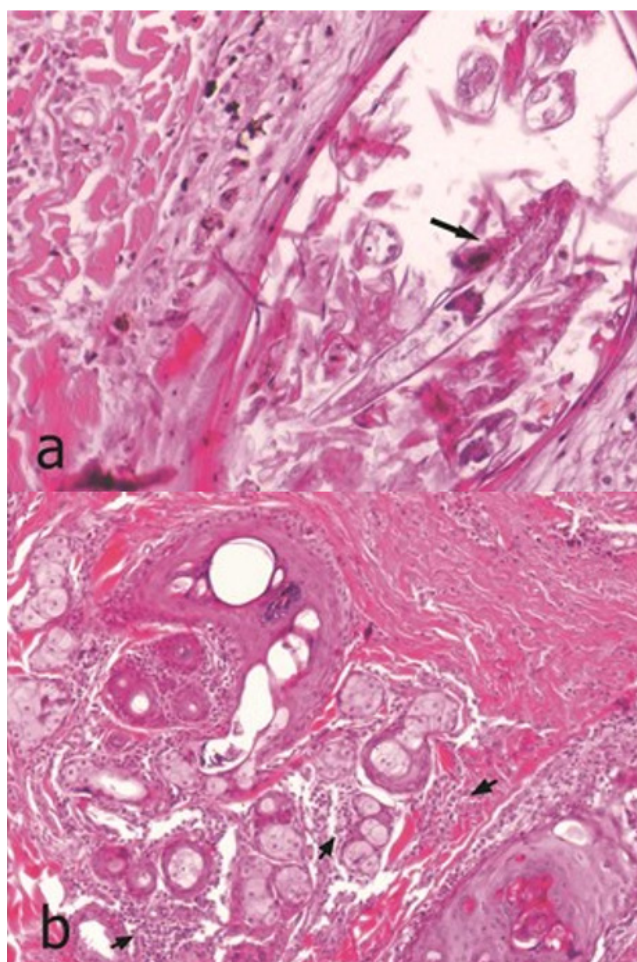


Figure 1. (a) Skin with generalized demodicosis showing follicular lumen containing mites (arrow) and keratinous debris (b) folliculitis infiltrated with plasma cells, lymphocytes and histiocytes (arrows). HE, a 280x; b 140x.

Immunohistochemical findings

Epidermal growth factor expression in the skin. EGF immunostaining was determined cytoplasmically in the keratinocytes of epidermis in skin sections taken before and after treatment. The cytoplasmic EGF expression was increased (22.7 ± 5.4) in the keratinocytes of epidermis before treatment (Figure 2a) but after the treatment, both the staining intensity and the number of stained keratinocytes was decreased (4.6 ± 0.6) ($p < 0.01$) (Figure 2b).

Vascular endothelial growth factor expression in the skin. VEGF immunopositivity was determined cytoplasmically in keratinocytes in the epidermis before and after treatment. In VEGF immunohistochemical stainings performed in pre-treatment sections of dog skin with GD, cytoplasmic VEGF expressions were high in

keratinocytes in the epidermis (Figure 3a) but after the treatment, both staining intensity and number of stained keratinocyte decreased (23.8 ± 4.3 vs. 3.2 ± 0.4 ; $p < 0.01$) (Figure. 3b). In addition, VEGF expressions were observed intensely in newly formed vascular endothelial cells and fibroblasts in dermis of the inflammatory area before the pretreated sections of dog skin with GD (Figure. 3c), while no expression was observed in vascular endothelial cells and fibroblasts in the dermis after the treatment (Figure. 3d).

