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Rad: 00-067923- -00011-0000
Fec: 2007-03-14 17:13:22 Dep. 2020 NUECREACIONES
Tra. 2 PATENTES
Eve: 1 REGDEPOSITO
Act 444 RESPUESTAREQUE Folios: 30

Re: **P1.-SOCIETE DES PRODUITS NESTLE S.A.-Solicitud de Patente de Invención para "MEJORAMIENTO DE LA CONDICIÓN DE MASCOTAS AÑOSAS".-Clase A.23K.-Expediente número 00-067.923.-**

1. Me refiero al auto dictado por ese Despacho notificado mediante fijación en lista de 28 de Diciembre de 2006.
2. De acuerdo con el Concepto Técnico No. **1988** se tiene que:

"Como resultado de la búsqueda de anterioridades se encontró que la solicitud en estudio corresponde con la solicitud europea con número de publicación EP1213971. En el examen de búsqueda se reportó como encontrados los documentos:

- **D1: J.E. BRANAM: "Dietary management of geriatric dogs and cats" VETERINARY TECHNICIAN, vol. 8, No. 10, 1987, pages 501-503, XP000978775.**
- **D6: E. KOLB ET AL.: "Zum Bedarf an vitaminen and an Ascorbinsäure beim Hund, mit Bemerkungen zur Publikation von M. Torel, TU 51, 785-790, 1996" TIERAERZTLICHE UMSCHAU, vol. 52, no. 12, 1997, pages 728-733, XP000978781 KONSTANZ, DE ISSN: 0049-3864.**
- **D8: KEARNS R J ET AL: "EFFECT OF AGE, BREED AND DIETARY OMEGA-6 (N-6) OMEGA-3 (N-3) FATTYACID RATIO ON IMMUNE FUNCTION, EICOSANOID PRODUCTION, AND LIPID PEROXIDATION IN YOUNG AND AGED DOGS" VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, AMSTERDAM, NL, vol.**

69, n . 2/04, 1999, pages 165-183, XP000924834 ISSN: 0165-2427 US ISSN: 0022-3166.

"Esta oficina requiere que el solicitante proporcione en un plazo que no excederá de 3 meses, la copia de los documentos mencionados anteriormente. Nos permitimos recordarle que si el solicitante no presentara los documentos requeridos dentro del plazo señalado, la oficina nacional competente denegará la patente".

3. Para responder a los requerimientos del señor Examinador de conformidad con el Artículo 46 de la Decisión 486, anexo me permito presentar a ese Despacho copia de los siguientes documentos solicitados:

- D1: J.E. BRANAM: "Dietary management of geriatric dogs and cats" VETERINARY TECHNICIAN, vol. 8, No. 10, 1987, pages 501-503, XP000978775.
- D6: E. KOLB ET AL.: "Zum Bedarf an vitaminen and an Ascorbinsäure beim Hund, mit Bemerkungen zur Publikation von M. Torel, TU 51, 785-790, 1996" TIERAERZTLICHE UMSCHAU, vol. 52, no. 12, 1997, pages 728-733, XP000978781 KONSTANZ, DE ISSN: 0049-3864.
- D8: KEARNS R J ET AL: "EFFECT OF AGE, BREED AND DIETARY OMEGA-6 (N-6) OMEGA-3 (N-3) FATTYACID RATIO ON IMMUNE FUNCTION, EICOSANOID PRODUCTION, AND LIPID PEROXIDATION IN YOUNG AND AGED DOGS" VETERINARY IMMUNOLOGY AND IMMUNOPATHOLOGY, AMSTERDAM, NL, vol. 69, no. 2/04, 1999, pages 165-183, XP000924834 ISSN: 0165-2427 US ISSN: 0022-3166.

