poultry

MDPI

Communication

Vitamin Compatibility with the Marek's Disease Vaccine †

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Abstract: *In ovo* injection of the Marek's disease (MD) vaccine (MDV) has been widely practiced in commercial US hatcheries. However, the MDV is very sensitive and may not be compatible with some nutrients when administered together by *in ovo* injection. When individually administered by *in ovo* injection, L-Ascorbic acid (L-AA) and 25-hydroxyvitamin D_3 ($25OHD_3$) have previously exhibited very promising results on the post-hatch physiological and immunological characteristics of broilers subjected to stressful commercial conditions. However, the compatibility of the MDV with these vitamins has not been previously explored. Their compatibility must first be established before their combined administration by *in ovo* injection can be considered. Therefore, the objective in this study was to determine the compatibility of the MDV with various levels of $25OHD_3$ or L-AA. The treatments employed were MDV-alone, MDV in combination with 0.6 (low) or 2.4 (high) µg doses of $25OHD_3$, or MDV in combination with 1.2 (low) or 12 (high) mg doses of L-AA. The live and dead ratio of primary chick embryo fibroblast cells infected by the MD virus (CEF-MDV) in each treatment was determined every 30 min for 2 h. The L-AA at both the low and high doses resulted in a 70% death of CEF-MDV within 1 h, but either dose of the $25OHD_3$ exhibited only an approximate 5% lower CEF-MDV survival as compared to those in the MDV-alone treatment. Therefore, it is suggested that the two designated doses of $25OHD_3$ have the potential to be effectively combined with the MDV for subsequent administration by *in ovo* injection.

Keywords: 25-hydroxycholecalciferol; compatibility; L-AA; *in ovo* injection; Marek's disease vaccine

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1. Introduction

Currently, vaccination is the common method in the modern commercial poultry industry to control infectious diseases, including infectious bursal disease (IBD), infectious bronchitis (IB), Marek's disease (MD), Newcastle disease (ND), and infectious laryngotracheitis (ILT) [1]. Effective immunization involving optimum levels of humoral and cellular immune responses is realized between 5 days and 2 weeks after vaccination [2]. Furthermore, this process can be accelerated by *in ovo* vaccination [3]. *In ovo* vaccination has emerged to promote early immunity [3,4]. Currently, *in ovo* vaccination has been developed to protect chickens against MD, ND, IBD, HPAI, and coccidiosis. In addition, the current use of *in ovo* vaccination has been largely limited to broiler hatching eggs. However, among the aforementioned *in ovo* vaccines, the MD vaccine (MDV) that is most commonly used in US hatcheries contains HVT alone, as the backbone virus in association with the MD virus, or as a combinatorial recombinant vaccine such as HVT-IBD, HVT-ND, and HVT-ILT [5]. It has been reported that *in ovo* injection of the MDV at 18 days of incubation (**doi**) results in an approximate 90% level of immune protection [4], an improvement in

intestinal histomorphology [6], and an increase in the expression of genes associated with humoral immunity [7] in early post-hatch broilers.

The *in ovo* administration of various nutrients [3] has been shown to promote subsequent growth in broiler embryos [8] and post-hatch chicks [9]. The second metabolite of vitamin D, 25-hydroxyvitamin D_3 ($25OHD_3$), is involved in many physiological and metabolic pathways such as bone development [10], embryonic growth [11], muscle development [12], and innate and adaptive immunity [13]. In previous research, it has been shown that the *in ovo* administration of various levels of $25OHD_3$ at 18 doi as compared to commercial diluent resulted in an increase in the bone quality [14] and breast meat yield [15] and an enhancement in the small intestine morphology [16], immunity [16], and live performance [15] of broilers. Furthermore, L-Ascorbic acid (L-AA) is well known as an antioxidant and immunocompetence agent. Also, as compared to a non-injected control treatment, the combination of 0.4 µg of $25OHD_3$ with 6 µg of vitamin K_3 has been shown to increase the humoral immunity, weight gain, and feed intake of 42-day-old broilers [17]. The *in ovo* administration of different doses of L-AA at 17 doi has resulted in an improvement in the antioxidant capacity [18], inflammatory response [19,20], and post-hatch live performance [17,21] of broilers. The *in ovo* injection of 0.6 µg of $25OHD_3$ combined with commercial diluent resulted in an increase in the hatchability of live embryonated embryos [22] and the subsequent bone quality of post-hatch broilers [14]. Furthermore, immunity [16,23,24], breast meat yield [25], and live performance [15,25] were improved in Ross 708 broilers in response to 2.4 µg of $25OHD_3$ as compared to non-injected or diluent control groups. However, in comparison to non-injected or diluent-injected control groups, there were no beneficial effects on hatch and post-hatch variables when lower than 0.3 µg or higher than 2.4 µg of $25OHD_3$ was *in ovo* injected [24,25]. As compared to saline or non-injected control treatments, L-AA at 12 mg resulted in an increase in the hatchability [26], body weight gain [20], and antioxidant capacity [16] of Ross 708 broilers, while there were no positive effects on their hatching process or post-hatch live performance when administered below 1.2 mg [17,26]. Moreover, as compared to a non-injected treatment, the amniotic *in ovo* administration of 6 mg of L-AA at 15 doi has been shown to improve the intestinal histomorphology and bone quality of 7-day-old broilers that were fasted for 36 h post hatch [27]. In addition, as compared to a sham treatment group, the *in ovo* injection of 3 mg of L-AA in the amino at 15 doi resulted in an increase in the expression of genes linked to heat stress control in 16 and 18 doi broilers [28]. Furthermore, amniotic *in ovo* feeding of 3 mg of L-AA at 15 doi has been shown to increase enzymatic antioxidant activity at 42 days of post hatch age, and result in an increase in humoral immunity at 1, 21, and 42 days of post hatch age [29].

