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Evaluation of Ascorbic Acid in Combination of Ivermectin in Augmentation the Recovery from Juvenile Generalized Demodicosis in Dogs: A Randomized Clinical Trial

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Abstract
The clinical form of canine juvenile generalized demodicosis is usually related genetically with immune deficiency. In this regard, in the current study we have evaluated the impact of immunostimulant agent as ascorbic acid (AA) on the rapid recovery of dogs naturally affected by generalized demodicosis. To achieve this purpose, twenty- eight German Shepherd male dogs were used in this study and divided randomly into two groups, then received daily oral dose of ivermectin (IVR) 1% (0.5 mg kg^{-1}) alone and IVR 1% in combination with AA (500 mg per animal, twice daily) for two months, respectively. Total mite numbers, egg counts, eosinophil counts and skin lesion score were used for assessment the treatment efficacy after 30, 60, 90, and 120 days subsequent to the initial treatment. Seventeen dogs (IVR (n = 8), and IVR + AA (n = 9)) completed the full length of experiment (4- months) as 11 dogs were withdrawn due to various causes. Out of 17 dogs completing the 4 months trial, two dogs treated with IVR + AA combination therapy achieved the parasitological cure. The results revealed rapid and marked reduction in the total mite numbers and eosinophil counts treated with combination therapy in comparison with those administrated ivermectin alone. These findings exhibited the potential anti-*Demodex* effect of ivermectin when administrated in combination with immunostimulant agent and highlight the impact of an inexpensive, available and immunomodulatory agent as ascorbic acid in treatment of canine juvenile generalized demodicosis.
Keywords: *Demodex spp.*, Demodicosis, Ascorbic acid, Treatment, Dog.

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INTRODUCTION

Canine juvenile generalized demodicosis (CanJGD) is an inflammatory skin disease caused by infestation of the normal hair follicle by mite *Demodex canis*, and in some cases, *Demodex injai* (Desch and Hillier, 2003; Ordeix *et al.,* 2009). The disease characterized by high prevalence in purebred young dogs and is considered a hereditary skin disease (Ravera *et al.,* 2015). The mechanism of CanJGD inheritance and genetic defect(s) associated with it, is still unknown (Wilkie, 1979; Ravera *et al.,* 2015). However, the key role in its pathogenesis is relied on the body immune system (Hirsh *et al.,* 1975; Ravera *et al.,* 2015; Beugnet *et al.,* 2016).

Two clinical forms are identified for canine demodicosis; localized and generalized ones as well as juvenile and adult onset (Ravera *et al.,* 2015). Presence of patches of alopecia with mild erythema in young dogs and lesions number less than four with a diameter of up to 2.5 cm is characteristic to the localized form and this form is regressed spontaneously without treatment (Guaguère and Beugnet, 2008; Mueller *et al.,* 2009 & 2012). Whereas, the generalized form of demodicosis is usually more severe than the localized one and may be complicated with secondary bacterial infection leading to death (Mueller *et al.,* 2012). Generalized demodicosis may develop from the localized condition or occur spontaneously in young and sub-adult dogs, but also recorded in older animals especially those under severe stress or with underlying diseases (Guaguère and Beugnet, 2008). The most frequent form in the field is the generalized one, which is characterized by presence of five or more affected areas (erythema, follicular papules to pustules, hair loss, and scales) in the body, and/or pododemodicosis involving two or more paws (Beugnet *et al.,* 2016). CanJGD is a thwarting disease that requires aggressive medical

