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Short Communication

Effect of N, N-Dimethylglycine (DMG) Supplementation on Haematological Parameters and Frequency of CD4+ and CD8+ T Cells in Cats Post-vaccination

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ABSTRACT

N, N-Dimethylglycine (DMG) is a commonly used nutraceutical in veterinary medicine, which is claimed to have immunomodulating properties. This novel study investigated the effect of DMG supplementation on feline haematological parameters and the percentages of CD4+ and CD8+ T cells after vaccination with a single-dose commercial core vaccine. A total of twelve neutered cats were divided into control (n=6) and treatment groups (n=6) and received one dose of feline core vaccine at day 0. The treatment group then received oral DMG supplementation (125 mg/mL) twice daily for 14 days. Blood samples were collected on days 0 and 15 for haematological, differential cell count, and T cell phenotyping analysis. Haematological analysis revealed no significant difference between the control and treatment groups after 14 days of the experiment. At post-treatment, the neutrophil percentage of the treatment group was significantly lower (p=0.0238) compared to the control group, while the lymphocyte percentage of the treatment group was significantly higher (p=0.013) compared to the control group. The reduction in neutrophil percentage could be due to bromelain, an anti-inflammatory enzyme, while an increase in lymphocyte percentage could be attributed to an increase in B cell or NK cell subsets instead of T cells. Future research is warranted to investigate the effects of DMG on B cells and natural killer cell activation and to explore the long-term effects of DMG supplementation on feline immune health.

Keywords: Dimethylglycine, feline, haematological parameters, immunomodulation

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INTRODUCTION

Nutraceuticals can be defined as food products or components of food that are postulated to be beneficial for human and animal health (Hayek et al., 2004). An example of a nutraceutical that is commonly used in veterinary medicine is N, N-Dimethylglycine (DMG). DMG is a naturally occurring tertiary amino acid and a by-product of the metabolism of choline (Cupp, 2003). DMG is claimed by its proponents to be able to support the production of both lymphocytes and antibodies via the OKT4 epitope that activates T-cells or by the inhibition of T-suppressor cells, thereby enhancing both cell-mediated and humoral immunity (Reap & Lawson, 1990). Correlating to that claim, human subjects given DMG orally exhibited a four times increase in antibody titres against pneumococcal vaccine antigen after vaccination with pneumococcal vaccine compared to human subjects in the control group (Graber et al., 1981). Similarly, in a study done on rabbits, animals supplemented with DMG showed a four times increase of neutralising antibodies titre towards influenza antigen post-vaccination with a killed influenza virus vaccine (Reap & Lawson, 1990). Interestingly, in contrast, DMG failed to exhibit its immunomodulating capabilities in a cat study as it shows that cats treated with DMG showed a lower titre of virus-neutralising antibodies against feline herpesvirus-1 when compared to the control group (Weisrs, 1992). In the same study, the feline lymphocyte blastogenic response towards T cell mitogens was assessed *ex vivo*, and no significant difference in T cell proliferation was observed between feline lymphocytes treated with DMG and those treated with control. It was postulated that the difference in the outcome could be due to species variability or suboptimal dose of DMG; however, to the author's knowledge, there were no other feline studies. Therefore, given the widespread promotion and usage of DMG by veterinarians and the limited data on its immunomodulating or immunoadjuvant effects in cats, this current study aims to evaluate the impact of DMG supplementation on haematological parameters as well as the percentages of CD4+ and CD8+ T cells in cats' post-vaccination. The results will provide updated insights that will potentially assist veterinarians in making informed decisions regarding DMG's immunomodulatory properties.