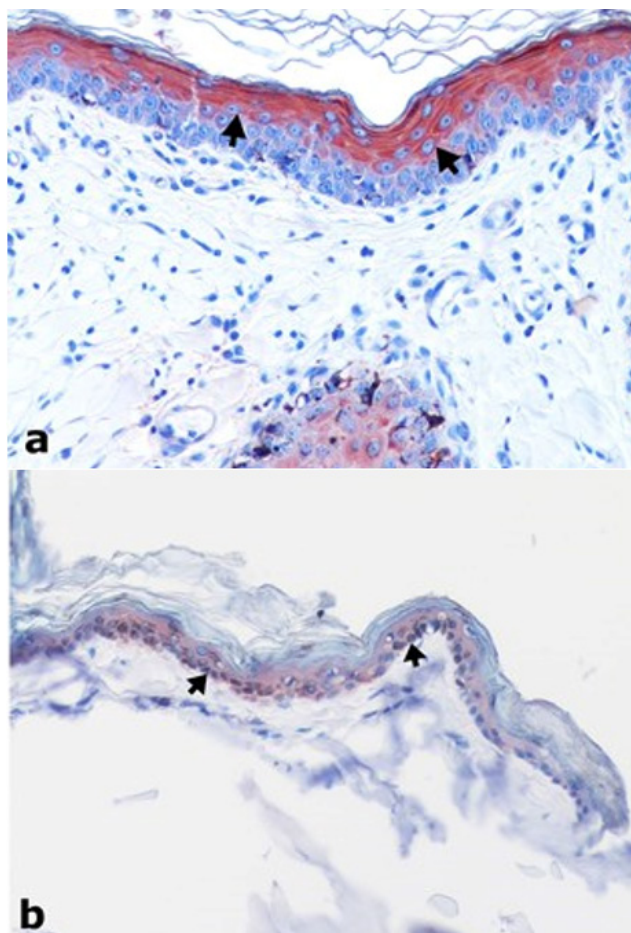


Figure 2. (a) Intense EGF immunostaining (arrows) in keratinocytes before treatment (b) weak EGF immunostaining (arrows) in keratinocytes after treatment. SABP immunostaining, AEC chromogen, Mayer's hematoxylin counterstaining, 560x.

Insulin growth factor-1 expression in the skin. Cytoplasmic IGF-1 immunopositivity in keratinocytes in the epidermis was observed both before and after treatment (Figure 4a). After treatment, IGF-1 expressions were significantly decreased in terms of both the intensity of staining and the amount of keratinocytes compared to the pretreatment levels (20.5 ± 3.8 vs. 5.7 ± 1.8 ; $p < 0.05$) (Figure 4b).