intervention with miticidal and supportive therapy (Paterson *et al.,* 2014). Although some cases self-curing particularly in young animals (Paterson *et al.,* 2009). In general, the course of CanJGD is unexpected as it is a potentially serious disease and many affected dogs are euthanized due to the severity of their disaster and their owner's frustration in managing the disease (Paterson *et al.,* 2009). The unpredictable course of the disease and the inheritance property of the disease with its main relation to the body immune status, makes the administration of immunostimulant agents to the diseased animals with studying their clinical response has a great priority in treatment of such clinical cases. In this concern, ascorbic acid (AA) is an essential water-soluble nutrient present in the extracellular fluid and the cytosolic compartment of the cell, which primarily exerts its effect on host defense mechanisms and immune homeostasis (Jacob and Burri, 1996). The potential immunostimulant effect of ascorbic acid causes enhancement of T-lymphocyte proliferation and inhibition of T- cell apoptosis signaling pathways in response to infection (Naidu, 2003). Moreover, previous study has reported the impact of ascorbic acid in collagen synthesis and modification (Dhama *et al.,* 2015). Furthermore, the preventative and therapeutic success of ascorbic acid in treatment of scurvy, viral infections, and common cold (Bowie and O'Neill, 2000); and the antioxidant effects of vitamin C on cancer (Mantovani *et al.,* 2003) and euglycemic- induced pateinets (Carroll and Schade, 2003) in various experimental studies were reported. In fact, ascorbic acid is produced normally in the animal body through L-gulconolactone oxidase enzyme (Fahey & Westmoreland, 2012). However, the efficacy of additional administration of ascorbic acid on the rapid recovery of dogs suffered from juvenile generalized demodicosis did not evaluated yet. Therefore, in this study we investigated the potential of ascorbic acid on the recovery of dogs naturally affected by generalized demodicosis with juvenile onset. To the best of author's knowledge, this is the first study explores the effect of such inexpensive and easily available immunostimulant agent on the recovery of immunodeficiency related disease; canine juvenile generalized demodicosis under natural field condition.

MATERIALS AND METHODS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animals and Clinical examination

Twenty- eight German Shepherd dogs were included in this study after diagnosed with CanJGD. Diseased dogs were selected for this study according to the inclusion criteria; a minimum of five affected areas (>10 cm2 each), a single-affected body region (>100 cm2) or two or more affected paws (Gortel, 2006). Detection of *D. canis* mites in scrapings and/or hair plucking samples collected from lesional skin was used for the diagnosis the infection. Presence of multi-focal areas of alopecia accompanied by fine scales on the face, pre-ocular area, commissures of the lips and the forelegs, erythema, follicular casts, scales and crusts indicate the presence of demodicosis (Radostits *et al.,* 2007). All dogs were belonged to private owners and all owners are signed owner permission for annexation their dogs in the study. The Dog's ages were ranged from 10 - 14 months old and their body weights were ranged from 18-26 kg. Ten blood sample were collected from apparently healthy German Shepherd dogs that were admitted either to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt or to private clinics for regular health-check, vaccination, regular de-worming or pre-neutering check-up. Sampling process was performed after obtaining written permission from the owners and get confirmation that these healthy dogs had no a previous history of sarcoptic mange or cutaneous hypersensitivity. The age of healthy dogs was ranged from 8-14 months.

Treatment protocol and efficacy assessment

The diseased animals under study were allocated into two groups each group contain 14- animals using a computer-generated randomization schedule. First group received daily treatment by (IVR) 1% orally (Merck AGVET, Pointe-Claire, Québec, Canada) at a dose of 0.5 mg kg^{-1}. The drug was administrated for continuous two months. While, combination therapy from ivermectin 1% (Merck AGVET) and ascorbic acid (iHerb.com) (IVR + AA) was administrated to the second group in the same inoculation period. Ascorbic acid was administered twice time per day orally at a dose of 500 mg per animal in the diet (Schwartz, 1996) for two months. A gradual increase in the dose rate of ivermectin was applied in order to recognize dog's sensitivity to ivermectin before reaching to the critical dosage that would induce life-threatening intoxication (Müller and Bettenay, 1999). Therefore, 8- successive doses of ivermectin were administrated to all dogs under study. First and second doses were 0.15 mg kg^{-1} and 0.25 mg/kg^{-1}, respectively. While, remained 6- doses were 0.5 mg kg^{-1}. During this regimen, initial symptoms of ivermectin-induced neurological toxicity, as tremor, ataxia, apparent blindness and profuse salivations were monitored. The efficacy of treatment for rapid recovery from canine demodicosis was assessed depending on the skin lesion score, skin scrapings with determination the total number of mites per scraping, egg accounts and hematological response before and after treatment by 30, 60, 90, and 120 days. The owners assigned an informed consent form and all the instructions regarding the treatment protocol were informed to them. Additional therapy other than IVR or AA was not permitted except in case of severe flea infestation or/ and significant secondary bacterial infection ectoparasiticidal therapy and antibiotics

were administrated, respectively as recommended by clinical decision and/or results of susceptibility testing.

