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Evaluation of antioxidant potential of vitamin D3 in geriatric dogs

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Abstract
The vitamin D3's potential significance as an antioxidant has not received enough attention. It has mostly been examined for its impact on the metabolism of calcium and phosphorus. This investigation aims to clarify the vitamin D3's antioxidant capabilities. Apparently healthy dogs (n = 16) were selected and divided into two groups based on their age, Group I: < 8 years and Group II: 8 years & above. Since there were age-related alterations in the oxidant-antioxidant profile, vitamin D3 (cholecalciferol drops) was used to amelioration. The changes in oxidant-antioxidant profile were measured on the day 0, day 15th, and day 30th of therapy. The mean values of lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) were significantly (p<0.05) higher in group II as compared to group I whereas the mean values of reduced glutathione (GSH) was significantly (P<0.05) lower in group II compared to group I on day 0. On 15th and 30th day, the mean values of LPO, SOD, CAT and GPX showed significant decline (p<0.05), while the mean values of GSH was significantly (p<0.05) higher in both vitamin D3 treated groups. The changes were more significant in older age group. Based on the findings of this study, it is concluded that vitamin D3 is having antioxidant potential especially in geriatric dogs.

Keywords: Antioxidant, ameliorative, free radical, haemolysate, spectrophotometer

1. Introduction
Being a fat-soluble vitamin, vitamin D is crucial for immune system health, bone health, and other physiological functions. Although vitamin D is not traditionally considered as an antioxidant, recent research has raised the possibility. According to a study that appeared in the International Journal of Molecular Sciences, mice given with vitamin D supplements had lower levels of oxidative components and higher antioxidant capacities in their serum and lung tissue homogenate (Adam *et al.* 2021) [1]. A significant imbalance between the generation of reactive oxygen species (ROS) and antioxidant defenses is referred to as oxidative stress (Mokhtari *et al.* 2016) [15]. Modifications in signaling cascades and potential tissue damage are brought on by oxidative stress. Polyunsaturated fatty acids, proteins, and nucleotides are all adversely affected by ROS, which can result in lipid peroxidation, inactive proteins, and damaged nucleic acids. In the absence of sufficient protection from ROS provided by enzymatic and non-enzymatic antioxidants, ROS may be harmful to cellular processes. Cholecalciferol is thought to serve as a membrane antioxidant by stabilising the membrane against lipid peroxidation through a hydrophobic ring binding (Wiseman 1993) [25]. Similar to the effects of vitamin E supplementation, vitamin D3 treatment resulted in a decrease in the level of lipid peroxidation (Sardar *et al.* 1996) [20]. By raising the intracellular pool of reduced GSH, decreasing malondialdehyde (MDA), and decreasing the activity of superoxide dismutase, catalase, and glutathione peroxidase in geriatric dogs, vitamin D3 may improve the mechanism of ROS elimination. Vitamin D3 regulates oxidative stress by inducing the expression of various antioxidant defence system components such as glutathione, glutathione peroxidase, catalase and superoxide dismutase and limiting the expression of NADPH oxidase (Görlach *et al.* 2000; Kono *et al.* 2013) [5, 11]. A growing body of research indicates that vitamin D3 has critical functions in promoting the development of various antioxidants and anti-inflammatory cytokines. This study was undertaken to study antioxidant potential of vitamin D3 in geriatric dogs.

2. Materials and Methods

2.1 Study area: The current study was carried out at Referral Veterinary Polyclinic, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, UP.

2.2 Experimental animals

Sixteen apparently healthy dogs which were brought for vaccination, deworming, and regular health checkups to the institute and dogs from neighboring areas of Bareilly were included in the study.

2.3 Experimental design

Dogs (n = 16) were divided into two groups based on their age, Group I: < 8 years (n = 8) and Group II: 8 years & above (n = 8). Dogs were evaluated for normal functioning of various systems of body namely respiratory, digestive, renal, musculoskeletal, skin etc. Thorough detailed anamnesis including housing, feeding habits, general health etc. was taken. Physical parameters like age, sex, breed and body weight were recorded. Recording of visible mucus membrane color, CRT (capillary refill time), body weight, heart rate, pulse rate, respiration rate, rectal temperature, lymph nodes examination etc. was done. Macroscopic and microscopic examination of feces was done. Urine samples were collected into sterile vials after catheterization of animal aseptically using a sterile catheter and 2% lignocaine jelly. Since there were age-related alterations in the oxidant-antioxidant profile, vitamin D3 (cholecalciferol drops) was used to ameliorate it (Zafalon *et al.* 2020) [26]. The changes in oxidant-antioxidant profile were measured on the day 0, day 15th, and day 30th of therapy.