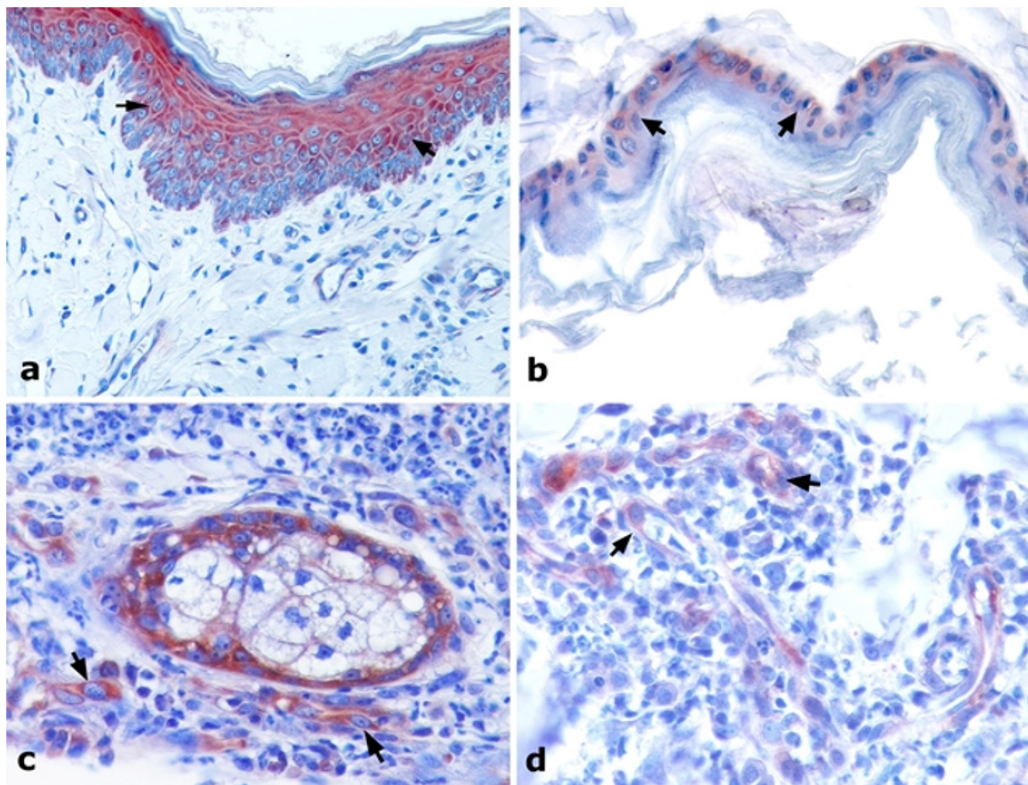


Figure 3. (a) Intense VEGF immunostaining (arrows) in keratinocytes before treatment (b) weak VEGF immunopositivity (arrows) in keratinocytes after treatment, (c) VEGF immunopositivity in epithelial cells around fibroblasts (arrows) in dermis and sebaceous glands (d) VEGF immunopositivity (arrows) in vascular endothelial cells in the dermis. SABP immunostaining, AEC chromogen, Mayer's hematoxylin counterstaining, a and b 20x; c and d 560x.

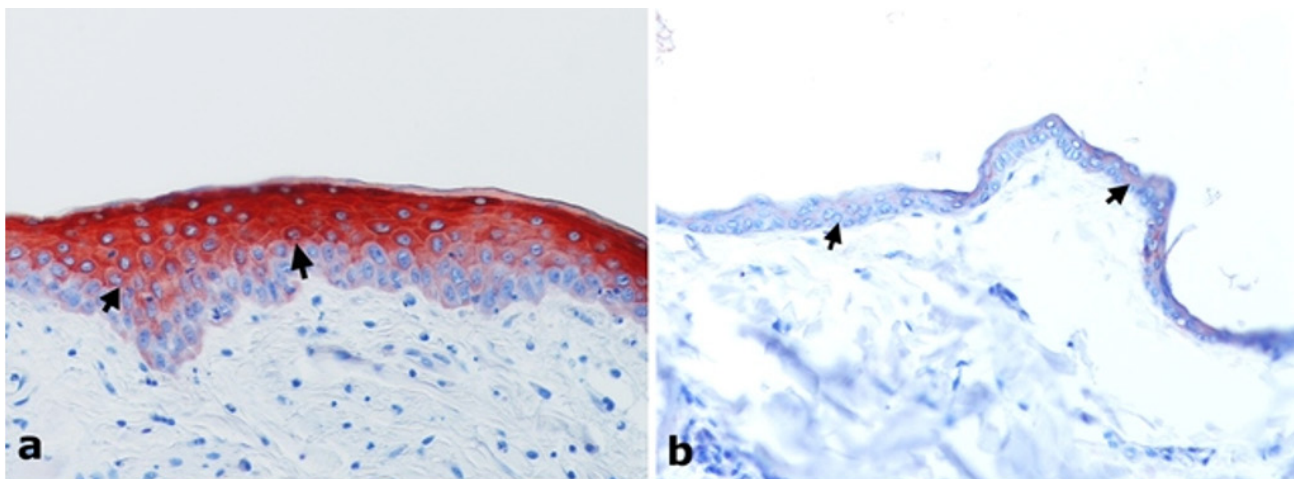


Figure 4. (a) Intense IGF-1 immunostaining (arrows) in keratinocytes in the epidermis before treatment, (b) weak IGF-1 immunostaining (arrows) in keratinocytes after treatment. SABP immunostaining, AEC chromogen, Mayer's hematoxylin counterstaining, a and b 280x.

Insulin growth factor-2 expression in the skin. Before and after treatment, cytoplasmic IGF-2 expressions in keratinocytes in the epidermis were observed. IGF-2 immunostaining intensity and amount in keratinocytes were increased before treatment (34.5 ± 4.0) (Figure 5a) and decreased after treatment (3.0 ± 0.4 ; $p < 0.001$)

(Figure 5b). In addition, before treatment, epithelial cells in the wall of the lumen of hair follicles and immunostaining of macrophages in the intersitial region were observed (Figure 5c) while in the skin sections after the treatment were not observed (Figure 5d).

