Safety evaluation of vitamin A in growing dogs

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Abstract

The safe upper limit for inclusion of vitamin A in complete diets for growing dogs is uncertain, with the result that current recommendations range from 5.24 to 104.80 μmol retinol (5000 to 100 000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME). The aim of the present study was to determine the effect of feeding four concentrations of vitamin A to puppies from weaning until 1 year of age. A total of forty-nine puppies, of two breeds, Labrador Retriever and Miniature Schnauzer, were randomly assigned to one of four treatment groups. Following weaning at 8 weeks of age, puppies were fed a complete food supplemented with retinyl acetate diluted in vegetable oil and fed at 1 ml oil/100 g diet to achieve an intake of 5·24, 13·10, 78·60 and 104·80 μmol retinol (5000, 12 500, 75 000 and 100 000 IU vitamin A)/4184 kJ (1000 kcal) ME. Fasted blood and urine samples were collected at 8, 10, 12, 14, 16, 20, 26, 36 and 52 weeks of age and analysed for markers of vitamin A metabolism and markers of safety including haematological and biochemical variables, bone-specific alkaline phosphatase, cross-linked carboxyterminal telopeptides of type I collagen and dual-energy X-ray absorptiometry. Clinical examinations were conducted every 4 weeks. Data were analysed by means of a mixed model analysis with Bonferroni corrections for multiple endpoints. There was no effect of vitamin A concentration on any of the parameters, with the exception of total serum retinyl esters, and no effect of dose on the number, type and duration of adverse events. We therefore propose that 104·80 μmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) is a suitable safe upper limit for use in the formulation of diets designed for puppy growth.

Key words: Puppies: Dogs: Retinol: Retinyl esters: Vitamin A

Vitamin A is an essential fat-soluble vitamin that has functions supporting vision, bone growth, reproduction, cellular differentiation and immune response in dogs⁴. The dog, unlike non-carnivorous species such as humans or rodents, transports vitamin A in the plasma predominantly in the form of retinyl esters, in both adequate and vitamin A-deprived states⁵. In human subjects, retinyl esters are only detected in the plasma in cases of intoxication or following a vitamin A-rich meal⁶. The concentrations of retinol found in dog serum are unaffected by dietary vitamin A intake, whereas the concentrations of serum retinyl esters have been shown to parallel the concentrations of vitamin A in the diet⁷. In the dog, excess vitamin A is stored in esterified form in lipid droplets contained within the hepatic stellate cells as well as the kidneys⁸. In addition to the unusual mechanism of vitamin A transport, dogs, unlike humans⁹, excrete vitamin A in the urine as both retinol and retinyl esters⁵. As a result of the species differences in metabolism and excretion, data from other species cannot be used to establish a dietary safe upper limit for retinol inclusion in the diets of growing dogs. Some studies in dogs, however, do exist. In growing dogs (10–12 months of age), prolonged activated prothrombin time, although not to levels outwith the

Abbreviations: BMD, bone mineral density; CTx, carboxyterminal telopeptides of type I collagen; DXA, dual-energy X-ray absorptiometry; FEDIAF, Federation Européenne de l’Industrie des Ailments pour Animaux Familiers; LR, Labrador Retriever, ME, metabolisable energy; MS, Miniature Schnauzer.

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reference range, and elevated retinol binding protein concentrations have been reported following feeding a diet containing 131·00 μmol retinol (125 000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME) for a period of 26 weeks potentially resulting from an interaction with vitamin K (11). Exceptionally high dietary intakes (>576·42 μmol retinol (550 000 IU vitamin A)/4184 kJ (1000 kcal) ME) were associated with severe side effects including reduced energy intake, reduced growth rates, pain responses in the carpal and tarsal joints as well as abnormal bone development and premature closure of the epiphyseal plate (8–10). These adverse effects are potentially a result of interactions with vitamin D, as one study reported a reduction in the severity of effects when retinol was co-administered with vitamins D and E (11). Much lower concentrations (10·48 μmol retinol (10 000 IU vitamin A)/kg/body weight) fed to three Cocker Spaniels from weaning to 10 months of age had no apparent adverse effects (12). The lack of published studies involving retinol concentrations above 10·48 μmol retinol (10 000 IU vitamin A)/kg body weight that have not observed adverse effects may explain the disparity in recommendations among key nutrition authorities. The National Research Council (13) recommends a safe upper limit of 13·10 μmol retinol (12 500 IU vitamin A)/4184 kJ (1000 kcal) for growing dogs, the American Association of Feed Control Officials (14) recommends 74·86 μmol retinol (550 000 IU vitamin A)/4184 kJ (1000 kcal) ME) were reported to 10 months of age. The hypothesis tested was that feeding diet concentrations up to 104·80 μmol retinol (12 500 IU vitamin A)/4184 kJ (1000 kcal) to puppies during growth does not result in a statistically significant change in biomarkers associated with health.

