

Evaluation of The Anti-Oxidative Activity and Trace Elements Concentrations in *Demodex Canis* Infected Dogs

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Abstract

The purpose of this prospective study was to evaluate the antioxidant status and plasma level of iron, zinc, copper, manganese and calcium levels in *Demodex Canis* infected dogs and the correlation between them, a total of 17 dogs clinically and laboratory diagnosed by skin scraping test and 6 healthy dogs as control were included in this study. Results showed a higher significant level of total antioxidant capacity (TAC) ($P < 0.01$), superoxide dismutase (SOD) ($P < 0.05$), malondialdehyde (MDA) ($P < 0.01$), and iron level ($P < 0.05$) in infected dogs than healthy ones, on the contrary; the level of zinc ($P < 0.05$), and copper ($P < 0.05$) were lower in infected than healthy dogs. For correlation analysis, there were a negative correlation between the activities of antioxidant enzymes; SOD with zinc and copper level ($P < 0.05$), also there was a negative correlation between MDA and iron level, the levels of calcium or manganese have on significant alteration between healthy control and diseased dogs, at the same time; sexes has no any significant effect on oxidative indices nor trace elements concentration in both healthy nor *Demodex Canis* infected dogs. This study proved that, *Demodex Canis* infected dogs has a high significant alteration in oxidative status and a significant imbalance in trace element as copper, zinc, and iron, and that can be considered as a cause or secondary consequences of oxidative stress-related pathogenesis in hair follicles and sebaceous glands in skin that associated with demodicosis, and antioxidant provision and zinc with copper is of high importance in treatment of demodicosis of dogs beside acaricide drugs.

Keywords: Oxidative status; Superoxide dismutase; Total antioxidant capacity; *Demodex Canis*; Trace elements; Dogs

Introduction

Demodex Canis (Leydig 1859) is the cause of local or generalized canine allergic dermatitis in dogs called demodicosis, because it spend the whole life cycle in the hair follicle, and sebaceous gland [1], and this resulted in oxidative stress that accompanied with increasing the production of free radicals that is incriminated in having a crucial role in production of a sever inflammation and allergy of skin that is appeared as erythema, non-pitting oedema, irritation with itching from immediate hypersensitivity reaction that followed by wrinkling, hyper keratinization of skin in affected area, and very bad smell of infected dog due to Oxidative stress in which the free radicals production exceed the detoxification by anti-oxidation process [2,3]. Total antioxidant capacity (TAC) is the analytic tests that applied to evaluate the antioxidant status of any biological samples and can determine the antioxidant response against the free radicals produced in a given disease, and organisms developed antioxidative mechanisms triggered by an increased the production of reactive oxygen species (ROS) to balance the oxidative damage that occurred [4].

In skin diseases, the body has several potent antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), GSH-peroxidase, and malondialdehyde (MDA), and the joined interactions between these antioxidants depend on the lysis of peroxides and free radicals [5] and for evaluation of the antioxidant status in dogs with skin diseases, it should be recommended that, it should be measure TAC. In addition, the combination of TAC assays with more specific analysis of individual antioxidants would provide a wider picture of the antioxidant status [6]. Iron, copper and zinc are a trace elements that have an important role as a cofactors in many enzymes to assume its proper function in the living organism as Cytoplasmic superoxide dismutase (SOD) enzyme that need zinc and copper and glutathione peroxidase enzyme contains selenium and catalase needs iron, and the levels of these elements are changed in skin diseases [7]. This prospective study was designed primarily to evaluate the antioxidant status, such as Total antioxidant capacity (TAC),

superoxide dismutase SOD enzyme, and malondialdehyde (MDA) levels in dogs with *Demodex Canis*, also trace element status such as iron, copper and zinc, and calcium, manganese, and correlation between them.

Material and Methods

Ethical consideration

This study followed the institutional ethical and animal care guidelines approved by supreme council of Egyptian universities, Egypt. All dogs belonged to private owners who signed an owner consent for the inclusion of their dogs in the study. All procedures were explained prior to sampling to dogs owners and veterinarians in their private clinics.

