Effects of vitamin A on antioxidant functions, immune functions and production performance in male sika deer (Cervus nippon) during the first antler growth period

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ABSTRACT
The present study examined the effects of dietary vitamin A (VA) on antioxidant functions, immune functions and production performance in farmed sika deer. Forty healthy male sika deer (initial body weight (BW): 47.07 ± 4.75 kg; 8 months of age) were randomly assigned to four treatments on the basis of BW. The dietary treatments included a basal diet (containing 330 U/kg VA) supplemented with 0 (control), 2500, 5000 or 10,000 U/kg retinol acetate (500,000 U/g, Rovimix A500, Roche, Basel, Switzerland). The results showed that deer fed a diet supplemented with 5000 U/kg VA had higher ($p < 0.05$) average daily gains and gain:feed values than those from the control group. VA supplementation significantly increased ($p < 0.05$) glutathione peroxidase and superoxide dismutase activities and total antioxidant capacity and decreased ($p < 0.05$) the concentrations of reactive oxygen species in the serum. Additionally, serum immunoglobulin A, interleukin-2 and soluble CD8 were significantly increased ($p < 0.05$) when dietary VA supplementation was increased from 0 to 5000 U/kg. However, a high dose of VA supplementation (10,000 U/kg) caused decreased ($p < 0.05$) concentrations of serum tumour necrosis factor-$\alpha$ and interleukin-1. Deer that received feed supplemented with 5000 U/kg VA had higher ($p < 0.05$) dry antler yield than the control deer. The present results indicated that VA supplementation improved growth performance, antioxidant functions, immune functions and dry antler yield. Taken together, the suitable level of VA supplementation was found to be 5000 U/kg (total VA content 5330 mg/kg dry matter) for male sika deer during the first antler growth period.

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Introduction
Vitamin A (VA), a fat-soluble vitamin, has been shown to be essential for many physiological processes, such as cell metabolism, reproduction, embryonic development, immunity and bone metabolism, in all vertebrates (Mora et al. 2008; Gutierrez-Mazariegos et al. 2011; Lind et al. 2011). As most mammals cannot synthesise VA \textit{de novo}, VA must be provided by the diet as retinol or provitamin A. Previous studies have shown that VA supplementation in the diet increased the body weight (BW) gain of lambs (Eldaim et al. 2015), antioxidant ability of beef cattle (Kleczkowski et al. 2004) and immune functions of dairy cows (Lu et al. 2014). The beneficial response to VA in ruminants has been recognised; however, the VA requirements for sika deer are poorly understood.

Sika deer are a source of animal medicine of great commercial value (e.g. antler production). The first antler growth begins when a deer approaches puberty (Gómez et al. 2006) and is characterised by pedicle initiation, followed by antler development, velvet cleaning and antler casting (Lincoln 1992). VA and its derivatives, retinoic acids (RAs), were shown to be a potential endogenous morphogen during antler growth. Kierdorf and Kierdorf (1998) reported that injection of all-trans RA into the incipient pedicle caused alterations in pedicle and first antler shape in a fallow buck. Allen et al. (2002) showed that RA receptors are expressed in antler tissues and that \textit{in vitro} activation of these receptors regulates the differentiation of antler chondrocytes and osteoclasts. In addition, VA has been reported to be easily degraded in the rumen (Weiss et al. 1995). We hypothesised that VA
supplementation may promote the growth of deer with better production performance and with increased health status.

Therefore, this study was designed to examine the effects of dietary VA supplementation on antioxidant functions, immune functions and production performance in farmed male sika deer during the first antler growth period.

Materials and methods

The experimental protocol used in this study was approved by the Wild Animal and Plant Subcommittee of the China Association of Agriculture Science Societies (WAPS CAASS). All experiments were performed in accordance with animal health and well-being regulation.

Experimental design, animals and diets

Forty healthy 8-month-old male sika deer averaging 47.07 ± 4.75 kg of BW were placed into four pens (15 m × 30 m), and animals were fed in groups of 10 for the 90 days of the experiment. The only independent factor in this experiment was the different levels of VA supplemented in the deer’s diets. VA was added to the basal diet (containing 330 U/kg VA) as retinol acetate (500,000 U/g, Rovimix A500, Roche, Basel, Switzerland) at 0, 2500, 5000 or 10,000 U/kg of dry matter (DM). The basal diet mainly consisted of corn, dry alfalfa grass, soybean meal and corn germ meal. The ingredient and nutrient composition of the basal diet are presented in Table 1. The diet was fed to the deer twice a day at 06:00 and 16:00 h, as total mixed rations with allowances made for 5% refusals, and the animals had free access to water. Orts were recorded and discarded before the next feeding each day. Individual deer BWs were recorded on days 0 and 90. Feed consumption was recorded daily on a group basis, and the average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) were calculated.