Experimental methods

The research protocol was evaluated and approved by the WALTHAM® Internal Ethics Committee. The study was a randomised, blinded, parallel design. A total of forty-nine puppies, from eight litters, born at the WALTHAM Centre for Pet Nutrition were entered into the study. The puppies were of two breeds, Labrador Retrievers (LR) and Miniature Schnauzers (MS). Dogs were housed in litter groups with their mother until 8 weeks of age and subsequently in pairs, in purpose-built, environmentally enriched kennels with free access to outdoor areas. The puppies could interact with other dogs in paddock groups, and all puppies participated daily in supervised group and individual exercise, training and socialisation. The puppies were habituated to all procedures from 4 weeks of age.

Diet and feeding

The base diet was a standard dry commercial recipe (Perfect Fit Junior; Mars GmbH) compliant with FEDIAF 2008 recommendations (15) for growth and reproduction (Table 1).

Table 1. Nutrient composition of the base diet

| Nutrient          | Mean (units/kg) | SD |
|-------------------|-----------------|----|
| PME (kJ)          | 16 640          | 582 |
| Protein (g)       | 302             | 6  |
| Fat (g)           | 169             | 4  |
| Ash (g)           | 69              | 3  |
| N-free extract (g)| 364             | 20 |
| Crude fibre (g)   | 23              | 17 |
| Linoleic acid (g) | 25·0            | 0·4 |
| α-Linolenic acid (g) | 1·7       | 0·1 |
| EPA and DHA (g)   | 0·9             | 0·1 |
| Ca (g)            | 13·1            | 1·1 |
| P (g)             | 9·2             | 0·7 |
| Ca:P              | 1·4             | 0·1 |
| Na (g)            | 5·4             | 0·7 |
| K (g)             | 6·0             | 0·9 |
| Mg (g)            | 0·86            | 0·1 |
| Fe (mg)           | 126             | 14 |
| Cu (mg)           | 12              | 2  |
| Mn (mg)           | 88              | 10 |
| Zn (mg)           | 142             | 34 |
| I (mg)            | 1·3             | 0·3 |
| Se (μg)           | 360             | 130|
| Chloride (g)      | 1·1             | 0·1 |
| Retinol (μg)      | 5870            | 1047|
| Cholecalciferol (μg) | 39·5       | 7·6 |
| α-Tocopherol (mg) | 529             | 204|
| Thiamin (mg)      | 2·5             | 0·6 |
| Riboflavin (mg)   | 20·7            | 5·4 |
| Niacin (mg)       | 139·4           | 44·4|
| Pyridoxine (mg)   | 10·0            | 0·8 |
| Pantothentic acid (mg) | 61     | 25 |
| Folic acid (mg)   | 1·12            | 0·8 |
| Cobalamin (mg)    | 0·13            | 0·1 |
| Choline (mg)      | 1222            | 689|
| Arg (g)           | 17·6            | 0·2 |
| His (g)           | 5·7             | 0·5 |
| Ile (g)           | 10·3            | 0·1 |
| Leu (g)           | 24·1            | 2·5 |
| Lys (g)           | 12·3            | 0·94|<|  Met (g) | 5·6 | 0·3 |
| Met + cystine (g) | 9·5             | 0·4 |
| Phe (g)           | 9·5             | 0·8 |
| Phe + Tyr (g)     | 2·17            | 2·7 |
| Thr (g)           | 9·1             | 0·4 |
| Val (g)           | 11·0            | 0·9 |

PME, predicted metabolisable energy.

* PME calculated according to the National Research Council (13).

For each group, ten batches of the diet were used throughout the study. The predicted ME content of the diet was calculated according to the National Research Council (13).

The maternal bitch was fed the base diet throughout lactation until the puppies were fully weaned to the base diet at 6 weeks of age. For 7 d following weaning (week 6–7), the puppies were group fed the base diet three times daily. For the following 7 d (week 7–8), the puppies were individually fed the base diet for three, 1 h periods daily. Feeding amounts were calculated from amounts consumed during the previous week and adjusted weekly for the remainder of the study with the aim of maintaining puppies on standard growth curves with ideal body condition scores (16, 17). At 8 weeks of age, puppies were randomised within litter, to one of four dietary concentrations of vitamin A, namely: group A 5·24 μmol retinol (5000 IU vitamin A)/4184 kJ.