Thus, it may be beneficial to combine MDV with multifunctional nutrients for efficient simultaneous delivery of each to benefit from their individual effects and possibly promote MDV efficacy. However, an evaluation of the effects of various nutrients that are candidates for *in ovo* administration on the functional properties of the MDV, including the viability of the chick embryo fibroblasts that constitute it, has not been previously reported. It has been previously demonstrated that the MD virus has the capability of transforming primary chick embryo fibroblasts [30]. Therefore, the objective of this study is to explore potential doses of $25OHD_3$ and L-AA that might be compatible with the commercial MDV by exhibiting negligible effects on the viability of primary chick embryo fibroblast cells infected by the MD virus (CEF-MDV).

2. Materials and Methods

2.1. Injection Solution Precreation

A water-soluble form of $25OHD_3$ (ROVIMIX® Hy-D® 1.25%; DSM Nutritional Products Inc., Parsippany, NJ, USA) was used. The $25OHD_3$ solution concentrations (0.6 (low) or 2.4 (high) µg/mL) that were used were prepared according to the procedures of Fatemi et al. [31,32]. A powder form of L-AA suitable for cell culture (Sigma-Aldrich Inc., St. Louis, MO, USA) was used. The solution concentrations of L-AA (1.2 (low) and

12 (high) mg/mL) were prepared according to the method described by Zhang et al. [17]. The $25OHD_3$ and L-AA solutions were prepared in commercial MDV diluent (Merial Co., Athens, GA, USA). Finally, according to the procedure described by Williams [4] and Gimeno et al. [7], a full dose of the CEF-MDV, possessing HVT serotype 3 and the FC-126 strain of the MD virus which is commercially available from licensed serial release (Merial, Inc. Duluth, GA, USA), was added to each *in ovo* injection solution that contained one of the treatment doses of $25OHD_3$ or L-AA. Approximately 25,000 chick embryo fibroblast cells from a standard lineage were delivered in each dose of the CEF-MDV (Merial, Inc. Duluth, GA, USA). The pH of each treatment solution was also measured using an Orion Star™ A211 analyzer (Thermo Fisher Scientific, Waltham, MA, USA) before compatibility analysis was performed.