Sample collection

On the final day, eight deer in each pen were anaesthetised with xylazine hydrochloride (Qing dao Hanhe Animal and Plant Medicine Co., Qingdao, China), which was administered by a blow-gun dart syringe at a dose of 0.5–3.0 mg/kg of BW in the morning (before watering and feeding). Samples of blood (5 mL) were taken from the jugular vein in disposable vacutainer tubes without anticoagulant. The blood was centrifuged for 5 min at 4500×g and 4 °C to obtain serum, and the serum was frozen at −20 °C for further analysis. Velvet antlers were removed at the same time according to the procedures described by Bao et al. (2017). For safety reasons, the first antlers were sawn off 2 cm above the pedicle. Fresh antler weight and length were measured by the same person after the blood was cleaned up with gauze. Antlers were stored at −80 °C and then processed by a vacuum freeze dryer. The DM content of antlers was calculated by the formula listed below: DM content (%) = (dry antler yield/fresh antler yield) × 100. The appearance of the pedicle was determined by the time of first relieved expression after palpation of the frontal bone (Gaspar-López et al. 2008).

Chemical analysis

The experimental diets were analysed in triplicate for DM (method 930.15; AOAC 2005), crude protein (CP, method 984.13; AOAC 2005), ash (method 942.05; AOAC 2005) and calcium and phosphorus (method 999.10; AOAC 2005). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed according to the methods described by Van Soest et al. (1991) with an Fiber Analyser (A2000I, Ankom Co., Macedon, NY, USA) and were expressed inclusive of residual ash.

The activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) and

| Table 1. Ingredients and chemical composition of the basal diet. |
|---------------------------------------------------------------|
| **Item** | **Content** |
|---------------------------------------------------------------|
| Corn flour | 20.00 |
| Soybean meal | 10.00 |
| Corn germ | 8.50 |
| Distiller dried grain soluble | 8.00 |
| Alfalfa meal | 52.00 |
| Salt | 0.50 |
| Premixa | 1.00 |
| Total | 100.00 |

**Measured nutrient concentration**

| Dry matter, % | 92.86 |
| Organic matter, % | 82.50 |
| Crude protein, % | 15.23 |
| Ether extract, % | 1.44 |
| Neutral detergent fibre, % | 46.84 |
| Acid detergent fibre, % | 25.05 |
| Calcium, % | 1.26 |
| Phosphorus, % | 1.14 |
| β-Carotene, mg/100 g | 0.50 |
| Vitamin A, µg/kg | 330.00 |

*Contained the following per kg of premix: Mg, 76 mg; Cu, 36 mg; Mn, 43 mg; Fe, 53 mg; Zn, 45 mg; Se, 31 mg; vitamin D₃, 496.8 U; vitamin E, 0.828 U; vitamin K₃, 0.23 mg; vitamin B₁, 10.092 mg; vitamin B₂, 0.69 mg; vitamin B₁₂, 1.38 mg; folic acid, 0.023 mg; nicotinic acid, 1.62 mg; calcium pantothenate, 1.15 mg; CaHPO₄, 5.17 g; CaCO₃, 4.57 g.*
total antioxidant capacity (T-AOC) in the serum were measured using a commercial colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), soluble CD4 (sCD4) and soluble CD8 (sCD8) were determined using commercially available deer ELISA kits (Shuangying Biological, Shanghai, China) according to the manufacturer’s protocol. The process was performed according to the ELISA kit’s protocol. The absorbance (OD value) was measured at 450 nm wavelength with an FLx800 Fluorescence Reader (BioTek, Winooski, VT, USA), and the concentration of ROS in the sample was calculated by standard curve.