Table 1: Experimental design for evaluation of vitamin D3 supplementation in various age groups of dogs

Groups	Description	Dosage regimen	No. of dogs (n)	Remarks
Group 1	Vitamin D3	10-20 IU/kg BW/d PO for 30 days	8	Apparently healthy
Group 2	Vitamin D3	10-20 IU/kg BW/d PO for 30 days	8	Apparently healthy

2.4 Collection of blood

Blood samples were taken from the cephalic or saphenous vein. After collection, 2mL of blood was placed in K3 EDTA (tripotassium ethylene diamine tetra acetic acid) vial and mixed thoroughly. Roughly 3 mL of blood was transferred to a plain anticoagulant free vial for serum extraction. After 15 minutes of centrifugation at 3000 rpm, serum was extracted and stored at -20 °C for further testing. Serum was used to evaluate a variety of biochemical parameters. For the oxidant-antioxidant profile estimation, 3 mL of venous blood was taken in heparinized vials.

2.5 Hematological analysis

Blood parameters were examined like haemoglobin (Hb) using Sahli's haemoglobinometer and expressed as g/dL, packed cell volume using Jain (1986) [7] method and was expressed in percent, total erythrocyte count (TEC) was calculated using a Neubauer's counting chamber and expressed as millions/mm^3 (Jain, 1986) [7], total leukocyte count (TLC) was calculated using a Neubauer's counting chamber and expressed as $10^3/mm^3$(Jain, 1986) [7], differential leukocyte count using Jain (1986) [7] method and the values were expressed in percentage, platelet count was calculated using Jain (1986) [7] method and expressed in lakhs per microliter.

2.6 Biochemical analysis

Liver function test (LFT), kidney function test (KFT) and urinalysis were performed using commercial kits (Span Diagnostics, India Using dipstick strips, urinalysis was performed on the urine of dogs. Urine pH, specific gravity and qualitative assessment of ketone bodies, protein, glucose, casts and cells were performed.

2.7 Oxidant-antioxidant profile

Approximately 3 mL of venous blood was taken in heparinized vials. After centrifugation for 10 minutes at 3000 rpm, the plasma and buffy coat were removed. The RBCs were centrifuged three times with ice cold solution of normal saline for 10 minutes at 3000 rpm and the supernatant was removed. To prepare a 10% haemolysate, about 2/3 of the RBC pellet was diluted in a 1:10 ratio with ice-cold distilled water assess lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). The rest 1/3rd of the RBC pellet was diluted in a 1:1 ratio with ice-cold normal saline solution to obtain RBC suspension. This RBC suspension was utilised to estimate reduced glutathione (GSH). The level of LPO in RBC's 10% haemolysate was determined as per Placer *et al.* (1966) [18]. This method works by forming a colored complex between lipid peroxidation products and thiobarbituric acid (TBA). 0.2 mL RBC haemolysate was added to 1.3mL Tris-KCl buffer (0.2 mol/L Tris, 0.16 mol/L KCl, pH 7.4), then also add 1.5 mL of 0.8% TBA reagent. The mixture was heated for 10 minutes in a boiling water bath using glass beads as a condenser. After cooling, 3 ml pyridine-n-butanol (pyridine and n-butanol as 3:1 v/v) and 1 ml NaOH (1 N) were added and mixed properly. In place of RBC haemolysate, 0.2 mL distilled water was used to make the blank simultaneously. At 548 nm, the absorbance was measured against the blank. The concentration of MDA per mg of haemoglobin (MDA/mg Hb) was calculated using an extinction coefficient of 1.56 X 10^5/mol/cm. GSH in RBC suspension was quantified using the DTNB (5, 5' –Dihiobis-2-Nitro Benzoic acid) methodology, according to Prins and Loos (1969) [19]. 0.2 mL of packed RBC suspension was thoroughly mixed with 4 ml of 0.08 N H2SO4. The mixture was allowed to cool for 10 minutes before adding 0.5 mL of tungstate solution to clear brown haemolysate. After closing the test tubes, the contents were vigorously stirred for 5 minutes. To prevent crust formation in the supernatant fluid in the subsequent centrifugation, the suspension was allowed to stand for 5 minutes without a stopper. The suspension was centrifuged for 20 minutes at 2000 rpm at room temperature. Then 2 mL of the supernatant was combined with 2.5 mL of tris buffer having pH 8.0. 0.2 mL of DTNB reagent was pipetted and carefully mixed just before measurement. OD was measured against blank at 412 nm when color develops completely. The Madesh and Balasubramanian (1998) [13] approach was used to determine SOD levels. The units were expressed as the amount of MTT [3-(4-5 dimethyl thiazol 2-yl) 2, 5 diphenyl tetrazolium bromide] formazan produced per mg Hb (haemoglobin). The method is based on the formation of superoxide by pyrogallol auto-oxidation as well as inhibition