2.2. MDV Integrity (Live/Dead CEF-MDV Ratio)

After thorough mixing, five 1 mL volumes of each solution were transferred to a cell culture plate designated for that particular treatment. Solution samples were kept at 4 °C for MDV integrity determinations every 30 min over a 2 h period (5 total determinations; 0, 30, 60, 90, and 120 min). At each 30 min time interval, 5 random 50 ul samples were taken using a single needle and syringe from each of the 5 individual solutions (1 mL total volume) belonging to a common cell culture plate designated for that particular treatment. Subsequently, each individual 1 mL sample taken from the culture plates was directly placed into a 5 mL cell culture tube designated for that specific treatment. Therefore, based on the original concentrations of the $25OHD_3$ and L-AA used in the 1 mL sample in each cell culture tube, the amounts of $25OHD_3$ tested in the low and high dose treatments were 0.6 and 2.4 ug, respectively, and the amounts of L-AA tested in the low and high dose treatments were 1.2 and 12 mg, respectively. The respective abbreviated treatment designations for the diluent and low and high doses of $25OHD_3$ and L-AA were: Diluent, $25OHD_3$-0.6, $25OHD_3$-2.4, L-AA-1.2, and L-AA-12. At each of the 5 time intervals, a 100 uL volume of each sample was mixed with 10 uL of Trypan blue dye (Sigma-Aldrich Inc., St. Louis, MO, USA) in a small capped conical serum vial for 5 min. A 20 uL volume of dyed sample was inserted, using controlled capillary action, under a cover slip mounted on a glass side. A live/dead CEF-MDV ratio was determined by the use of a Cellometer AutoT-4 device (Nexcelom Bioscience, Lawrence, MA, USA), and the CEF-MDV survival percentage in each solution was calculated. Because ruptured or dead CEF-MDV exhibited an increased absorption of dye, differentiation between live and dead CEF-MDV concentrations was detected. Only 20–25% of chick embryo fibroblasts are infected with HVT in the MDV. However, the viability of both infected and non-infected chick embryo fibroblasts in the MDV was detected. The data for this study were generated by a Cellometer AutoT-4 device, and the values indicated the percentages of live cells. The device did not provide replication at each individual time point to allow for statistical analysis. Statistical analysis could only be performed if the data were pooled across time. Because the timing of the responses to treatment was important, the authors elected to plot the progression of cell viability with time so that the results could be reported more meaningfully and with more relevance and accuracy. Thus, the extracted information for a Cellometer AutoT-4 device was plotted to illustrate the percentages of live cells without conducting a statistical comparison between treatments, which allowed us to numerically compare the treatments.

3. Results

The pH values of the treatment solutions were: Diluent = 6.91; $25OHD_3$-0.6 = 6.91; $25OHD_3$-2.4 = 6.90; L-AA-1.2 = 4.33; and L-AA-12 = 2.55. The compatibility results of the MDV with the two doses of $25OHD_3$ and L-AA are illustrated in Figure 1. The low or high levels of L-AA in combination with MDV resulted in a 65 to 80% respective death of CEF-MDV after 30 min, which remained low during the total 2 h period. However, both levels of $25OHD_3$ over the 2 h period had similar effects to that of MDV-alone, in which the

percentage of viable CEF-MDV in the MDV-alone, MDV+ 25OHD$_3$-0.6, and 25OHD$_3$-2.4 treatments were 88, 86, and 84%, respectively.