MATERIALS AND METHODS

Animals and Study Design

This study was approved by the Universiti Putra Malaysia Institutional Animal Care and Use Committee (UPM IACUC) with approval number U032/2023. The study was conducted at an animal shelter, and 12 healthy neutered cats of both genders (male; $n = 5$, female; $n = 7$) were recruited for this study. Two weeks before the start of the study, recruited cats were given ectoparasite control and dewormed (Inovet, Belgium) at a dosage of 1 tab/10 kg of body weight. All the cats were conditioned in the same room they had been in before

the start of the study. Food in the form of kibbles and water was provided *ad libitum* throughout the study. Since this study aims to evaluate the immune response of the cats, only neutered cats were included in this study to minimise any hormone-related effects (Hellard et al., 2013). The cats (n=12) were randomly assigned to two groups, control and treatment, consisting of six cats per group. All cats were physically examined for clinical signs and tested negative for FeLV/FIV prior to vaccination. At day 0, both groups were administered a single dose of Purevax® Feline 4 Vaccine (Boehringer Ingelheim, Germany) containing modified live feline viral rhinotracheitis, feline calicivirus, feline panleukopenia virus and *Chlamydia psittaci* subcutaneously. Cats in the treatment group (n=6) were then orally supplemented with N, N–Dimethylglycine (DMG) in the liquid form (VetriDMG 125 mg/ml, VetriSciences, Vermont, USA) at a manufacturer's recommended dosage of 0.5 mL twice a day daily (BID) for 14 days. All 12 cats were monitored for 14 days post-vaccination and DMG supplementation for adverse reaction signs.

Sample Collection

All cats were subjected to blood sampling on days 0 (pre-treatment) and 15 (post-treatment) for haematological parameters, CD4 and CD8 T-cell phenotyping analysis. Approximately 3 mL of blood samples from the cats in both groups were collected and transferred into 3 mL tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were then placed in a cool box and transported to the lab for processing. Samples were processed within 24 hours, starting with haematological analysis, followed by peripheral blood mononuclear cell isolation and flow cytometric analysis.

Haematological Analysis

Haematological parameters such as haematocrit (HCT), red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), and platelet (PLT) counts were determined using Celltac Alpha VET MEK-6550K Haematological Analyser (Nihon Kohden, Japan). A thin blood smear was performed on all blood samples and stained with modified Wright's stain. A differential white blood cell count was carried out on each stained blood smear, whereby 100 cells were counted per slide to determine the percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

CD4 and CD8 Phenotyping from PBMC Using Flow Cytometry

Peripheral blood mononuclear cell (PBMC) isolation was performed using a previously published protocol (Megat et al., 2022). Upon isolation, the PBMC pellet was resuspended and adjusted to a concentration of 2×10^6 cells/mL for flow cytometric analysis. Briefly, 2×10^6 cells/mL of PBMC were washed with 1 ml of buffer containing 2% FBS and 0.5 mM EDTA prior to antibody staining. Mouse anti-feline CD4-FITC (clone 34F4; Southern

Biotech, USA) and mouse anti-feline CD8-PE (clone: fCD8; Southern Biotech, USA) of 1 µL each were added to the cells and incubated at 4°C for 30 minutes in the dark. The cells were washed again with staining buffer containing 2% FBS and 2M EDTA, resuspended in 200 µL of 1% paraformaldehyde, and stored at 4°C for up to 72 hours before flow analysis. Data were acquired using BD FACSCanto (BD Biosciences, USA) for 50,000 events and analysed using BD FACSDiva software (BD Biosciences, USA). Compensation was carried out using fluorochrome minus one (FMO) CD4+ and FMO CD8+ controls. The positive CD4+ and CD8+ T lymphocyte population was determined by gating a similar population of unstained cells.

Statistical Analysis

All data were presented as median (M) and standard deviation (SD) except for haematological parameters where median and interquartile range (IQR) were used. As the data were not normally distributed, non-parametric tests were used using GraphPad Prism 9 software (GraphPad Software Inc., USA) to compare differences within (Wilcoxon matched pairs test) and between groups (Mann-Whitney test) at different time points. $p \leq 0.05$ was considered statistically significant, and for the Wilcoxon matched pairs test, Spearman's correlation coefficient (rs) was indicated if the result was statistically significant.