Sample collection and preparation

Over the period from November 2017 to December 2018 from local veterinary clinics of pet animals of different governorates in Egypt (Assiut, Sohag, Behera, Alexandria). A total of seventeen positive *Demodex Canis* infected dogs and six non infected well examined healthy control dogs of both sexes of which 10 female (7 infected, 3 healthy), and 13 males (10 infected, 3 healthy control), weight from 4 to 16kgs, and ages ranged from 1-2 years were considered in this prospective study. For mite examination, at first an overview of the skin of each dog took place a multi skin scraping at the margin of the lesion with a disposable scalpel blade and all that had clinical signs of *Demodex* infestation such as erythema, hair loss, seborrhea, follicular casts, scales, and crust in at least five spots or on an entire body region were considered positive, skin scraping was collected in petri dish of 10 cm and sent to laboratory for mites examination. 2 ml of blood samples were collected from each dog either from cephalic or recurrent tarsal vein and using heparin (10IU/ml of blood) as anticoagulant. Blood samples were centrifuged at 2000rpm for 5min in a refrigerated centrifuge to separate plasma. The plasma was collected in to a clean eppendorf tube. The packed RBC was re-suspended in PBS and was centrifuged for 5 min at 5000rpm and the supernatant was discarded. The process was repeated for three times. Finally, 1% diluted RBC hemolysate was prepared in distilled water for estimation of total anti-oxidant capacity (TAC), superoxide dismutase (SOD), and 33% diluted RBC hemolysate for malondialdehyde (MDA), spectrophotometrically [8], another blood sample from recurrent tarsal vein without anticoagulant in 5 ml plain sterile test tube that were centrifuged at 700×g for 15min., then collected and stored at -20 °C until use in detection the levels of copper, zinc, iron, calcium, and manganese concentration in plasma.

Parasitological examination

For mite's examination, skin scrapings were put in 5ml centrifuge tube after adding 3ml sodium hydroxide 10% to make lysis of hair and scrape, then samples were centrifuges at 1500rpm. For 10min. supernatant were excluded and one drop of sediment were put on glass slide and examined at 40x. for the presence of

mites, then identification of the morphological characteristics, measurements were made using a previously calibrated eyepiece micrometer, and they were clearly identified them as *Demodex Canis* type [9].

Determination of antioxidant status

Total antioxidant capacity (TAC), and superoxide dismutase (SOD) activity were estimated in erythrocyte by using commercial kits admitted from Bio diagnostic company for diagnostic reagent, Egypt. The level of SOD was evaluated in plasma samples with a detected exogenous amount of hydrogen peroxide (H_2O_2) as described by [8,10]. Erythrocytes malondialdehyde (MDA) level estimation was done according to [11,12].

Analysis of minerals concentration

For mineral analysis, 2ml of plasma sample was analyzed for trace elements; zinc (Zn), copper (Cu) and iron (Fe) using spectrophotometer (X-ma-Model 6100/6300/6100S (Double beam)-UV/VIS Spectrophotometer, Seoul, South Korea) as per the method described by [13] with little modification. Two-ml plasma sample was mixed with equal volume of concentrated nitric acid and kept at low heat on hotplate (below 90 °C) for digestion till volume reaches 1.5ml. then 2.0ml of hydrogen peroxide was added to this volume and the sample was again digested till volume reaches 1.5ml. The final volume of 10ml was made by adding distilled water. The concentration of zinc, iron and copper calcium, manganese in the digested samples was then determined by plotting their absorbances against the absorbances of a standard curve of each appropriate element constituted in a similar matrix to that of the dog digested samples [14].

Statistical Analysis

The statistical analysis was performed using IBM-SPSS Statistics software (Windows Release 10.0). Results were expressed as means \pm standard error and $p < 0.05$ was taken as the level of significance. Comparison and significant correlation between different groups was performed using the Paired Samples T test using the P value ≤ 0.05 is considered statistically significant at 95% [15].