4. En vista de todo lo anterior, ruego a usted proceder de conformidad y conceder el privilegio de patente solicitado.

Señor Superintendente,


GUSTAVO TAMAYO ARANGO
T.P. 37.172

D1

XP-000978775

CLINICAL NUTRITION

Dietary Management of Geriatric Dogs and Cats

P.D. 1987
p. 501-503 = 3

Edward Branam, DVM, is Technical Services Manager for Hill's Pet Products, Far West United States. Dr. Branam received his veterinary degree from Michigan State University. After two years of private practice, he completed a small animal internal medicine residency at the Veterinary Medical Teaching Hospital, University of California, Davis. He remained on staff at Davis for two years conducting research, teaching, and specializing in clinical geriatric medicine. For three years, Dr. Branam was associated with a referral practice in Sacramento, California, before accepting a position with Hill's Pet Products. Dr. Branam has authored scientific papers and consulted with veterinarians and pet owners throughout the western United States regarding a wide variety of small animal nutritional and medical conditions.

During their aging process, dogs and cats experience significant alterations in nutrient utilization and develop a heightened sensitivity to both nutrient excesses and deficiencies as well as abrupt dietary changes. At five to seven years of age in either species, these nutritional changes become clinically important. Larger-breed dogs typically experience the need for dietary alterations earlier than do cats and smaller dogs. Inadequate consideration of these changing requirements increases the risk of nutritionally related disease in older dogs, directly reducing quality and length of life.

Altered Nutrient Requirements

Current medical evidence indicates that nutrient requirements in older cats approximate those of their young adult counterparts. Geriatric cats are more sensitive to nutrient imbalances, however, and suffer from many of the same life-shortening illnesses that affect geriatric dogs. Therefore, the quality and quantity of the diet fed to older cats have important medical ramifications.

Several important physiologic changes are responsible for the altered nutrient requirements that occur with aging. Reductions in physical activity and lean body mass result in a decreased metabolic rate

and reduce caloric needs for maintenance by approximately 20%. These changes represent the primary reasons that nearly 40% of our aged pet population is obese. Animals weighing more than 15% above their optimum body weight have been linked with startling increases in the incidence of life-threatening diseases. One recent study showed increases of 74% in cases of heart disease, 67% in cases of musculoskeletal disease, 50% in tumors diagnosed, and 40% in skin problems seen in obese dogs.

In contrast, some older pets have reduced appetite and reduced digestive-absorptive capacity, resulting in inability to maintain optimal body weight. Smell and taste may be diminished with aging. These changes reduce an animal's desire to eat. Maintenance of good oral hygiene is also important. Gingival inflammation and recession from excessive accumulation of plaque as well as oral infections can also reduce food intake. Older animals that experience a reduction in saliva production may show further decreases in appetite and oral health.

Aging affects the function of virtually every organ system. Reductions in digestive secretions, absorption, and motility contribute to constipation, flatulence, and abnormal assimilation of nutrients. Reductions in thyroid, adrenal, and exocrine pancreatic functions are also common sequelae of the aging process. In addition, kidney, skin, musculoskeletal, heart, lung, and nervous disorders increase with age. Feeding geriatric animals optimal levels of critical nutrients can reduce the incidence and severity of illnesses related to dysfunction of these organ systems.

Management of Older Pets

There are three major objectives to the management of older animals. They include:

- To slow or prevent onset of disease
- To ameliorate existing problems
- To maintain optimal body weight.

All three objectives can be achieved by medical or surgical intervention, physical activity, and dietary management. Medical or surgical intervention or both can be essential but may also be invasive and hazardous and require continual professional

attention. Physical activity, if properly handled, maintains muscle tone, enhances circulation, improves elimination of waste, and reduces obesity. If medically judicious, one or two 15-minute walks daily can be of great benefit to the older dog. For cats, games requiring moderate physical exertion are recommended.

Proper dietary management can have a significant impact on older pets. Ensuring maintenance of optimal nutrient levels in older dogs and cats reduces stress on major body organs and metabolic processes. When properly formulated, diets for geriatric pets contain moderate levels of high-quality protein and have essential fatty acids, vitamins, calcium, zinc, and fiber in amounts greater than suggested for younger adult animals. These diets should not contain excessive amounts of phosphorus and sodium (Table 1). In addition, cats require restriction of dietary magnesium.