Statistical analysis

Data were analysed using the GLM procedure of SAS (SAS Institute Inc. 2008). Each deer was independently used for all experiments and considered as the experimental unit (n = 10). Results for growth performance, antioxidant function, immune function and antler growth was conducted with a n = 8 per treatment group. Determination of significant statistical differences among the mean values of the four treatment

Table 2. Effects of VA supplementation on growth performance of sika deer.

| Item                  | Supplemental levels of VA, U/kg of diet | SEM | p value |
|-----------------------|------------------------------------------|-----|---------|
|                       | 0            | 2500 | 5000  | 10,000 |       |
| Initial BW, kg        | 48.07        | 46.09| 47.28 | 46.93  | 0.94  |
| Final BW, kg          | 57.04        | 56.51| 59.83 | 57.81  | 1.04  |
| ADG, g/day            | 99.62b       | 115.77a| 139.45a| 120.88ab| 6.29  |
| ADFI, kg/day          | 2.12         | 2.12 | 2.13  | 2.10   | 0.01  |
| G:F, g/g              | 0.04b        | 0.05ab| 0.06a | 0.05ab | 0.00  |

BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; G:F: gain to feed ratio; VA: vitamin A; SEM: Standard error of the mean.
Means with different lowercase of superscripts were significantly different at p < .05; n = 8 per treatment.

Table 3. Effects of VA supplementation on antioxidant functions of sika deer.

| Item                  | Supplemental levels of VA, U/kg of diet | SEM[4] | p value |
|-----------------------|------------------------------------------|--------|---------|
|                       | 0            | 2500 | 5000  | 10,000 |       |
| SOD, U/mL             | 48.17b       | 78.20*| 81.95*| 66.09ab| 5.91  |
| MDA, nmol/mL          | 1.12         | 1.03 | 1.04  | 1.24   | 0.01  |
| GPx, U/L              | 425.12b      | 678.25*| 679.75*| 630.75*| 38.89 |
| T-AOC, U/mL           | 4.16b        | 5.23ab| 5.90* | 6.86*  | 0.58  |
| CAT, U/mL             | 2.76         | 2.56 | 3.39  | 3.20   | 0.37  |
| ROS, fluorescence intensity/mL | 435.76ab | 412.09*| 356.62ab| 369.50*| 34.66 |

SOD: superoxide dismutase; MDA: malondialdehyde; GPx: glutathione peroxidase; T-AOC: total antioxidant capacity; CAT: catalase; ROS: reactive oxygen species; VA: vitamin A; SEM: Standard error of the mean.
Means with different lowercase of superscripts were significantly different at p < .05; n = 8 per treatment.

Results

Growth performance

The effects of VA supplementation on growth performance are shown in Table 2. The ADFI and final BW did not significantly differ among the four treatment groups (p > .05). However, deer fed diets supplemented with 5000 U/kg VA had higher (p < .05) ADG and G:F than those of the control deer. There was no significant difference in growth performance among the VA supplementation groups (p > .05).

Antioxidant functions

Deer that received a diet supplemented with 5000 U/kg VA had higher (p < .05) serum GPx, SOD and T-AOC activities than those of the control deer but had activity levels similar to those of deer in the other two groups (Table 3). VA supplementations of 5000–10,000 U/kg decreased (p < .05) the concentration of serum ROS. There was no significant difference in serum MDA or CAT among all the groups (p > .05).

Immune functions

The effects of VA supplementation on immune functions are shown in Table 4. Serum IgA, IL-2 and sCD8 were significantly increased (p < .05) when the dietary VA supplementation increased from 0 to 5000 U/kg. However, 10,000 U/kg VA significantly decreased (p < .05) the contents of IL-1 and TNF-α in the serum. Serum IgG, IgM, IL-4, IL-6 and sCD4 were not affected by the levels of VA supplementation (p > .05).

First antler growth

Deer that received feed supplemented with 5000 U/kg VA had higher (p < .05) dry antler yield compared with
the control deer, but deer in this treatment had similar dry antler yields to those of the other two groups (Table 5). There was no significant difference in fresh antler weight, antler length pedicle emergence among all the groups \((p > .05)\).

**Discussion**

Proper dietary concentrations of microminerals are essential for animal maintenance and productivity, but the requirements for most wild species, including deer, are poorly understood. Since VA has a role in regulating growth hormone (GH) gene expression (Bedo et al. 1989) and energy homeostasis (Kumar et al. 1999), it has positive effects on growth promotion. Data from the current study showed that deer that received a diet supplemented with 5000 U/kg VA had higher ADGs and G:Fs compared with the control animals. Analogous results of VA having significant growth-promoting benefits were found in Holstein steer calves (Salinas-Chavira et al. 2014) and sheep (Soliman 2015). It has been proposed that VA has an important role in promoting the differentiation of pituitary cells toward GH-secreting cells and in the stimulation of GH secretion (Bedo et al. 1989). The improvement in growth performance was likely associated with increased GH secretion, which consequently stimulates the longitudinal growth (Mauras 2003). However, Alessia Sagazio et al. (2007) reported that VA deficiency does not influence longitudinal growth in mice. Further studies are required to confirm this in sika deer. Furthermore, VA enhances absorption and utilisation of nutrients. A VA-deficient diet has been shown to harm the small intestinal epithelium of lambs, while VA supplementation minimises these harmful effects (Holland et al. 1993). Nevertheless, a significant difference in ADG was not observed among the VA supplementation groups in the current study. Analogous results were reported by Gibb et al. (2011), who observed that VA supplementation levels had no effect on growth performance in feedlot heifers. In general, body size is highly correlated with antler size and reproductive success in stags (Kelley et al. 2000). Our findings indicated that VA supplementation may have the potential to improve the production performance and economic benefits of sika deer.