RESULTS

Haematological Parameters and Differential White Blood Cell Percentage

There were no significant differences in RBC, HCT, HGB, PLT, and WBC values between the control and treatment groups at post-treatment (Table 1). However, within each group,

Table 1
The effect of N, N-Dimethylglycine Supplementation on haematological parameters

Parameters	Time point	Control Group (*n*=6)	Treatment Group (*n*=6)
Red blood cells (RBC) (10^{12}/L)	D0	9.56 (9.99–8.62)	10.50 (10.83–9.49)
	D15	9.02 (9.45–8.64)	9.01 (10.75–8.27)
Haematocrit (HCT) (%)	D0	42.50 (44.50–40.10)	45.50 (47.53–41.13)
	D15	39.80 (41.10–39.25)	39.70 (47.40–34.73)
Haemoglobin (HGB) (g/dL)	D0	139.00 (146.75–133.75)	145.00 (156.00–131.25)
	D15	135.00 (137.00–131.75)	134.00 (159.25–117.00)
Platelet (PLT) (10^9/L)	D0	226.00 (323.50–184.00)	171.50 (195.50–96.00)
	D15	265.00 (334.50–207.50)	135.50 (210.25–122.25)
White blood cells (WBC) (10^9/L)	D0	12.150 (20.55–10.45)	11.90 (17.48–10.65)
	D15	14.05 (20.83–9.30)	18.30 (23.98–14.68)

Note. Data is presented as median (IQR). Haematological parameters were obtained at pre-treatment (day 0) and post-treatment (day 15) of the study

the cats exhibited a decrease in RBC, HCT, and HGB and increased in WBC numbers. Nevertheless, all these haematological changes were not statistically significant ($p \geq 0.05$). It is also important to note that most of the values for haematological parameters of both groups for both day 0 and day 15 were within the normal reference range or slightly above the upper normal limits (RBC value for treatment group on day 0; median: 10.50 x 10^{12}/L [IQR: 10.83–9.49], and HCT value for treatment group on day 0; median: 45.5 [IQR: 47.53–41.13]). However, the values for PLT for both groups on both day 0 and day 15 were below the reference range (Latimer, 2011; Weiss & Wardrop, 2010).

At post-treatment, values for parameters such as monocyte and eosinophil did not differ significantly between the control and treatment groups (Figure 1). Basophils were not detected in both cohorts at the two different time points (data not shown). Interestingly, cats in the treatment group exhibited significantly lower neutrophil percentage ($p = 0.024$; $M = 60.67$, $SD = 4.63$) compared to the control group ($M = 67.50$, $SD = 4.93$) at post-treatment. Conversely, cats in the treatment group exhibited significantly higher lymphocyte percentage ($p = 0.013$; $M = 33.83$, $SD = 4.49$) compared to the control group ($M = 25.50$,