Protein

Proteins are the major source of tissue-building elements in the diet. Specifically, proteins function as components of enzymes, hormones, and antibodies as well as protective and structural tissues and, as such, are essential throughout all phases of an animal's life, even old age. While protein is an essential nutrient, fed in excess it can be detrimental to the older animal, particularly as a major organ function, such as kidney or liver, begins to deteriorate.

When fed in excess of bodily needs, protein cannot be stored but must be used as energy or converted to fat for storage. Dietary protein is metabolized by the liver to a substance called urea or urea nitrogen. Measurement of urea nitrogen in blood (blood urea nitrogen, or BUN) is a

TABLE 1
Nutrient Requirements
for the Older Dog

Nutrient	Maintenance (% dry matter)	Geriatric (% dry matter)
Protein	18-25	15-18
Calcium	0.5-0.9	0.5-0.8
Phosphorus	0.4-0.8	0.4-0.7
Sodium	0.2-0.5	0.2-0.4

routine procedure. Dogs and cats undergoing liver failure may have unusually low BUN values, while dogs and cats with chronic renal failure may have unusually high BUN values (Figure 1). Feeding high quantities of protein to animals with hepatic disease or renal failure results in development of clinical signs and can seriously compromise the quality of a pet's life.

Because cats are true carnivores, they require larger amounts of dietary protein than do dogs. As a result of the high incidence of kidney disease in older cats, however, their diet should contain high-quality protein at somewhat reduced levels.

Calcium

Calcium requirements may increase moderately with age in dogs because of reduced calcium-absorptive capabilities in the small bowel. Despite the increased dietary requirements, calcium deficiencies are usually only seen in pets fed home-made diets.

Calcium excesses are typically derived from diets formulated for growth (multi-purpose diets), diets high in meat and bone meal, or dietary supplements. Clinical signs associated with calcium excess in older dogs include zinc deficiency, reduced thyroid function, and possibly gastric blox and torsion.

Gastrin secretion is increased by excessive ingestion of calcium, resulting in thickening of the gastric mucosa and delayed emptying of food from the stomach. Recent studies support this hypothesis that chronic excessive dietary calcium may be a risk factor for blox and torsion in large-breed dogs.³

Phosphorus

Improper ratios of calcium to phosphorus can cause secondary nutritional hyperparathyroidism. Low-calcium, high-phosphorus foods include meat or organ tissues (calcium-phosphorus ratio of at least 1:3). Clinical signs of hyperparathyroidism include lameness, spontaneous fractures related to calcium reabsorption from bone to offset high serum phosphorus levels, and renal calcification. Excess dietary phosphorus promotes kidney disease by increasing glomerular filtration rate (increased blood flow to the glomerulus over a sustained period results in glomerular damage and sclerosis) and by promoting calcium and phosphorus deposition in the kidney (resulting from the increased serum levels of these minerals).

Sodium

Many commercial pet foods typically provide sodium at 80 to 150 mg/kg of body weight or from 10 to 20 times the amount required for older animals. Excess sodium promotes retention of fluids, hypertension, and cardiovascular and renal