Results

Clinical finding and micrometry

The infected dogs that admitted in veterinary clinics was clinically suffered from generalized form of demodicosis that involve larger area of the body as abdomen, legs, face with alopecia, redness, inflammation and crusting of skin, and rubbing their face and head, also the paws were swollen as seen in (Figures 1-3), the skin scraping test from affected lesions and morphometry was done on 17 samples, as follow; gnathosoma ($18.42 \pm 0.10 \mu\text{M}$), podosoma ($64.48 \pm 0.33 \mu\text{M}$), opisthosoma ($128.72 \pm 2.07 \mu\text{M}$), total body length (μM), width ($39.48 \pm 0.21 \mu\text{M}$), and ratio of prosoma to opisthosoma was 0.65 ± 0.05 . and these measurements confirm that samples are *Demodex Canis* as shown in (Table 1) (Figure 4).



Figure 1 & 2: Generalized form of demodicosis that involve larger area of the body as abdomen, legs, face with alopecia, redness, inflammation and crusting of skin, and rubbing their face and head, also the paws were swollen.



Figure 3: Generalized demodicosis.



Figure 4: *Demodex canis*. 40 X.

Table 1: Morphometry of *Demodex canis* mites (μm).

Parameters	Demodex canis (n=17)	
	Mean \pm SD	Range
Gnathosoma	18.42 \pm 0.10	17-19
Podosoma	64.48 \pm 0.33	63-66
Opisthosoma	128.72 \pm 2.07	115-157
Total body length	221.81 \pm 10.86	146-259
Width	39.48 \pm 0.21	36-43
Ratio of prosoma to opisthosoma	0.65 \pm 0.05	

Data were expressed as Mean \pm standard deviation.

Anti-oxidative enzymatic activity

As shown in (Table 2), in the *Demodex Canis* infected dogs, there were a significant increase in the level of total antioxidant capacity (TAC) at ($P<0.05$), it was elevated up to $0.25\pm 0.01\text{k/mg Hb}$. in females dogs in comparison to healthy one that was $(0.16\pm 0.01\text{k/mg Hb})$, also in males, there were a significant difference between healthy $(0.19\pm 0.02\text{k/mg Hb})$, and infected dogs $(0.26\pm 0.02\text{k/mg Hb})$ at ($P<0.05$), and there is no significant difference between males and females in both healthy and infeted dogs. For superoxide dismutase enzyme (SOD), there were a high significant difference between healthy and infected dogs at ($P<0.05$), it were $(0.44\pm 0.01\text{U/mg Hb})$ and $0.46\pm 0.01\text{U/mg Hb}$

in both females and male infected dogs respectively, but there was non-significant difference in results of SOD between sexes, also there was a significant correlation at ($P<0.05$) between SOD level and both copper, and zinc concentration in blood but not correlated to other trace elements as seen in (Table 2). For malondialdehyde (MDA), there was a significant difference between the healthy and infected dogs at ($P<0.05$), it was $(4.94\pm 0.01\text{nmol/mg Hb})$, and $(3.84\pm 0.01\text{nmol/mg Hb})$ in both infected female and males respectively, where in healthy ones it was $(2.36\pm 0.01\text{nmol/mg Hb})$ in females dogs and $(1.29\pm 0.01\text{nmol/mg Hb})$ in males but of no significance at ($P<0.05$). At the same time, there was a significant correlation between MDA level and iron concentration at ($P<0.05$), but of no correlation to other trace elements (Table 2).

Table 2: Total antioxidant capacity (TAC), Superoxide dismutase (SOD), Malondialdehyde (MDA) status in canine erythrocytes, and trace elements (copper, zinc, iron, calcium, and magnesium) levels in serum of both sexes of healthy and infected dogs with *Demodex canis*.