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Food intake was recorded immediately following each meal as the difference between the mass of food presented and the mass of food returned (Sartorius UK Limited). Body weight was recorded weekly using calibrated scales (Sartorius UK Limited). Body weight and food intake were recorded immediately following each meal as the difference between the mass of food presented and the mass of food returned (Sartorius UK Limited).

Vitamin A supplementation

The base diet was supplemented to achieve the required level of vitamin A by the addition of retinyl acetate (DSM Nutritional Products Limited) diluted to the required concentration in vegetable oil. Stock solutions were prepared weekly for each group according to the manufacturer’s instructions and were refrigerated in light-proof bottles under N2 until required. Individual supplements were prepared in 1 ml syringes from the stock solution and kept refrigerated in light-proof containers before being added to the diet at a ratio of 1 ml supplement/100 g of diet immediately before feeding and mixed thoroughly. A 1 ml aliquot of each stock solution was analysed within 48h of the stock solution preparation for retinyl acetate concentrations using modified gradient reversed-phase HPLC analysis (3) to allow for the calculation of actual vitamin A intake.

Measurement of food intake and body weight

Food intake was recorded immediately following each meal as the difference between the mass of food presented and the mass of food returned (Sartorius UK Limited). Body weight was recorded weekly using calibrated scales (Sartorius UK Limited) immediately before the first meal of the day. Although not fully validated in puppies, the body condition score was recorded weekly using calibrated scales (Sartorius UK Limited). Body weight and food intake were recorded immediately following each meal as the difference between the mass of food presented and the mass of food returned (Sartorius UK Limited).

Bone mineral density and bone mineral content

Bone mineral density (BMD) was assessed at 26 and 52 weeks of age by means of dual-energy X-ray absorptiometry (DXA, total body software package, Lunar Hologic QDR-1000W; GE Healthcare). Dogs were sedated following a minimum 16 h fast, with a combination of Torbugesic (0.3 mg/kg; Pfizer Animal Health), Medetomidine (MS: 20 μg/kg, LR: 5 μg/kg; Pfizer Animal Health) and Midazolam (MS: 0.25 mg/kg, LR: 0.20 mg/kg; Roche Limited), and reversed with Atipamezole (0.1 mg/kg; Pfizer Animal Health).

Clinical examinations and adverse event reporting

The puppies underwent physical examination before the start of the trial and every 4 weeks thereafter. Particular attention was paid to signs of joint or muscle pain. In addition, any illness or injury
between the examinations that required veterinary attention was considered an adverse event. Adverse events were classified into ten categories, namely: poor faeces quality, vomiting, foreign body ingestion, lameness, accident/injury, skin conditions, eye conditions, ear conditions, dental conditions and urinary conditions. On each occasion, the type and duration of treatment were recorded. Any blood parameters outside of the puppy reference range\(^{18}\) were referred to the veterinarian for investigation and re-tests were conducted within 24 h and repeated as required for diagnosis. The veterinarian was blinded to the dietary treatment groups.

### Data analysis and statistics

The primary outcome variable was total serum retinyl ester concentration. Retinyl ester concentration was selected as the primary outcome variable to demonstrate a biological impact of feeding various levels of retinol to growing dogs. The study was powered at 90% to detect differences between the groups based on a previously described log-linear relationship between dietary vitamin A intake and serum retinyl ester concentration\(^3\). The power calculation determined a minimum of nine dogs per group; this was increased to eleven to account for potentially greater variability in puppies compared with adult dogs. Following the conclusion of the study, post hoc power analyses were conducted on each of the safety biomarkers to determine whether there was sufficient power (>80%) to detect a clinically relevant effect size with the variation associated with the dataset. A clinically relevant change was defined as a change from the mean of each variable to a mean outside the reference range\(^{18}\).

Data were analysed by means of linear mixed model analysis including the fixed terms sample number, sex, breed and dietary group, and also the baseline measurement of the variable being modelled. The model included the random terms dog and litter (to take account of possible similarities between littermates), with a correlation between successive samples (within an individual dog) accounted for via use

### Table 2. Population demographics of dogs completing the trial

|        | Group A | Group B | Group C | Group D |
|--------|---------|---------|---------|---------|
| n      | 15      | 11      | 11      | 11      |
| Breed  |         |         |         |         |
| MS     | 8       | 6       | 6       | 5       |
| LR     | 7       | 5       | 6       | 6       |
| Sex    |         |         |         |         |
| Male   | 8       | 5       | 6       | 6       |
| Female | 7       | 6       | 6       | 5       |

MS, Miniature Schnauzer; LR, Labrador Retriever.