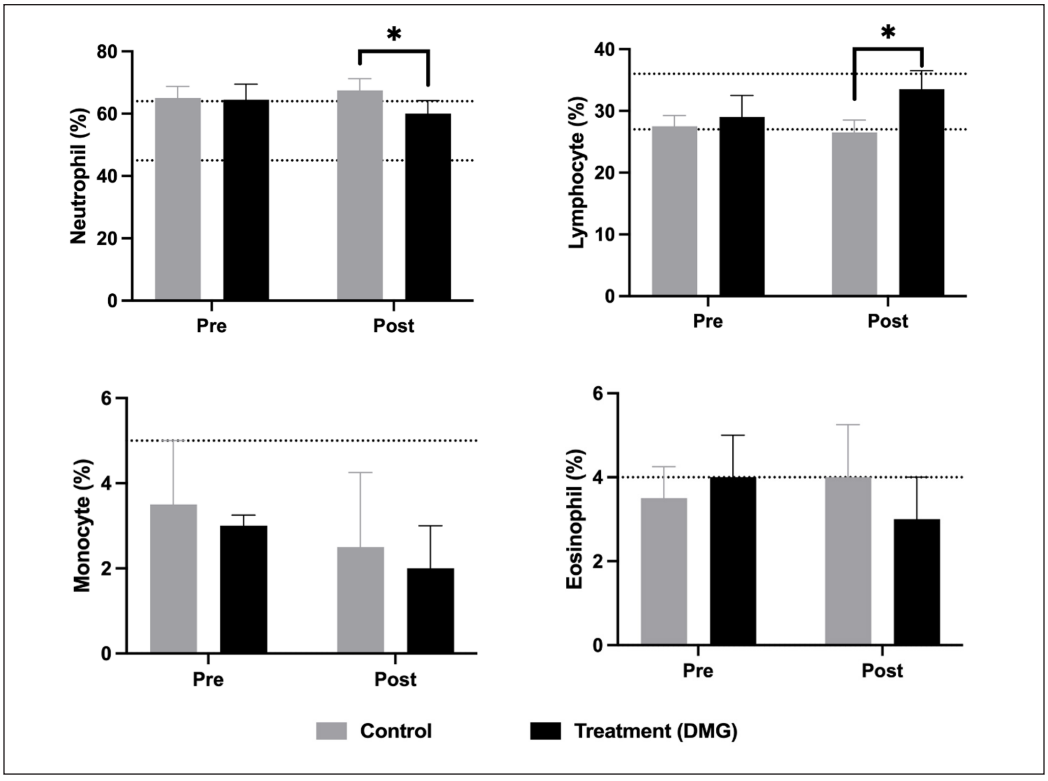

Figure 1. Effect of N, N-Dimethylglycine supplementation on differential white blood cell percentages. Data was analysed using a non-parametric Mann-Whitney test to determine significant differences between groups at day 15 (*) and a Wilcoxon matched pairs test to determine significant differences within groups at day 0 versus day 14. $p \leq 0.05$ is considered to be statistically significant

SD = 3.94). As shown in Figure 1, except for the lymphocyte percentage of the control group post-treatment, all the values for differential white blood cell percentage parameters of both pre-and post-treatment are within the normal reference range or slightly above the upper normal limit.

CD4+, CD8+ T Cells Phenotypic Analysis

Figure 2 presents all results and statistical analyses of differences between the control and treatment groups regarding lymphocyte subsets (CD4+ T cell percentage, CD8+ T cell percentage, and CD4+ to CD8+ ratio). The Wilcoxon matched pairs test indicated that cats in the control group exhibited a significant increase in CD4+ T cell percentage from day 0 ($p = 0.03$; *rs*: -0.086; *M*: 16.75 *SD*: 8.00) to post-treatment (*M*: 29.90, *SD*: 8.063).

In contrast, cats in the treatment group showed a decrease in CD4+ T cell percentage from pre- to post-treatment, albeit non significantly. Due to this, the control group had a significantly higher mean CD4+ T cell percentage post-treatment than the treatment group (p: 0.0022; $M = 29.90$, $SD = 8.063$ vs $M = 6.933$, $SD = 3.681$). As for CD8+ T cell percentage, both groups exhibited a non-significant ($p \geq 0.05$) decrease from pre- to post-treatment, although the decrease in CD8+ T cell mean percentage was prominent in the treatment group. Lastly, the control group showed a slight increase in the mean CD4:CD8 ratio post-treatment, as determined by the reference range, represented by the dotted line (Byrne et al., 2000). Conversely, the treatment group exhibited a non-significant reduction of the CD4:CD8 ratio from pre- to post-treatment. It is important to