Skin lesion score

Skin lesion score between 0 (normal) and 6 (extremely severe) for each case was determined according to the extent and range of the lesion (erythema, scales, crusts ⁄ comedones, papules ⁄ pustules and alopecia). The extent and severity of lesion was calculated firstly of each affected area, and then the sum of all affected areas was determined to produce the final score.

Total mite and egg counts

Skin scrapings were taken from five sites using blade with capillary oozing followed. Then the scraping from each animal was labeled with the animal ID, group and body region. Next, the total number of mites (adult, larvae ⁄ nymphs, egg) was examined under microscope after mixed with mineral oil on glass slide and counted separately in each scraping. The same-recorded five sites and/or sites of new lesions were scraped in each examination on which mites were counted. Two consecutive skin scrapings with 1- month interval with evidence of absence of any life stage, indicate parasitological cure

Hematological analysis

Blood samples were collected from each animal via cephalic vein puncture after hair clipping and disinfectant the puncture area with ethyl alcohol 70% to perform eosinophil counting using an electronic cell counter (MS9; Rhône Mérieux, France). The blood collection was performed from each dog at 5 occasions; before treatment as well as 30, 60, 90, and 120- days post treatment by specific drug as earlier descried.

Statistical analysis

A statistical software program (JMP for windows Version 5.1; SAS Institute, Cary, NC, USA) was used for data statistical analysis and for calculation the median and range of the assessed variable. For the evaluation of treatment results, the main effect of drug and time was determined using MANOVA repeated measures on treatment and time. Moreover, Wilks' Lambda test was selected to evaluate within group interactions and the evidence of time group interactions and to indicate the statistically significant difference between groups. While, one-way ANOVA with Tukey-Kramer HSD *post-hoc* multiple comparison tests were used to identify which group was statistically different from the rest. $P < 0.05$ was considered significant.

RESULTS

Dogs under study were exhibited severe clinical signs with high total mite counts. Seventeen dogs (IVR (n = 8), and IVR + AA (n = 9)) completed the full length of experiment (4- months) as 11- dogs were withdrawn due to various causes including relocation (n = 4), lost to follow up (n = 3), death due to unrelated causes (n = 2), and two dogs were showed hypersensitivity to IVR. Out of 17- dogs completed the 4- months trial, two dogs treated with IVR + AA combination therapy were achieved the parasitological cure.

Clinical response

The improvement in skin lesions was observed at 30-days post treatment in all treated groups. Three months later, complete recovery (Walk's Lambda for drug x time interaction, $P < 0.0001$) was exhibited in IVR- treated dogs and IVR combined with AA- treated group (Table 1). The results revealed no statistical significant difference ($P > 0.05$) in the skin lesion score between both groups through the full length of the experiment (Table 1). Additionally, the percent of reduction in the mean of skin lesion score didn't exhibited statistical significant difference ($P > 0.05$) between both groups at 30, 60, 90, and 120 days post-treatment (Figure 1).

Total mite counts

Deep skin scraping in the affected dogs revealed huge total mite counts in dogs under study were clarified upon enrolment with range 300–850 (Table 2). Non-significant differences ($p > 0.05$) were observed in the total number of mites between groups prior treatment administration (Table 2). Four months' post treatment, the mite counts were statistically significantly reduced (MANOVA, $P < 0.0001$; Walk's Lambda for drug x time interaction, $P < 0.0001$) in all treated dogs compared to those at outset (Table 2). Interestingly, the mite counts were statistically significantly reduced ($p < 0.05$) in dogs received treatment by combination therapy than dogs treated by ivermectin alone at one and two- months post treatment (Table 2). Moreover, statistical significant difference ($P < 0.05$) in the percent of reduction in the mean of total mite counts between both groups was observed at 30 days post-treatment (50 % for IVR vs 88% for IVR+AA). In contrary, no statistical significant difference ($P > 0.05$) was observed between both groups at 60 days (93 % for IVR vs 96 % for IVR+AA), 90 days (97.25 % for IVR vs 98.49 % for IVR+AA), and 120 days (99.21 % for IVR vs 99.65 % for IVR+AA), post-treatment (Figure 1).