Analysing serum biochemistry is a reliable means for evaluating animals’ health and nutritional status (Gupta et al. 2007). SOD, GPx and MDA are enzymes that play important antioxidative functions by preventing the formation of free radicals, scavenging them, or promoting their decomposition (Young and Woodside 2001). VA has been considered to enhance the antioxidant defence system against oxidative stress. Ma et al. (2005) have shown that supplementation with 3300–4400 U/kg VA in beef cattle diets could significantly increase the activities of serum GPx and T-AOC. In goats, dietary supplementation of VA at a level 2000–3000 U/kg DM has been shown to increase serum TAC and GPx activities (Yang et al. 2010). In this study, a similar phenomenon occurred at 5000–10,000 U/kg VA levels. Compared to the control, the activities of SOD were significantly increased when deer were fed diets supplemented with 2500–5000 U/kg VA. This result is in agreement with a previous study by Zhao et al. (2008), who reported that supplementation with VA (250,000 U/day) slightly increased the activities of serum SOD in dairy cows. The

### Table 4. Effects of VA supplementation on immune functions of sika deer.

| Item | Supplemental levels of VA, U/kg of diet | SEM | \(p\) value |
|------|----------------------------------------|-----|------------|
| IgA, \(\mu g/mL\) | 3.43<sup>b</sup> 4.30<sup>c</sup> 4.34<sup>c</sup> 3.05<sup>b</sup> | 0.26 | .329 |
| IgG, \(\mu g/mL\) | 35.66 31.77 31.89 26.45 | 2.14 | .039 |
| IgM, \(\mu g/mL\) | 1.68 1.20 1.34 1.79 | 0.11 | .036 |
| IL-1, \(ng/L\) | 647.66<sup>b</sup> 637.65<sup>b</sup> 578.56<sup>a</sup> 514.81<sup>c</sup> | 42.76 | .041 |
| IL-2, \(ng/L\) | 206.52<sup>b</sup> 255.06<sup>ab</sup> 283.55<sup>a</sup> 251.77<sup>ab</sup> | 17.05 | .036 |
| IL-4, \(ng/L\) | 198.43 194.64 208.02 166.52 | 10.28 | .025 |
| IL-6, \(ng/L\) | 10.76 10.43 10.69 12.37 | 0.96 | .026 |
| sCD4, \(U/L\) | 33.67<sup>b</sup> 44.57<sup>a</sup> 44.09<sup>a</sup> 36.53<sup>b</sup> | 2.57 | .038 |
| sCD8, \(U/L\) | 33.67<sup>b</sup> 44.57<sup>a</sup> 44.09<sup>a</sup> 36.53<sup>b</sup> | 2.57 | .038 |
| TNF-\(\alpha\), \(ng/L\) | 91.56<sup>a</sup> 99.91<sup>a</sup> 110.02<sup>a</sup> 79.68<sup>b</sup> | 6.14 | .025 |

IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; IL-1: interleukin-1; IL-2: interleukin-2; IL-4: interleukin-4; IL-6: interleukin-6; sCD4: soluble CD4; sCD8: soluble CD8; TNF-\(\alpha\): tumour necrosis factor-\(\alpha\); SEM: Standard error of the mean. Means with different lowercase of superscripts were significantly different at \(p < .05\); \(n = 8\) per treatment.