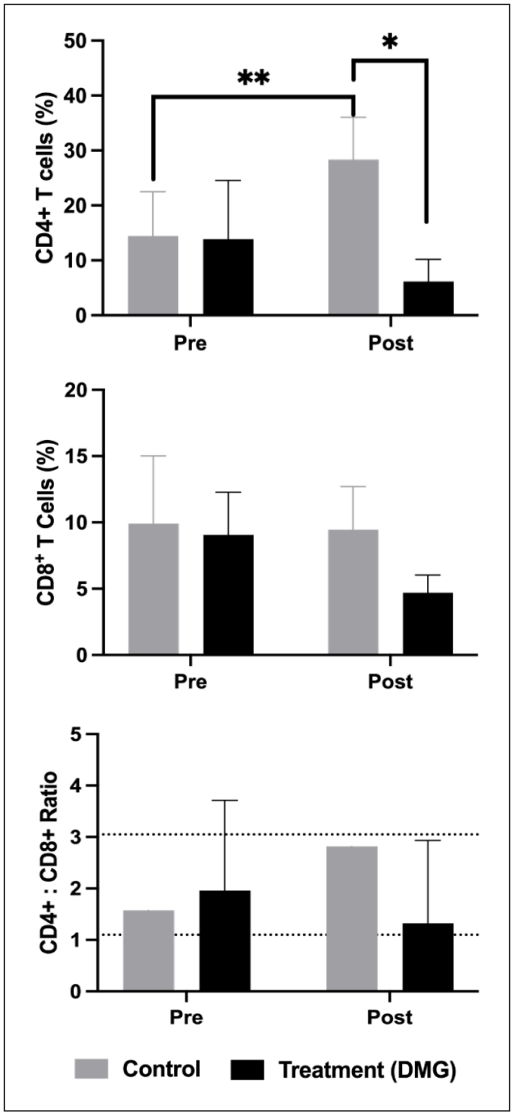

Figure 2. Effect of N, N-Dimethylglycine supplementation on Lymphocyte Subsets (CD4+ and CD8+ T cell). Data was analysed using a non-parametric Mann-Whitney test to determine the significant differences between groups at day 15 (*) and a Wilcoxon matched pairs test (**) for within-group analysis at day 0 versus day 15. $p \leq 0.05$ is considered to be statistically significant

note that, as seen in Figure 2, the CD4:CD8 ratios of both groups on day 0 and day 15 were within the normal range.

DISCUSSION

The impacts of oral supplementation of various nutraceuticals on the immune response towards infectious diseases and their influence on vaccine response are still being studied in various animal species (Mayer et al., 2019; Mohamed et al., 2019). In the context of DMG, studies pertaining to the relationship between DMG supplementation and its effects on the immune response to this day are still relatively scarce and infinitesimal, especially for the feline species. As noted earlier, the only study that investigated the effects of DMG supplementation on the immune response of cats was conducted back in the early 90s (Weiss, 1992). As a result of this, further explanations regarding the DMG's mechanism in affecting the feline immune system can only be extrapolated from earlier research conducted on other species, such as poultry (Kalmar et al., 2012), and a model of human keratinocyte (Lendvai et al., 2023). This study focused on the effects of DMG supplementation on haematological parameters (complete blood count including differential white cell count) and lymphocyte subset**.** The results showed there were no significant differences between pre-treatment (Day 0) and post-treatment (Day 15) for both groups of cats for RBC, HCT, HGB, PLT, and WBC (Table 1). Additionally, both groups' RBC, HCT, HGB, and WBC values were within the normal range or slightly above the upper normal limit. The RBC and HCT values were slightly high on Day 0 for the treatment group, most probably due to haemoconcentration. This could be due to dehydration prior to the start of the study, as the RBC and HCT values were normalised at the end of the study. This is unsurprising as the cats were from an animal shelter, and according to Miller and Zawistowski (2015), many shelters must work within tight budgets, often opting for the most affordable food options or depending on donations to meet their needs.

Hence, the cats' nutrition and hydration status would probably not be optimal. Considering these findings, we can extrapolate based on our study that short-term supplementation of DMG does not affect the normal values of haematological parameters. This finding supports the claims of the manufacturer, whereby it is claimed that DMG does not cause any side effects (Weiss, 1992). Clinically, throughout the entire study, cats that received daily bi-supplementation of DMG also did not exhibit any abnormal clinical signs and behaviours. Indeed, studies have shown that supplementation of DMG was proven to be safe when consumed in large doses and for long-term duration (Kalmar et al., 2012). For instance, chickens that were supplemented with DMG in the form of dimethylglycine sodium salt (Na-DMG) at a dosage of 10 g/kg for 39 days did not cause significant alteration to the liver enzyme activities such as aspartate aminotransferase activity or impaired their broiler performance parameters (Kalmar et al., 2012).