of superoxide dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-yl) 2, 5 diphenyl tetrazolium bromide] to its formazan. The process was stopped when dimethyl sulfoxide (DMSO) was added. The solubilization of the produced formazan is aided by DMSO. The colour that results is long-lasting (for many hours). 1280 µL of PBS (1 M, pH 7.4), 60 µL litres of MTT, 20 µL litres of haemolysate, and 150 µL litres of pyrogallol make up the assay mixture. Pyrogallol was freshly produced and added to the reaction mixture after all other reagents had been added. After properly mixing the contents, they were incubated for 5 min. at 30 degrees Celsius. Eventually, the process was stopped by adding 1500 µL of DMSO. By adding distilled water, the blank was prepared. Rather than haemolysate, all other reagents were used to make the control. The absorbance was measured at 570 nm. The amount of SOD produced is measured in µmol MTT formazan generated per mg of Hb. CAT levels in haemolysate were calculated using hydrogen peroxide (H_2O_2) as a substrate, according to Aebi (1986). 1980 µL of phosphate buffer (50 mM, pH 7.0) was poured to 20 µL of stock haemolysate to make 2 ml of working hemolysate. Then 2 ml of working hemolysate was appropriately mixed with 1 ml of phosphate buffer hydrogen peroxide solution. This reaction mixture was taken in a cuvette with a capacity of 3 ml. A spectrophotometer was used to measure absorbance against phosphate buffer for one minute at 240 nm wavelength. Initial and final absorbances were recorded (at 0 second and 60 seconds respectively). Finally the difference between two absorbances was calculated. The activity of catalase was measured in µmol H_2O_2 decomposed/min./mg Hb. Paglia and Valentine (1967) approach was used to calculate GPX. 2.48 mL K_2HPO_4 (pH 7.0, 50mM) along with EDTA (5mM), glutathione reductase (4.6 U), 100 µL 150mM reduced glutathione, 10 µL 112.5mM sodium azide, 8.4mM reduced NADPH and 0.5 mL haemolysate were added to the spectrophotometer cuvetee along with 100 µL H_2O_2. The absorbance was determined at 340 nm for 4 min. GPX activity was standardised against protein concentration by using molar extinction coefficient of 6200 at wavelength of 340 nm. The results were measured in µM of NADPH oxidised per minute per mg of Hb (mU/mgHb). The results for linear NADPH oxidation by hydrogen peroxide alone were correlated, without the use of enzyme protein.

2.8 Statistical analysis

SPSS 16.0 software JMP 9.0 was used to Organise and statistically analyse the data. The Tukey test (at significance level of 5%) was used to make comparisons between time intervals, periods, and groups as well as their relationships with other parameters. Pearson's correlation coefficient method was used to conduct an investigation of correlation with age for multiple attributes. ANOVA with two-way repeated measures was used to evaluate the data from the ameliorative study. When P value < 0.05 was used with 95 percent confidence intervals, the differences were deemed significant (Snedecor and Cochran, 1994) [21].