Total egg counts

The mean egg counts in treated dogs were ranged from 10 to 49 at initiation (Table 3). Before drug administration, the egg counts weren't exhibited significant differences ($p > 0.05$) between treated dogs (Table 3). One month later, significant reduction ($p < 0.05$) the egg counts were observed in all treated groups (Walk's Lambda for drug x time interaction, $P < 0.0001$) (Table 3). The effect of IVR alone or combined with AA was similar on the reduction of egg counts (Table 3). Of note, the egg counts were statistically significantly reduced ($p < 0.05$) in dogs

received treatment by combination therapy than dogs treated by ivermectin alone at one- month post treatment (Table 3). Additionally, the percent of reduction in the mean egg counts exhibited a statistical significant difference ($P < 0.05$) between both treated groups was at 30 days' post-treatment (89 % for IVR vs 95.55 % for IVR+AA). In contrary, no such statistical significant difference ($P > 0.05$) wasn't observed between both groups at 60 days, 90 days, and 120 days, post-treatment (Figure 1).

Eosinophil counts

Dogs with juvenile generalized demodicosis exhibited significant elevation ($P < 0.05$) in the eosinophil counts in comparison with the control group (Table 4). From one month after IVR administration either alone or combined with AA, a significant reduction (MANOVA fit, $P < 0.0001$, Wilks, Lambda test for drug x time interaction, $P < 0.0001$) in eosinophil count was observed (Table 4). Interestingly, statistical significant difference ($P < 0.05$) in the percent of reduction in the mean of eosinophil counts between both groups was observed at 30 days (46.89 % for IVR vs 61.72% for IVR+AA), 60 days (49.54 % for IVR vs 69% for IVR+AA), and 90 days (58.65 % for IVR vs 71.87% for IVR+AA) post-treatment. In contrary, no statistical significant difference ($P > 0.05$) was observed between both groups at 120 days (67.44% for IVR vs 69.92 % for IVR+AA), post-treatment (Figure 1). Taken together, the earlier significant reduction ($P < 0.05$) in the mite counts that observed in dogs received ascorbic acid in combination with ivermectin, highlights the superiority of this cheap and available immunostimulant agent in treatment of dogs naturally infected with juvenile generalized demodicosis.

Table 1. Skin lesion score in dogs treated with ivermectin 1% alone (IVR) or ivermectin 1% combined with ascorbic acid (IVR + AA)

Treated groups	Time post-treatment (day)*				
	0	**30**	**60**	**90**	**120**
IVR (n=8)	60 (40-120)^{a}	40 (20-60)^{b}	10 (0-40)^{c}	0 (0-40)^{c}	0 (0-20)^{c}
IVR + AA (n=9)	70 (40-120)^{a}	40 (20-80)^{b}	20 (0-40)^{c}	0 (0-20)^{c}	0 (0-20)^{c}

*$P > 0.05$ no statistically significant differences between the skin lesion score in IVR- treated dogs and IVR+AA combination-treated dogs either before treatment or at days post treatment. Variables with different superscript letters in the same row are significantly different at $P < 0.05$. The obtained values represent the median and range (minimum and maximum) of dogs in each treated group. MANOVA fit, $P = 0.12$. Wilks, Lambda test for drug x time interaction, $P < 0.0001$

Table 2. Total mite counts in dogs treated with ivermectin 1% alone (IVR) or ivermectin 1% combined with ascorbic acid (IVR + AA)

Treated groups	Time post-treatment (day)*				
	0	**30**	**60**	**90**	**120**
IVR (n=8)	590 (300-800)^{a}	130 (75-170)^{b}	35.5 (23-48)^{bc}	11.5 (9-20)^{c}	4 (1-10)^{c}
IVR + AA (n=9)	625 (450-850)^{a}	71 (60-80)^{b}	15 (10-33)^{c}	10.5 (5-16)^{c}	2 (0-6)^{c}