Although no side effects were observed, post-mortem examination, and a toxicity study could only rule out any toxicity effects. As for the PLT values, both groups showed lower PLT values compared to the reference range on both day 0 and day 15. One possible explanation for this is due to a 12–24 24-hour interval between sample collection and processing, of which a significant decrease in apparent platelet count could occur if sample processing were delayed (Hardy et al., 2020). However, the treatment group had a significantly lower neutrophil percentage and significantly higher lymphocyte percentage compared to the control group. There are a few possible explanations for the lower neutrophil percentage of the treatment group. First, the decrease in neutrophils in the treatment group may signify DMG's ability to reduce the ongoing inflammatory process in the cats. It is common knowledge that increased neutrophil count may signify an ongoing inflammatory process. In the context of our study, immunisation or vaccination could cause the recruitment and mobilisation of neutrophils from blood vessels to the site of the vaccination leading to an inflammatory process (Wang et al., 2022).

However, the reduced neutrophil count was not observed in the control group that also received vaccination. When studied in models of human keratinocytes, mimicking inflammatory diseases such as contact dermatitis, dimethylglycine exerts robust anti-inflammatory as well as antioxidant properties (Lendvai et al., 2023). Therefore, the reduction in neutrophils in the treatment group may also signify the anti-inflammatory properties of bromelain, which is present in DMG supplements at a concentration of 2 mg/mL. Bromelain is an enzyme extracted from pineapples and is considered an alternative to non-steroidal anti-inflammatory drugs (Rathnavelu et al., 2016). Bromelain has been shown to have anti-inflammatory properties in various *in vivo* and *in vitro* studies (Pavan et al., 2012). Although the vaccine would cause an increase in lymphoproliferative effects due to T and B cell activations (Vojtek et al., 2021), the control group that received vaccination did not show a significant increase in their lymphocyte percentage compared to the treatment group.

The significant increase in the treatment group can be attributed to the synergistic effects of both the vaccine and DMG (Graber et al., 1981; Vojtek et al., 2021). Interestingly, in contrast, in the cat study of Weiss (1992), the supplementation of DMG did not result in a lymphoproliferative effect towards T cell mitogens. However, the lymphoproliferative effect was not tested in our study. In several studies done in the past, DMG has been shown to have lymphoproliferative properties when studied in *in vitro* and *in vivo* (Graber et al., 1981; Reap & Lawson, 1990). One of the proposed mechanisms is inhibiting T-suppressor cells and enhancing the T-cell presentation of the OKT4 antigen (Real & Lawson, 1990). However, in this study, DMG appears to have no significant impact on the percentages of CD4+ and CD8+ T cells.

Moreover, there was a significant reduction in CD4+ after the DMG supplementation, which could also be due to the effects of bromelain in the DMG supplementation (Secor et al., 2009). Treatment of lymphocytes with bromelain *in vitro* caused a reduction in the activation of CD4+ T cells and lowered the expression of CD25 in a mouse study. We speculate that the lymphocyte increase upon DMG supplementation might cause an increase in other lymphocyte subsets, such as B cells and natural killer cells. However, these subsets were not measured in the current study. Hence, based on our results, we can extrapolate that DMG supplementation does not significantly affect the percentages of CD4+ and CD8+ T cells. In addition, the study only evaluated the effects of DMG supplementation for 14 days. It is possible that long-term supplementation could produce a pronounced effect on the immune system parameters, which was seen in the human study where subjects were supplemented with DMG for 10 weeks (Graber et al., 1981). As for the long-term supplementation duration, we suggest extending the study to 40 days or longer, similar to the previous DMG study done in cats and broiler chickens (Weiss, 1992; Kalmar et al., 2012).

CONCLUSION

Cats supplemented with DMG displayed lower neutrophils and higher lymphocytes compared to the control group, suggesting potential anti-inflammatory and lymphoproliferative effects. However, DMG did not significantly influence the percentages of CD4+ and CD8+ T cells. Therefore, a comprehensive and longer longitudinal study is warranted to look at the effect of DMG in B cell and NK cell activations and long-term supplementation in cats to fully understand DMG's immunomodulatory properties.

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