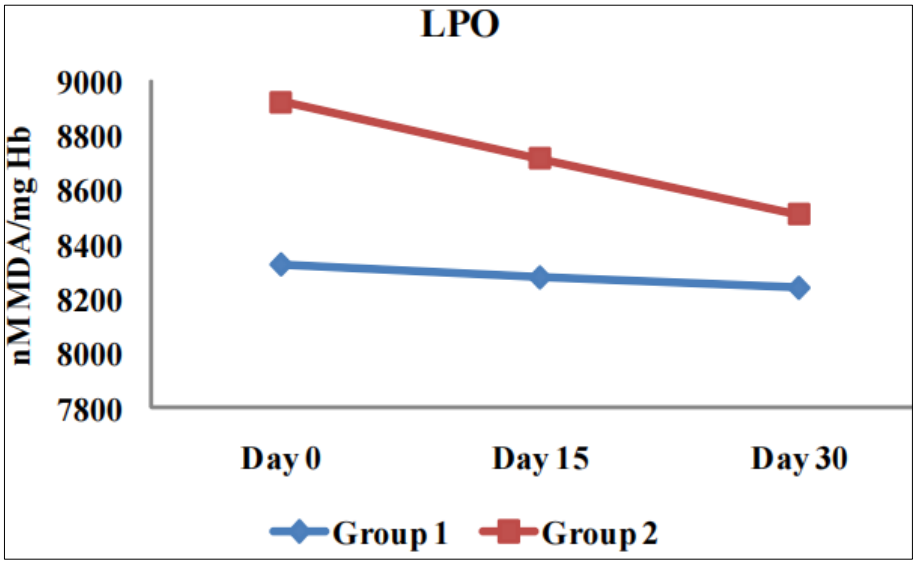
3. Results and Discussion

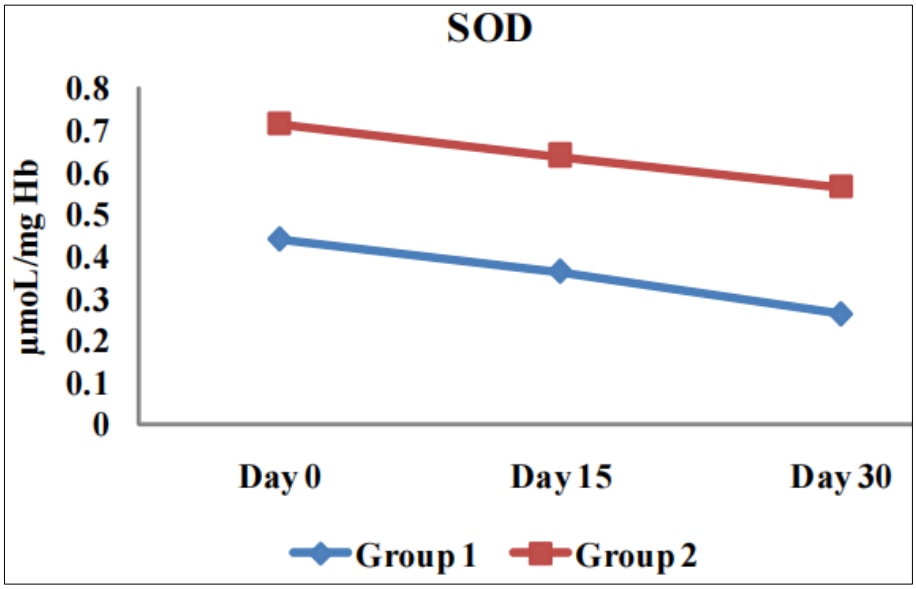
The LPO levels and activity of SOD rises with age, and the difference was significant. On days 15 and 30, there was a significant (p<0.05) drop in LPO levels in both groups after receiving vitamin D3. In comparison to group I, dogs in group II had significantly greater levels (p<0.05) of SOD. On day 30 after vitamin D3 treatment, the levels of superoxide dismutase in both groups were considerably (p<0.05) lower. GSH levels declined with age, and the difference between groups I and II was significant (p<0.05). On day 30, there was a significant (p<0.05) rise in reduced glutathione group I after vitamin D3 supplementation. On day 15, there was a significant (p<0.05) rise in GSH levels in group II. Catalase levels rise with age, with a significant (p<0.05) rise in group II compared to group I. On day 15 after receiving vitamin D3, a significant (p<0.05) decline in catalase was seen in group I. On day 15 and 30 after receiving vitamin D, a significant (p<0.05) decline in catalase was seen in group II. GPX levels rise with age and both groups varied significantly (p<0.05). On day 15 and 30, after receiving vitamin D, there was a significant (p<0.05) drop in GPX levels in both groups.