* Group A, 5.24 µmol retinol (5000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME); group B, 13.10 µmol retinol (12 500 IU vitamin A)/4184 kJ (1000 kcal) ME; group C, 78.60 µmol retinol (75 000 IU vitamin A)/4184 kJ (1000 kcal) ME; group D, 104.80 µmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) ME.

Fig. 1. Actual vitamin A intake of puppies from 8 to 52 weeks of age. Values are means, with 95% CI represented by vertical bars. Mean values were significantly different between the groups (\(P<0.05\)). Group A (——), 5.24 µmol retinol (5000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME); group B (—■—), 13.10 µmol retinol (12 500 IU vitamin A)/4184 kJ (1000 kcal) ME; group C (—△—), 78.60 µmol retinol (75 000 IU vitamin A)/4184 kJ (1000 kcal) ME; group D (—□—), 104.80 µmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) ME.
of an appropriate correlation structure (determined using graphical methods of residuals from a model with identity correlation structure). Where necessary, data were loge transformed to improve the distribution of the data, as assessed by residual plots. Non-significant terms were removed from the model. Data analyses were performed using R.2.10.1 (R Foundation for Statistical Computing).

All primary and secondary endpoints were separately subjected to Bonferroni correction to account for the presence of multiple endpoints; the overall significance level used was 0.05. Data are presented as means with their 95% CI unless otherwise stated, and P values are reported as Bonferroni-corrected P values.

**Results**

**Population demographics**

The study population demographics are described in Table 2. A total of forty-eight dogs completed the study. A total of forty-eight dogs completed the study. A single LR, allocated to group C, was removed from the study at 9 months of age following diagnosis of a congenital kidney defect. All data from this dog were removed from the analysis. There were no differences in breed or sex distribution between the groups.

**Dietary intake and growth**

There were no significant differences in energy intake on a per kg body weight or per kg metabolic body-weight basis between the groups. As intended, there were significant differences in dietary vitamin A intake between the groups (P<0.0001). When expressed on a per kg body-weight basis (P=0.0001) and on a per kg metabolic body-weight basis (P=0.0001) (Fig. 1), both vitamin A and energy intake significantly decreased over time with maximal intake recorded at week 8. Body weight significantly increased in all groups with time (P=0.0001; Fig. 2), and all dogs maintained an ideal body condition score (score D) throughout the trial. There were no significant differences in body weight between the groups at any time point (P=1.00) nor any significant differences in the rate of growth (P=1.00). There was a significant effect of breed, with LR having significantly higher body weights than MS (P=0.0008).

![Fig. 2. Body weight of two breeds of puppies from 8 to 52 weeks of age. Values are means, with 95% CI represented by vertical bars. Mean values were significantly different between the groups (P<0.05). Group A, 5.24 μmol retinol (5000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME); group B, 13.10 μmol retinol (12 500 IU vitamin A)/4184 kJ (1000 kcal) ME; group C, 78.60 μmol retinol (75 000 IU vitamin A)/4184 kJ (1000 kcal) ME; group D, 104.80 μmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) ME. †—, Labrador Retriever (LR) group A; —△—, LR group B; —△—, LR group C; —△—, LR group D; —△—, Miniature Schnauzer (MS) group A; —△—, MS group B; —△—, MS group C; —△—, MS group D.](https://doi.org/10.1017/S0007114512000128)
**Table 3.** Change in clinical chemistry parameters with age and treatment group*  
(Mean values with their 95% confidence intervals)

| Age (weeks) | Group A† | Group B‡ | Group C§ | Group D¶ |
|-------------|----------|----------|----------|----------|
|             | Mean 95% CI | Mean 95% CI | Mean 95% CI | Mean 95% CI |
| 8           | 48.3 47.2, 49.4 | 49.5 47.7, 51.3 | 48.3 46.4, 50.1 | 47.9 46.3, 49.5 |
| 26          | 55.7 53.9, 57.5 | 55.8 53.3, 58.3 | 55.7 54.0, 57.5 | 55.5 53.8, 57.2 | 0.526 |
| 52          | 54.9 53.1, 56.6 | 57.3 55.3, 59.3 | 58.0 56.5, 59.5 | 57.1 55.4, 58.8 |

**Blood sample analysis**

There were no significant differences in any of the haematological or biochemical parameters between the groups at any time point. However, some variables (Table 3), namely erythrocyte count, mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, mean platelet volume, number of monocytes, number of granulocytes, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and cholesterol, showed statistically significant changes with age. Bone-specific alkaline phosphatase significantly decreased (P<0.0001) and cross-laps (CTx) significantly increased (P<0.0001) with age. There were no significant differences, however, in the concentrations of bone-specific alkaline phosphatase (P<1.00) or CTx (P<1.00) between any of the groups at any time point.
Both serum retinol ($P=0.0001$) and serum total retinyl ester concentrations ($P=0.015$) increased with time. There was no significant difference in serum retinol concentration between the groups ($P=1.00$), whereas total retinyl ester concentrations were significantly different (Fig. 3) between each group and all the other groups at all time points ($P=0.0001$) with the exception of baseline (week 8).