*$P < 0.05$ statistically significant differences between the total mite counts in IVR- treated dogs and IVR+AA combination-treated dogs. Variables with different superscript letters in the same row are significantly different at $P < 0.05$. The obtained values represent the median and range (minimum and maximum) of dogs in each treated group. MANOVA fit, $P < 0.0001$. Wilks, Lambda test for drug x time interaction, $P < 0.0001$

Table 3. Egg counts in dogs treated with ivermectin 1% alone (IVR) or ivermectin 1% combined with ascorbic acid (IVR + AA)

Treated groups	Time post-treatment (day)*				
	0	**30**	**60**	**90**	**120**
IVR (n=8)	33 (18-49)^{a}	3 (1-8)^{b}	0.5 (0-3)^{b}	0 (0-2)^{b}	0 (0-0)^{b}
IVR + AA (n=9)	32.5 (10-45)^{a}	0 (0-4)^{b}	0 (0-1)^{b}	0 (0-1)^{b}	0 (0-0)^{b}

*$P < 0.05$ statistically significant differences between the egg counts in IVR- treated dogs and IVR+AA combination- treated dogs. Variables with different superscript letters in the same row are significantly different at $P < 0.05$. The obtained values represent the median and range (minimum and maximum) of dogs in each treated group. MANOVA fit, $P = 0.0115$. Wilks, Lambda test for drug x time interaction, $P < 0.0001$

Table 4. Eosinophil counts (%) in dogs treated with ivermectin 1% alone (IVR) or ivermectin 1% combined with ascorbic acid (IVR + AA)

Treated groups	Time post-treatment (day) *				
	0	30	60	90	120
IVR (n=8)	9.40 ± 1.89 [a]	5.21 ± 2.09 [b]	4.26 ± 1.54 [b]	3.95 ± 1.28 [b]	3.20 ± 1.31 [b]
IVR + AA (n=9)	10.40 ± 1.57 [a]	4.13 ± 1.59 [b]	3.40 ± 1.67 [b]	3.00 ± 0.94 [b]	3.21 ± 1.02 [b]
Healthy dogs (n=10)	4.40 ± 1.64 [a]	3.60 ± 1.57 [a]	3.52 ± 1.26 [a]	3.20 ± 1.03 [a]	4.11 ± 1.59 [a]

*$P < 0.05$ statistically significant differences in the eosinophil counts between the diseased dogs and healthy control dogs. Variables with different superscript letters in the same row are significantly different at $P < 0.05$. The obtained values represent the mean and standard deviation (SD) of dogs in each group. MANOVA fit, $P < 0.0001$. Wilks, Lambda test for drug x time interaction, $P < 0.0001$