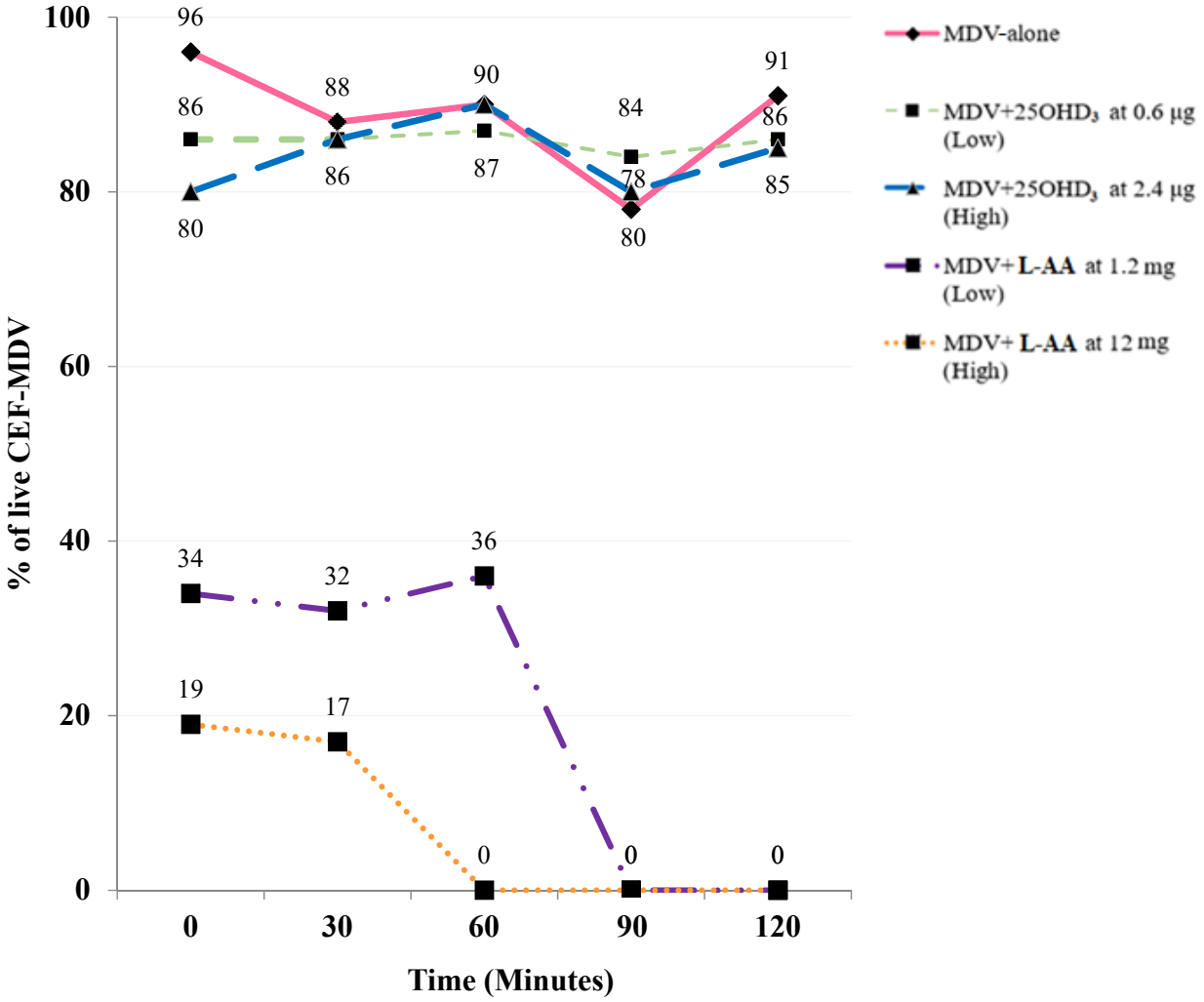

Figure 1. Percentage of live primary chick embryo fibroblast cells infected by the Marek's disease virus (**CEF-MDV**) every 30 min (0, 30, 60, 90, and 120 min) when the MD vaccine (**MDV**) was combined with low (1.2 mg) and high (12 mg) doses of L-ascorbic acid (**L-AA**) or low (0.6 µg) and high (2.4 µg) doses of 25-hydroxyvitamin D_3 (**25OHD$_3$**) during a 2 h period.

4. Discussion

To date, the viability of CEF-MDV upon its exposure to various vitamins has not been evaluated. Without statistical comparison, the plots of the progression of cell viability with time for each treatment clearly indicated that at the levels of inclusion used, L-AA had a detrimental effect on the viability of CEF-MDV. However, low and high levels of the vitamin D source (25OHD$_3$) did not show significant negative effects on CEF-MDV viability, indicating that 25OHD$_3$ at the doses tested may be suitable for *in ovo* application in combination with MDV. The negative impact of L-AA may be linked to its acidic characteristics and the high level of sensitivity of the MDV to drastic changes in pH. Therefore, despite the buffering capacity of the MDV diluent, a lowering of the pH of the MDV solution may have decreased CEF-MDV viability. In addition, the lowest dose of L-AA used in the current study was 1.2 mg, which is 500 times more than the highest dose of 25OHD$_3$ (2.4 µg) that was tested. The hatch and post-hatch performance results of broilers have not been promising when they were administered concentrations of L-AA lower than 1.2 mg/mL by

in ovo injection [18]. This was the basis for choosing a 1.2 mg/mL concentration of L-AA as the low-level dose in this study.

Vitamin D metabolites, including vitamin D_3 and $25OHD_3$, are normally required to be suspended in ethanol to be soluble for *in ovo* administration [14]. However, in this study, the $25OHD_3$ source was water soluble and did not require alcohol suspension. There are several solutions that have been used to suspend various nutrients for *in ovo* injection purposes. These have included distilled water [33], saline [18–21,26], ethanol [17,34], soybean oil [35], and glycerol [36]. Nevertheless, commercial diluent has been used in more recent studies involving the *in ovo* injection of $25OHD_3$ [15,16,23–25,32]. Furthermore, CEF-MDV can only survive in commercial diluent, and the diluent is easily accessible. In the current study, both vitamin solution concentrations were prepared in a commercial diluent to which the full dose of MDV was added. Thus, commercial diluent should be used in further studies investigating the combinatorial administration of MDV and vitamins.

5. Conclusions

In conclusion, different doses of two bioactive vitamins (L-AA and $25OHD_3$) were separately combined with MDV in commercial diluent in order to determine the compatibility of MDV with the aforementioned vitamins. The compatibility results reported in this study using plots of cell viability demonstrated that both low and high doses of L-AA had negative effects on CEF-MDV survival. However, low and high doses of $25OHD_3$ exhibited minimal detrimental effects on CEF-MDV survival, indicating that $25OHD_3$ is a potential candidate for *in ovo* administration at 18 doi in combination with the MDV.

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