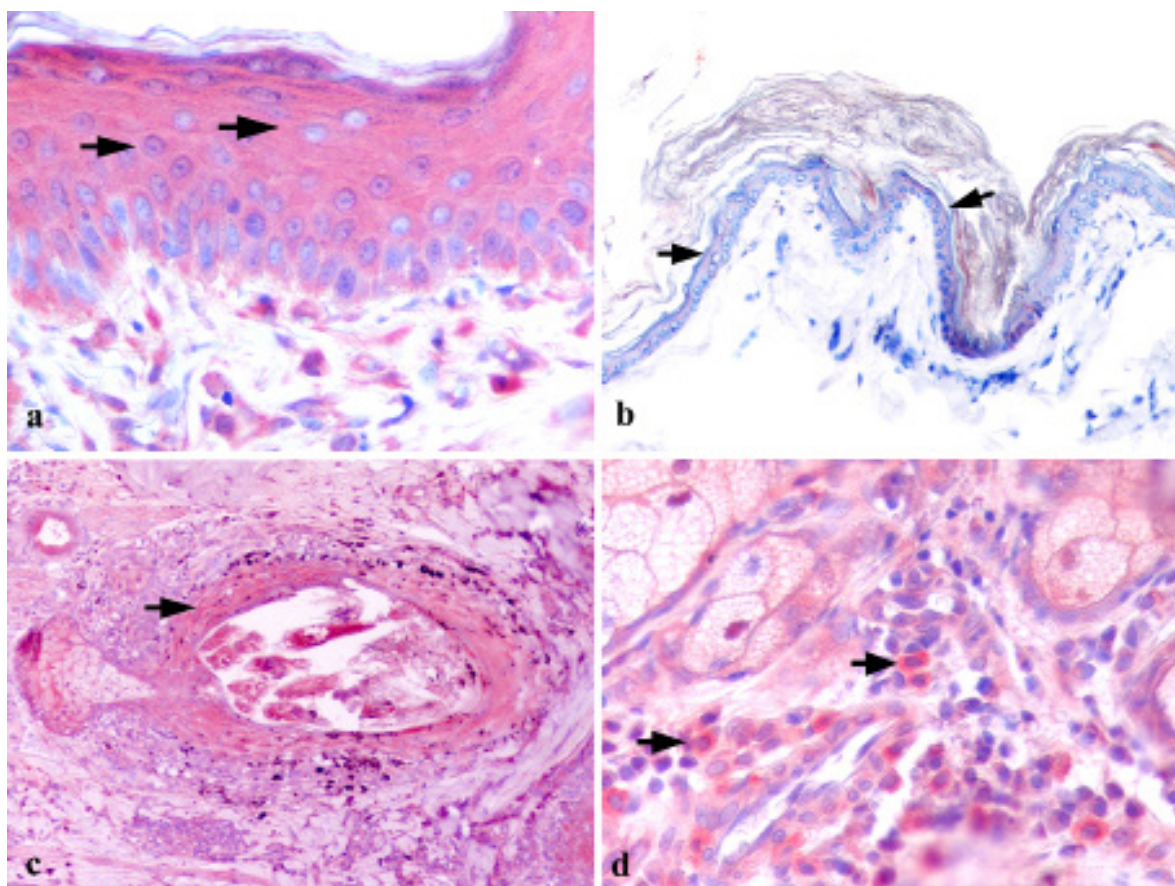


Figure 5. (a) Intense IGF-2 immunostaining (arrows) in keratinocytes before treatment, (b) weak IGF-2 immunostaining (arrows) in keratinocytes after treatment, (c) IGF-2 immunopositivity in hair follicle epithelium (arrow) and (d) IGF-2 immunopositivity in macrophages in the dermis (arrows). SABP immunostaining, AEC chromogen, Mayer's hematoxylin counterstaining, a, b and c 280x; d 560x.

DISCUSSION

Generalized demodicosis is one of the most common dermatological problems in dogs often associated with nodular dermatitis, perifolliculitis, folliculitis, and furunculosis (2). Local cutaneous immune response is stimulated in dermal lesions progressing from perifolliculitis to furunculosis in dogs with GD (3). The relationship between the immune responses in the skin and the different cellular and molecular mechanisms have crucial role in the repair of skin and healing of the disease. Although studies have been carried out to elucidate the molecular mechanisms in the healing process of skin wounds (10,12), complex mechanisms are still highly unknown. Despite an increasing evidence for an effects of growth factors in the skin repair process, the possible effect of treatment on change in circulating and/or skin growth factors in dogs with GD has not been fully investigated yet. The purpose of the current study was to demonstrate both the circulating and skin expression levels of IGF-1,

IGF-2, EGF, and VEGF in the dogs with GD that treated with amitraz plus *IPPVO*.