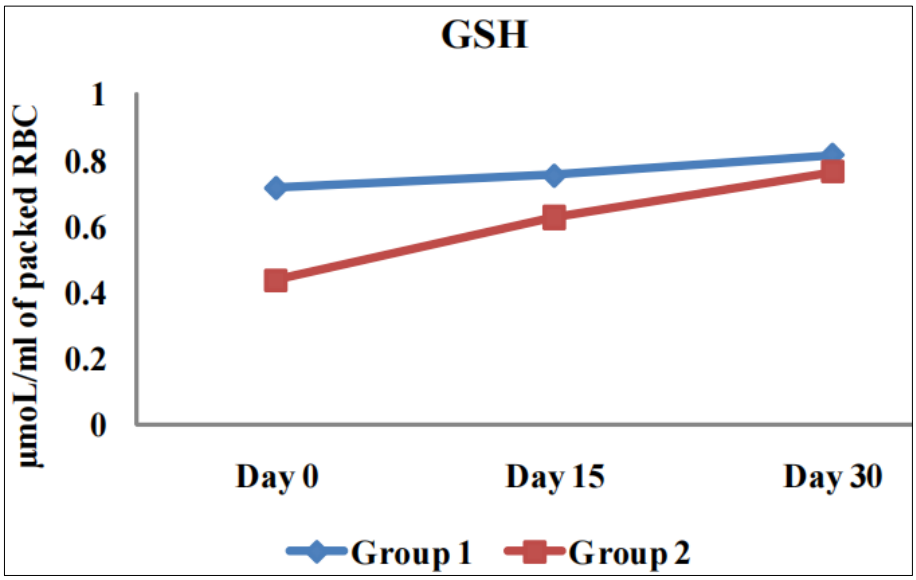
Table 2: Effect of vitamin D3 supplementation on oxidant-antioxidant profile in different age groups of dogs on days 0, 15 and 30 (Mean ± SE)

Parameters	0 day	15 day	30 day
LPO (nM MDA/mg Hb)			
Group I (n=8)	1.12±0.02^{c}	0.88±0.02^{d}	0.65±0.02^{e}
Group II (n=8)	1.78±0.02^{a}	1.55±0.01^{b}	1.11±0.01^{c}
SOD (µmol/mg Hb)			
Group I (n=8)	0.44±0.02^{c}	0.36±0.02^{e}	0.26±0.01^{d}
Group II (n=8)	0.71±0.01^{a}	0.63±0.02^{ab}	0.56±0.03^{b}
GSH (µmol/ml of packed RBC)			
Group I (n=8)	0.71±0.02^{b}	0.75±0.01^{ab}	0.81±0.01^{a}
Group II (n=8)	0.43±0.02^{d}	0.63±0.03^{c}	0.76±0.02^{ab}
CAT (µmol H_2O_2 decomposed/min/mg Hb)			
Group I (n=8)	114.80±0.64^{d}	110.83±0.68^{e}	109.50±0.69^{e}
Group II (n=8)	158.07±0.68^{a}	147.21±0.80^{b}	129.60±0.45^{c}
GPX (mU/mgHb)			
Group I (n=8)	8326.50±1.47^{d}	8280.41±1.17^{e}	8244.03±1.13^{f}
Group II (n=8)	8922.38±1.64^{a}	8716.15±1.53^{b}	8511.46±1.70^{c}

'Values within a row, having different superscripts, differ significantly (p<0.05) with each other


LPO
nM MDA/mg Hb
9000
8800
8600
8400
8200
8000
7800
Day 0
Day 15
Day 30
Group 1
Group 2


SOD
µmoL/mg Hb
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 0
Day 15
Day 30
Group 1
Group 2


GSH
µmoL/ml of packed RBC
1
0.8
0.6
0.4
0.2
0
Day 0
Day 15
Day 30
Group 1
Group 2