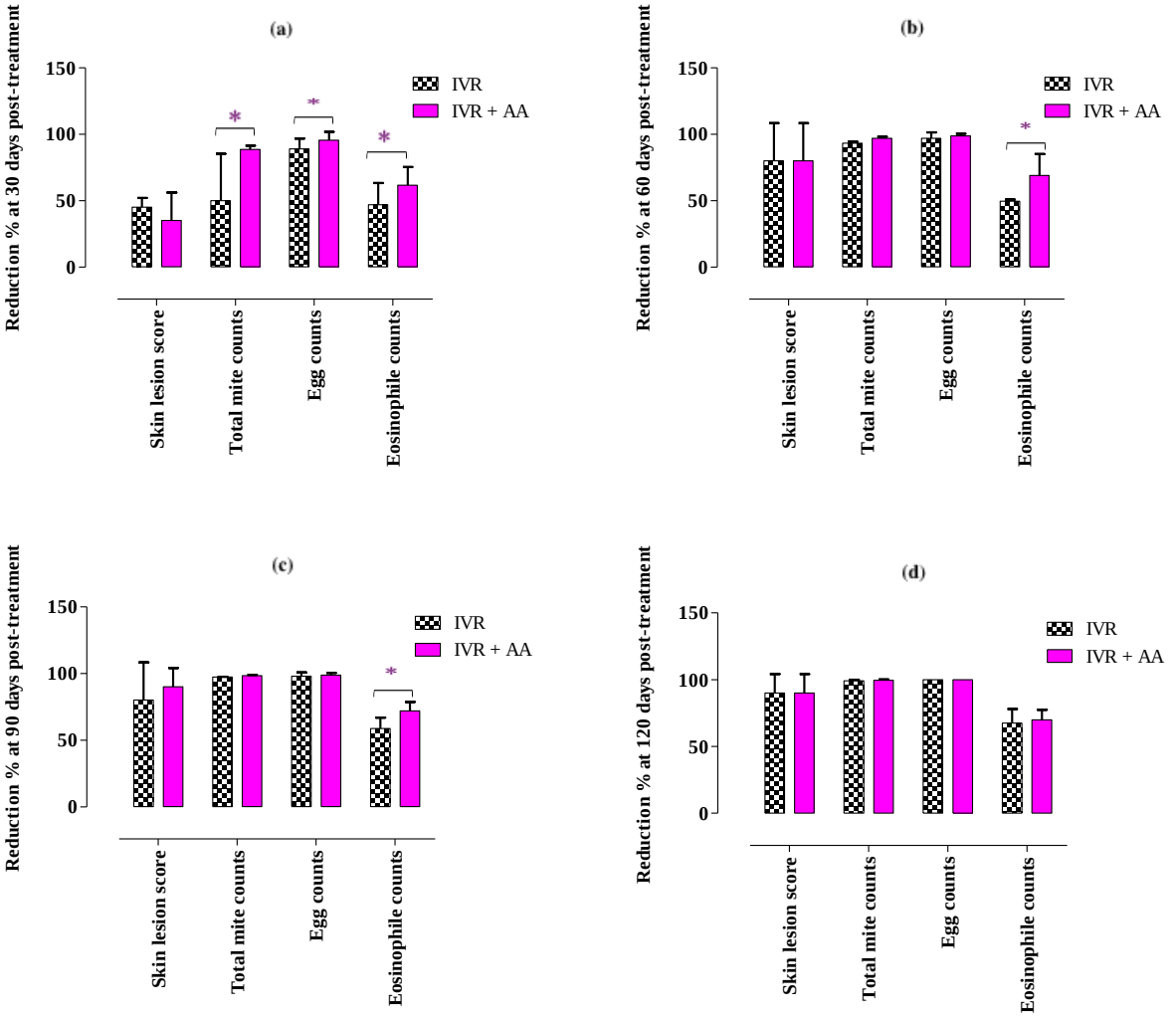

Fig. 1. Reduction percent (± standard deviation) in mean of skin lesion score (clinical response), total mite counts, egg counts and eosinophil counts after different periods of treatment by ivermectin alone (IVR) or ivermectin combined with ascorbic acid (IVR + AA). (a) 30- days post-treatment. (b) 60- days post-treatment. (c) 90- days post-treatment. (d) 120- days post-treatment. *Significant differences between IVR and IVR + AA ($P < 0.05$).

DISCUSSION

Juvenile generalized demodicosis is one of the most common form of demodicosis in the field, whereas, characterized clinically by presence of casts, crusts, scales, erythematous areas and hair losses in affected body region (Ravera et al., 2015). One of the few choices licensed for treatment of such disease condition is Amitraz (Medleau and Willemse, 1995; Hugnet *et al.,* 2001). Although, the potential toxicity resulted from its administration and its inconstant efficacy, there has been increasing attention in the use of the macrocyclic lactones. In this regard, the most widely used drug from macrocyclic lactone is ivermectin which is administrated at 400–600 µg/kg orally once per day till parasitological cure (Mueller, 2004; Mueller *et al.,* 2012).