Our immunohistochemical findings in the dogs with GD showed that high levels of immunopositivity in the keratinocytes before the treatment were significantly decreased after the treatment. Moreover, in the present study, high serum EGF levels in the dogs with GD was significantly ($p < 0.05$) decreased after the treatment. Therefore, these findings indicate that EGF has a contribution effect of the epithelial regeneration process in dogs with GD.

VEGF plays role in physiological functions such as hematopoiesis, bone formation, growth and wound healing (20,21), it's relations about tumor progression was also reported (31). The VEGF shows its effect on wound healing via enhancement of neovascularization (11,22). The relationship between VEGF expression and angiogenesis in healing process was also reported in rat wound model (32). Our

immunohistochemical results in the present study showed that high level of immunopositivity of VEGF observed in the keratinocytes before the treatment showed a significant decrease following the treatment. Similarly, serum VEGF value, compared to VEGF level obtained in the pretreatment (164.9 ± 29.5 pg/ml), was also decreased by 9.64-fold after the treatment (17.1 ± 3.5 pg/ml) with a significant ($p < 0.001$) difference. Following the treatment, the serum VEGF level in dogs was close to the previously reported values of healthy dogs (11.14 pg/ml and 14.9 pg/ml) (33,34). Mukorera et al. (35) postulated that the circulating VEGF concentration of dogs with neoplastic spirocercosis was higher than the nonneoplastic and healthy ones.

An association of IGF-1 with the skin disorders and wound healing was proved (23,36). In our study, serum IGF-1 concentration, was decreased in the post-treatment compared to pretreatment ($p < 0.01$). This finding suggests that successful treatment with a combination of amitraz plus *IPPVO* has a decreasing effect on the circulating protein level of IGF-1 in dogs with GD. Furthermore, it has been suggested that IGF-1 has a mitogenic activity in the epidermal cell growth and regeneration (37). Recently, it has been reported that PMSCs expressing IGF-1 contribute to wound healing in mouse model of burn wound by promoting cell proliferation and epithelial differentiation, inhibiting inflammation and collagen deposition (38). Studies have shown that IGF-1 and IGF-2 expressions increase during the wound healing process at the time of fibroblasts which have dominance (8). Moreover, our immunohistochemical findings showed a significant decrease in the IGF-1 expression after the treatment which was increased before treatment in the skin of the dogs with GD. We previously reported that the increased tissue expression and serum IGF-2 concentration could be due to newly proliferating cells such as keratinocytes and fibroblasts during skin repair process in canine GD (28). The immunohistochemical analysis of the present study showed strong intracytoplasmic localization of the IGF-2 in the keratinocytes, histocytes, and fibroblasts of the

skin tissue in the dogs with GD. Furthermore, locally synthesized IGF-2 is known to contribute to postnatal vasculogenesis by proliferation of endothelial progenitor cells (39). As a result of this study, it was determined that IGF-2 levels were both decreased in serum concentration ($p < 0.01$) and the skin expression after treatment in dogs with GD. Indeed, increased pretreatment levels of serum and tissue expression levels of IGF-2 in dogs with GD may be explained by the proliferating new cells of keratinocytes and fibroblasts related to the skin damages.

Present study revealed that keratinocytes in the healthy epidermis express growth factors studied including EGF, VEGF, IGF-1 and IGF-2. However, their expressions were increased significantly in dogs suffering from GD and their expression levels were decreased following the treatment. Consequently, this study demonstrated that *IPPVO* and amitraz treatment in dogs suffering from GD resulted in a significant reduction in serum protein levels and skin expressions of EGF, VEGF, IGF-1 and IGF-2. The elevated serum protein levels and their simultaneous elevation in the epidermis of these growth factors could be a part of healing process of epidermis affected by GD. It could be postulated that, following the treatment, these factors were no longer needed for the healed epidermis and therefore they were regressed to their normal level. These changes were not considered to be specific to the active substance used for treatment or related to the disease itself. Thus, the study presented gives important data about the effects of growth factors on this challenging disease in dogs and requires further research.

Conflict of interests

The authors of this article declare that there is no conflict of interest.

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