Parameters	Groups(n=23)				P value ^a	P value ^b	P value ^c	P value ^d
	Females (n=10)		Males (n=13)					
	Healthy (n=3)	Infected(n=7)	Healthy (n=3)	Infected(n=10)				
TAC (k/mg Hb)	0.16±0.01	0.25±0.01	0.19±0.02	0.26±0.02	0.029*	0.323 ^{NS}	0.028* ^c	0.69 ^{NS}
SOD (U/mg Hb)	0.39± 0.04	0.44± 0.01	0.35± 0.02	0.46± 0.01	0.003**	0.983 ^{NS}	0.002* ^c	1.09 ^{NS}
MDA (nmol/mg Hb)	2.36±0.01	4.94±0.01	1.29±0.01	3.84±0.01	0.038*	1.013 ^{NS}	0.998 ^{NS}	0.036* ^d
Copper (ppm)	0.82±0.04	0.52±0.01	0.79±0.03	0.48±0.03	0.043*	3.033 ^{NS}	0.031* ^c	0.932 ^{NS}
Zinc (ppm)	0.72±0.06	0.62±0.05	0.70±0.04	0.58±0.03	0.041*	1.033 ^{NS}	0.042 ^c	0.788 ^{NS}
Iron (ppm)	1.52±0.04	1.82±0.03	1.47±0.04	1.92±0.06	0.029*	0.932 ^{NS}	0.889 ^{NS}	0.048* ^d
Calcium (ppm)	94.51±5.09	95.91±3.09	90.01±5.09	92.01±2.49	3.991 ^{NS}	3.032 ^{NS}	4.299 ^{NS}	3.983 ^{NS}
Magnesium (ppm)	40.65±0.36	41.55±0.30	43.67±0.33	42.07±0.70	4.675 ^{NS}	3.232 ^{NS}	3.908 ^{NS}	4.980 ^{NS}

Plasma trace elements concentration

Results in (Table 2) showed a higher significant decrease in serum level of copper (0.52±0.01ppm) in females, and (0.48±0.03ppm) in males, also for zinc level it was decreased in both females and males (0.62±0.05 ppm), and (0.58±0.03 ppm) respectively, at (P<0.05), in all examined *Demodex Canis* infected dogs than healthy control dogs, and there was no significant effect of sex on the copper and zinc in infected dogs. On contrary for iron serum level, there was a significant increase at (P<0.05), in its level in infected dogs (1.82±0.03ppm) in females, and (1.92±0.06ppm) in males, than healthy dogs, the level of iron in healthy and infected both sexes animals are differs but of no significant difference at (P<0.05). The serum level of calcium was (95.91±3.09ppm) in infected females and (93.51±2.49 ppm) in infected males, but manganese level was (41.55±0.30 ppm) in females and (92.01±2.49ppm) in infected males and they were not significantly altered and they were of no value in this study and there were no any significant correlation between calcium or manganese and TAC, SOD, MDA levels as oxidative enzymatic activity of *Demodex Canis* infected dogs at (P<0.05). results of trace elements concentrations in both male and female infected dogs were not significantly differs, and so; the sex factor was not significantly affect the serum level of trace elements concentrations at (P<0.05) as shown in (Table 2).

Discussion

In this study; we would like to elucidate the picture of antioxidant activity and trace element status in cases of demodicosis in dogs infected with *Demodex Canis* that is responsible for the clinical picture of the disease, there are several studies were done on the oxidative stress phenomena and anti-oxidant activity in cases of skin diseases and methods for their quantification by Kohen et al. [16] and Raman et al. [17], and also studies on minerals concentration in dogs have been described by Adamama Moraitou et al. [1], but low studies were done on demodicosis till now in Egypt. Bartosz G [3], stated that; all studies that concerned with the evaluation of anti-oxidant status of any parasitic infection commonly depends on the detection of total anti-oxidant capacity (TAC) which is measures the antioxidant components of a sample in a general way, and its technique is simple, and of low cost per each sample,