The relation of CanJGD with the deficiency in the animal body's immune status and the hair losses resulted from the disease in the affected dogs, encourage us to evaluate the efficacy of immunostimulant agent that has a great role in collagen synthesis with hair-regrowth (Dhama *et al.,* 2015), subsequently as ascorbic acid in the treatment of such cases in the present study. Previous studies reported the significant role of micronutrients in immunomodulation and their impact on treatment of immunodeficiency diseases (Mahima *et al*., 2013, Rahal *et al*., 2014, Dhama *et al.,* 2015). Interestingly, vitamin C is one of the most imperative micronutrients that used as immunostimulant immunomodulatory agent (Dhama *et al.,* 2015). Vitamin C exerts its immunomodulatory effect through stimulation of innate immune cells, cell mediated immunity and humoral immunity (Dhama *et al.,* 2015). Taken together, the antioxidant activity of vitamin C and its role as cofactors in the cytokine production and regulation beside its significant role in immune homeostasis (Hartel *et al*., 2004; Kunisawa and Kiyono, 2013).

The obtained data in the current study revealed the improvement in clinical response with statistical significant reduction ($P > 0.05$) in the total mite, egg, and eosinophil counts in all treated dogs. While, the rapid and marked reduction in the total mite numbers and eosinophil counts were observed in dogs treated with ascorbic acid combined with ivermectin. In fact, usage of skin scraping as indicator to complete recovery from demodicosis is a matter of depate as the life cycle of the mite extends over a period of 18–24 days and the scraping is performed on a limited area of the lesion (Paterson *et al.,* 2009; Mueller *et al.,* 2012). Therefore, a single negative skin scarping is not actual indication to the complete recovery from mite infestation. Subsequently, the complete recovery is achieved based on two consecutive negative skin scrapings at a one-month interval (Fourie *et al.,* 2013). In the current study, two out of nine- dogs (22.22%) and one out of eight- dogs (12.50%) treated with IVR+AA and IVR alone, respectively were exhibited two successive negative skin scrapings at a one-month interval after 4- months treatment. Indeed, recurrence of canine generalized demodicosis is considered one of the main critical problems of this disease due to the non-complete removal of mite from the skin of affected dogs. Of note, the obtained data in this study denote the possible efficacy of ivermectin and ascorbic acid combination in overcoming the relapse of demodicosis in dogs. Interestingly, recent similar study (Beugnet *et al.,* 2016) reported the efficacy of anew-developed antiparasitic drug; afoxolaner in complete removal of mite from skin of treated dogs at day 84 post-treatment. Therefore, other studies are required to evaluate the possible synergistic effect of ascorbic acid and afoxolaner in complete removal of mite from animal skin.

Administration of ivermectin combined with ascorbic acid resulted in 88.62 %, 96.94 %, 98 %, and 99.65 % inhibitions in the total mite counts at 30, 60, 90, and 120 days post-treatment. These results are higher than the 12.5% inhibition for imidacloprid/ moxidectin on day 84 (Beugnet *et al.,* 2016), 45%, 64% and 89% inhibition in total live adult mite counts for Advocate administrated at once per month, 2-weeks, and weekly on day 120 (Paterson *et al.,* 2009), 49%, 93% and 95% inhibition in total juvenile mite counts for Advocate administrated at once per month, 2-weeks, and weekly on day 120 (Paterson et al., 2009). In the present study, administration of IVR alone causes 99% reduction in the total mite counts at 120 days, which was nearly similar to those observed in study of (Paterson et al., 2009), who reported 98% and 97% reduction in the total live adult and total juvenile mite counts after administration of IVR by 120 days. The study limitations should be mentioned. However, the potential anti-*Demodex* effect of IVR and AA was exhibited in the present study, further studies are required to explore the synergistic relation between both hits (IVR and AA). Moreover, other studies are warranted to evaluate the anti-parasitic efficacy of ascorbic acid in combination with other potential anti-*Demodex* agent. Furthermore, the sample size used in the current study was small and the treatment period was short, which may not allow obtaining a concrete conclusion. Therefore, future studies should perform on larger size samples with evaluation the skin scrapings every 30 days after the stopping the treatment.

CONCLUSION

In conclusion, administration of a cheap, immunostimulant and immunomodulatory agent as ascorbic acid in combination with commonly used anti-*Demodex* agent as ivermectin resulted in rapid and marked reduction in the total mite and eosinophil counts in treated dogs in comparison with administration of ivermectin alone. This high level of efficacy offers new perspectives to veterinarians for the control of demodicosis.

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CONFLICT OF INTEREST

The writers have announced that no contending interest exists.

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