**Bone mineral density and bone mineral content**

There was no significant effect of dietary vitamin A concentration on BMD ($P=0.137$) or bone mineral content ($P=0.936$) as assessed by DXA (Table 4). There was a significant effect of breed, with LR having greater BMD ($P=0.0001$) and bone mineral content ($P=0.0020$) than MS at both time points.

**Urine sample analysis**

Excretion of retinol and retinyl esters in the urine was negligible up to 26 weeks of age and was not consistently detected until 35 weeks of age (Fig. 4). There were no significant differences in the time to excretion of retinol ($P=1.00$) or retinyl esters ($P=1.00$) between the groups nor were there differences between the groups in concentrations of retinol ($P=1.00$) and retinyl esters ($P=1.00$) excreted at weeks 35 and 52.

**Clinical examination and adverse events**

There were no significant differences between the treatment groups in the number and type of adverse events reported. Poor faeces quality was the most commonly reported adverse event ($n=22$), with twenty individual dogs affected. All cases were resolved within 3 d. In LR, two cases of lameness without apparent cause were observed, one in group A and one in group C; both cases were resolved following treatment in less than 7 d.
**Discussion**

The hypothesis tested was that feeding dietary vitamin A at concentrations up to 104.80 μmol retinol (100,000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME) to puppies during growth would not result in any significant changes in biomarkers associated with health. No significant differences were found in any of the biomarkers of health including biochemical markers or haematological parameters. Previous data from human subjects and other animal species indicate that excess vitamin A is associated with a stimulation of bone resorption and an inhibition of bone formation leading to a reduction in BMD potentially resulting from interactions between vitamins A and D\(^2\). No significant differences were found in the biomarkers of these processes namely bone-specific alkaline phosphatase and CTx, and there was no significant effect of dose on either BMD or bone mineral content as assessed by DXA. There are no specific studies examining the interaction between vitamins A and D in the dog. In rats and broiler chickens, biochemical studies have suggested an antagonism between vitamins A and D at the receptor level\(^{12,20}\) as well as an interaction with Ca-regulating hormones such as parathyroid hormone\(^2\). There was no significant effect of dietary vitamin A concentration on plasma Ca or P levels, which suggests that vitamin D deficiency did not occur, although the levels of cholecalciferol in the diet were approximately three times the FEDIAF’s recommended allowance\(^3\). Further studies are required to understand whether there is an interaction between vitamins A and D in the dog and if so, at which dietary concentrations an interaction occurs.

Feeding high levels of retinol has also been shown to prolong activated prothrombin time in the dog, although not outwith the reference range\(^1\). This is potentially due to the interactions with vitamin K. Activated prothrombin time was not directly measured in the present study; however, no signs of clinical vitamin K deficiency such as increased bleeding tendency were observed.

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**Fig. 4.** Total urinary retinyl ester excretion. Values are means, with 95% CI represented by vertical bars. Mean values were significantly different between the groups (\(P<0.05\)). Group A (○), 5.24 μmol retinol (5000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy (ME); group B (■), 13.10 μmol retinol (12,500 IU vitamin A)/4184 kJ (1000 kcal) ME; group C (■), 78.60 μmol retinol (75,000 IU vitamin A)/4184 kJ (1000 kcal) ME; group D (■), 104.80 μmol retinol (100,000 IU vitamin A)/4184 kJ (1000 kcal) ME.
Unlike previous studies in young dogs fed much higher dietary concentrations of vitamin A (314.4 IU retinol (300 000 IU vitamin A)/kg body weight) (9), there were no differences in the rate of growth, energy intake or body weight at any time between the groups. No differences were observed in the number, type or duration of adverse effects were observed and those that were observed could not be attributed to the variation in dietary vitamin A concentration. The present study was limited to the first 52 weeks of life and suggests that dietary vitamin A at the concentrations tested is safe to feed during this growth phase.

As with observations in adult dogs (3), increased dietary retinol concentration was associated with increased serum retinyl ester concentrations (Fig. 3) but not with serum retinol concentrations. Until 26 weeks of age, the concentrations of retinolfored, any differences between the groups at time to

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