less time consuming, and can be performed either by automated or manual methods, as described by Marques et al. [18], and Erel O [11]. But, on the other hand; Sies [19], and Fraga et al. [12], stated that; only TAC analysis cannot provide enough information about the anti-oxidative status due to TAC not measures all the antioxidant components, for example, it do not measures the level of superoxide dismutase, glutathione peroxidase, or catalase, or MDA as mentioned by Nemeč et al. [20]. Based on these previous studies, it is recommended that the combination of TAC assay with other specified analysis of any antioxidant as plasma level of SOD and MDA can provide a larger and précised image about the antioxidant status of the infected animals. Results of the present study has determined the significant elevation of plasma level of TAC in infected dogs in comparison with healthy controls dogs, and this was in agreement with Camila et al. [4], who stated that; it can be observed that TAC determined with the method developed by Erel [11] decreased after surgery, anesthesia, in visceral leishmaniosis, and after vaccination against canine monocytic ehrlichiosis; and increased in demodicosis and parvoviral enteritis. Results of the previous study that was done by Dimri et al. [8] revealed that; the SOD enzyme activity increased in parasitic dermatitis and in dogs, suffering from demodectic mange, because SOD activities represent the first line of anti-oxidant activity of free radicals side effects especially for skin affections as mentioned by Raman et al. [17], as SOD catalyze the formation of O₂ from reactive oxygen species, the possible reason of increase in SOD activity in the present study, could be then due to its up-regulation in its synthesis to counteract free radicals Dimri et al. [8]. Significant decrease in the zinc and copper concentration also supported this hypothesis, since it may be, at least partially, due to enhanced utilization of these elements for synthesis of SOD. So increased SOD activity indicates increased oxidative stress in these cases. SOD is a metalloenzyme and hence, requires a metal cofactor for its activity. On the basis of the type of metal ion required as cofactor by SOD, MDA various forms of the enzyme exist consequently rendering the potentially harmful superoxide anion less hazardous [8,9]. Muller E [21] stated that; elements such as metals (particularly iron, which exists in abundance in the body) have been reported to be catalysts of oxidative damage. The metal ions which are normally bound by SOD are iron (Fe), zinc

(Zn) copper (Cu) and manganese (Mn). In this regards, SODs are classified into three forms and these include (i) Fe-SOD which is commonly found in prokaryotes and chloroplasts of some plants (ii) Mn-SOD which is present in prokaryotes and mitochondria of eukaryotes and (iii) Cu/Zn-SOD is predominant in eukaryotes and more distributed, localized basically in cytosol but also found in chloroplasts and peroxisomes as stated by Halliwell, & Gutteridge [15]. Adamama-Moraitou et al. [1] postulated that; there are some products that derived from nitrogen called reactive nitrogen species (RNS), such as sulfur, copper, and zinc, manganese that is also considered free radicals in biological systems and causing cell damage through oxidative stress, and it is was in agreement with the results of the present study as the plasma level of zinc and copper were significantly decreased in *Demodex Canis* infected dogs than healthy control dogs, at the same time; there was a significant negative correlation between the plasma level of TAC, and SOD with zinc and copper, and it was agreed with study elucidated by Dimri et al. [8], who conducted a study on the status of certain oxidative stress indices and zinc and copper concentrations in blood were estimated in dogs with localized demodicosis (LD) and generalized demodicosis (GD) [22].

In comparison to healthy control, erythrocytic lipid peroxides level and superoxide dismutase activity were significantly higher in both LD as well as GD, and the blood zinc and copper levels in dogs with LD and GD were significantly lower than healthy control [23]. From the present study, it was concluded that demodicosis is associated with oxidative stress and antioxidant supplementation may be beneficial in management of canine demodicosis, and no any significant correlation between manganese, calcium with TAC or SOD enzymes. Sexes of infected animals do not significantly affected the plasma level of total antioxidant activity nor superoxide dismutase enzymatic activity, and it was in agreement also with [17]. On the other hand; results showed a significant increase in plasma malondialdehyde (MDA) enzymes as a lipid peroxidation enzyme, with a significant increase in iron plasma level of *Demodex Canis* that provoke dermatitis in infected dogs than healthy control dogs and it was in agreement with [6] who postulated that; MDA was increased in skin affections as vitiligo patient. Results of this study showed that; the level of plasma MDA level was high without the significant correlation of animals sex between infected and healthy dogs, also there was no a significant correlation between plasma MDA and manganese nor calcium and this might be due to these elements not a cofactors for MDA metabolism [24-27].

From the results of this study and the previous studies; we can concluded that; total anti-oxidant activity, plasma superoxide dismutase and malondialdehyde were significantly increased in demodectic mange, but zinc, copper were significantly decreased, and iron level was increased, on the other hand; manganese, and calcium not significantly altered, sex of infected animals not affect the plasma level of enzymes nor minerals. There is a negative correlation between plasma level of SOD, and zinc, copper level, also positive correlation between MDA and iron level and so exogenous parenteral or oral supply of zinc, copper but not iron for dogs infected with demodicosis is necessary beside the acaricide drugs [28,29].

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Conflict of Interest

Authors declares that; there are no any conflict of interest in this study.

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