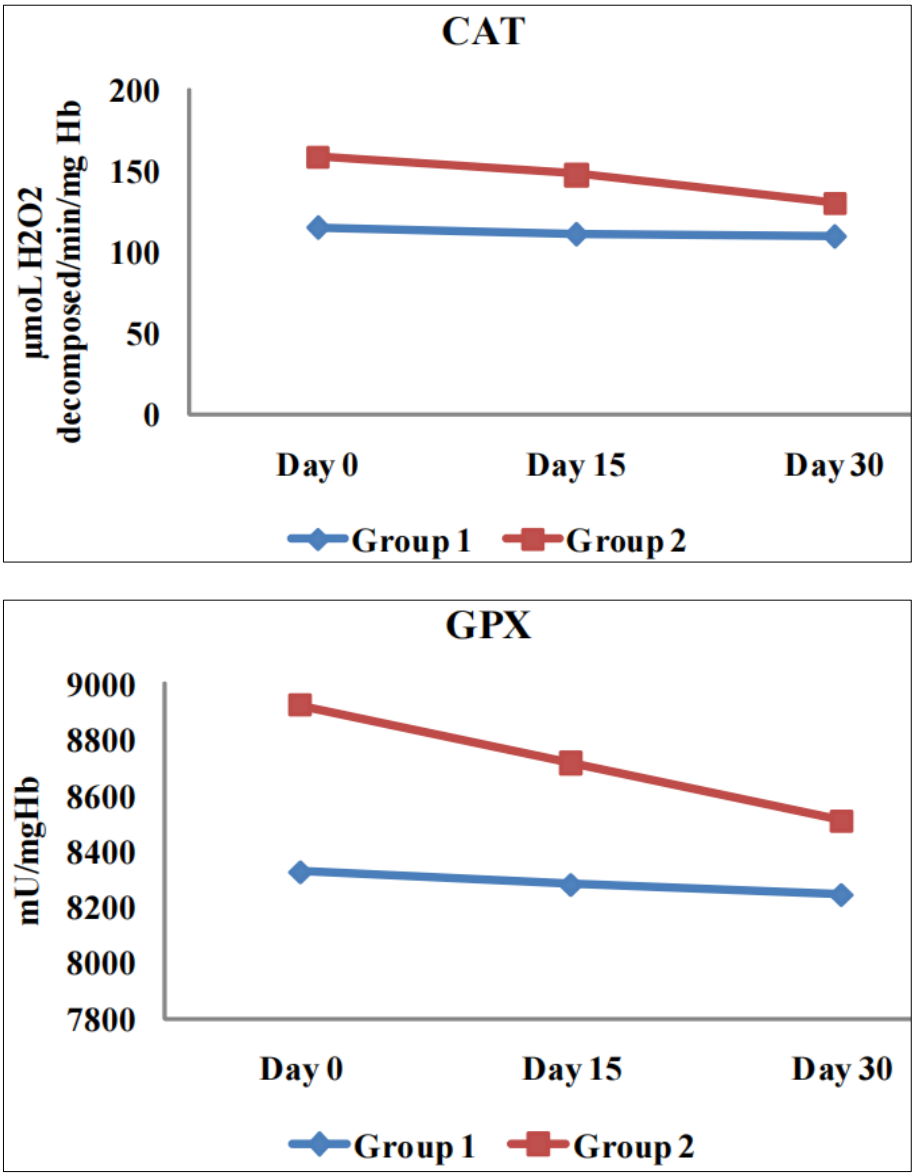


Fig 1: Effect of vitamin D3 on lipid peroxidation (LPO), superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT), and glutathione peroxidase (GPX) levels in different age groups of dogs

Our research demonstrates that older dogs had lower levels of oxidative stress after supplementation of Vitamin D3. Both age groups showed significantly (p<0.05) greater values of lipid peroxidation and superoxide dismutase. Older animals had greater MDA and SOD levels than younger animals (Vajdovich *et al.* 1997) [24]. Older tissues and cells are more susceptible to free radicals. Peroxidation rate of older tissues is higher when cell homogenates are exposed to rapid oxidation, implying that RBC enzymatic activity are largely age driven (Kasapoglu *et al.* 2001) [10]. The enzyme's enhanced antioxidant activity in older animals seems to be a compensatory strategy for the increased levels of ROS associated with ageing (Tomsic *et al.* 2016) [23]. The present work found a considerable drop in reduced glutathione levels with ageing. There was a significant rise in glutathione peroxidase and catalase in this study (Vajdovich *et al.* 1997) [24]. One of the causes for decreasing enzymatic activities is that enzyme activity declines in ageing tissues. Supplementing with vitamin D3 had a beneficial effect in the ameliorative phase. At varied time intervals, significant (p<0.05) decreases in changed levels of lipid peroxidation, catalase, superoxide dismutase, and glutathione peroxidase were observed. Although, at various time periods, significant (p<0.05) greater amounts of reduced glutathione were reported. Administration of vitamin D3 improved glutathione levels (Ansari *et al.* 2020) [3]. Vitamin D3 reduces lipid peroxidation and increases superoxide dismutase activity (Zhong *et al.* 2014) [27]. The present oxidative stress assessment finding confirms Vitamin D3's role as an important antioxidant in preventing free radical damage.

4. Conclusion

The present oxidative stress assessment finding confirms Vitamin D3's role as an important antioxidant in geriatric dogs. Vitamin D3 is essential for a wide variety of non-traditional effects in addition to maintaining adequate calcium homeostasis. One of the latest identified non-calcemic uses for vitamin D3 is its antioxidant function. To explore the antioxidant capacity of vitamin D3 in animals, additional investigations are needed.

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