### Table 5. Effects of VA supplementation on first antler growth of sika deer.

| Item | Supplemental levels of VA, U/kg of diet | SEM | \(p\) value |
|------|----------------------------------------|-----|------------|
| Fresh antler yield, g | 118.52 128.64 133.55 124.01 | 13.29 | .087 |
| Dry antler yield, g | 40.80<sup>b</sup> 43.12<sup>ab</sup> 47.50<sup>a</sup> 43.18<sup>ab</sup> | 4.17 | .044 |
| Dry matter content, % | 34.49 33.75 35.62 34.76 | 1.50 | .120 |
| Left branch length, cm | 11.82 12.91 14.58 13.13 | 2.43 | .621 |
| Right branch length, cm | 12.15 13.23 14.20 12.80 | 2.05 | .571 |

VA: vitamin A; SEM: Standard error of the mean. Means with different lowercase of superscripts were significantly different at \(p < .05\); \(n = 8\) per treatment.
increasing serum SOD, GSH-Px and T-AOC activities are likely due to the powerful free radical scavenging and lipo-peroxyl radical-quenching function of VA (Kontek et al. 2014). Under physiological conditions, a homeostatic balance exists between the formation of ROS and their removal by endogenous antioxidant scavenging compounds (Gutteridge and Mitchell 1999). Oxidative stress occurs when this balance is disrupted by excessive production of ROS. In the present study, 5000–10,000 U/kg VA supplementation significantly decreased the contents of ROS in the serum, which is consistent with previous results observed in dairy cows (Shi et al. 2016). We speculate that the reduction of ROS is associated with increased GPx. Papp et al. (2007) found that GPx converts ROS to less reactive metabolites and thus protects tissues against oxidative damage. Data from the current study confirmed that VA supplementation may enhance the antioxidant functions of sika deer.

VA has long been considered important for maintaining and stimulating the immune system. VA affected the immune response in both lines of immunity (Mora et al. 2010). In innate immunity, it is important for many cells including neutrophils, macrophages and natural killer cells to function normally (Stephensen 2001). In adaptive immunity, VA plays a role in the development of T and B lymphocytes (Raverdeau and Mills 2014). However, few studies have been performed to understand the effects of VA on the immune response of sika deer. In the current study, changing dietary VA supplementation from 0 to 5000 U/kg resulted in an increase in serum IgA, IL-2 and sCD8. Lu et al. (2014) reported that a higher dose of VA (220 U/kg of BW) supplementation significantly increased the concentrations of IgA, IgG, IgM and sCD4 in the serum of dairy cows, which is in agreement with our findings. Additionally, VA supplementation significantly increased the serum IgG concentrations and lymphocyte percentages in lambs (Soliman 2015). The reasons may be explained as follows: first, VA improved the antioxidant status thereby protecting the immune cells against oxidant stressors (Yang et al. 2010); furthermore, the VA metabolite, RA, directly and indirectly influences the development and effector functions of various immune cell types and potentiate antibody production (Manicassamy and Pulendran 2009). The enhancing effect of VA on immune function could be particularly important for deer held in captivity because the close contact among animals and stress of confinement are likely to increase their exposure and susceptibility to disease. The results of the present study also showed that 10,000 U/kg VA significantly decreased the contents of serum IL-1 and TNF-α. These results indicate that VA supplementation within a certain range may enhance the immune functions of sika deer. The reduction in serum IL-1 and TNF-α observed in the 10,000 U/kg VA group should be interpreted after further investigation.

Antlers are bony appendages developed from outgrowths of the frontal bone of the skull, referred to as pedicles, in most species of the deer family (Li et al. 2009). As VA and its derivatives, play important roles in embryonic skeletal development and in regeneration (Hayes and Morriss-Kay 2001), it is likely that VA and its derivative will also affect antler growth. Indeed, Bubenik (1990) showed that the growth rate and lateral deviation of the antlers increased when all-trans RA was injected into the tip of an early antler bud of a young white-tailed buck. In fallow deer, treatment of the growing pedicle with RA increased the size of the first antlers (Kierdorf and Bartos 1999). In the current study, no significant differences were found in antler length and pedicle emergence; however, deer that received feed supplemented with 5000 U/kg VA had higher dry antler yield compared with the control deer. It has been reported that individuals that develop larger antlers may have a better ability to buffer developmental stress than individuals that develop smaller antlers (Markusson and Folstad 1997), and antler length is functionally related to body mass (Gómez et al. 2006). We therefore predict that in this study, VA supplementation improved the antioxidant functions and immune functions, as well as BW gain, which in turn increased antler yield.

Conclusions

VA supplementation improved growth performance, antioxidant functions, immune functions and dry antler yield. A suitable level of VA supplementation was found to be 5000 U/kg (total VA content 5330 mg/kg DM) for male sika deer during the first antler growth period. However, the supplemental levels of VA that were tested were limited, and further investigation is required to examine the exact mechanism by which VA regulates the immune functions, antioxidant functions and antler growth.

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