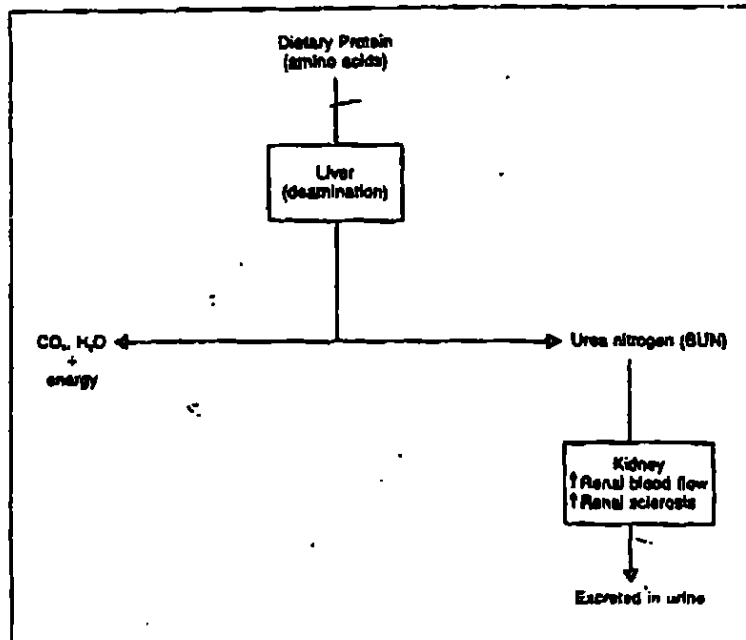


Figure 1—Shown is the normal pathway of energy production indicating the changes that occur in conversion of protein to energy.

disease. Sodium intake should be restricted to required levels in older animals.

Zinc

Intestinal absorption of zinc declines with age in many dogs. Clinical signs of deficiencies most commonly include reduced appetite and changes in skin and haircoat. Adequate zinc is necessary for normal sensations of taste and smell as well as integumentary health.

Magnesium

Feline urologic syndrome (FUS) represents one of the major medical health care concerns of cat owners. It is estimated that 1 of 10 cats will develop the syndrome in its lifetime. Excessive dietary magnesium is a significant factor in the pathogenesis of feline urologic syndrome. Obesity, reduced physical activity, and neutering have been identified as predisposing factors that increase the incidence of feline urologic syndrome when levels of dietary magnesium are elevated. Because the percentage of cats exhibiting these predisposing factors increases with age, dietary magnesium restriction becomes more important in older cats. The recommended dietary magnesium content should not exceed 0.10% on a dry-matter basis or 20 mg/100 kcal of diet. (Many commercial foods contain one and one-half to three times this amount of magnesium.)

Fiber

Greater dietary fiber content promotes gastrointestinal health in older dogs. In addition, increased fiber moderately reduces total food digestibility, assisting glucose tolerance and promoting proper stool consistency and maintenance of body weight in dogs prone to obesity.

Vitamins

Increased intake of vitamins A, B₁, B₂, B₆, and E is indicated in older animals because of changes in the digestive system and metabolism associated with aging. These vitamins also counteract certain aspects of the aging process. SPECIFIC NUTRIENT ALTERATIONS REQUIRED BY OLDER PETS SHOULD NOT BE ATTEMPTED BY SUPPLEMENTING POOR DIETS.

A diet specifically formulated to meet the nutritional needs of older animals should be fed. Because of reduction in digestive and absorptive capabilities in older dogs and cats, a single fixed-formulation diet is recommended.

Indoor pets should be fed in a location separate from the family eating area during the owner's morning and evening meals. This reduces begging behavior as well as the desire of family members to feed table scraps. Older animals that are having difficulty maintaining adequate body weight or showing decreased appe-

lite should be fed a palatable, high-caloric diet specifically formulated for older animals and should be fed frequently. Hand-feeding, warming the food, and adding water to the food will improve palatability. Feeding a proper diet and maintaining optimal body weight throughout an animal's older years can profoundly affect both quality and length of life.

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This article on clinical nutrition is one of a regular series written specifically for veterinary technicians. The series is designed for the practicing technician as an introduction to and update on the role of nutrition in management and treatment of a variety of clinical disorders that are commonly seen in dogs and cats.

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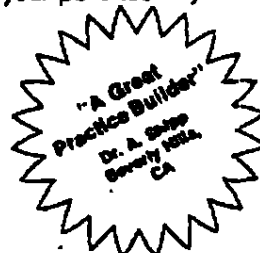
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tum, *M. scrofulaceum*, *M. chelonae*, *M. avium* und *M. intracellulare* (zit. nach Štoudrek u.a., 1994). In der Slowakei und in Rußland stieg die Tuberkulosemorbidity seit 1991 sogar wieder an (Tabelle 2). Im Ural, in West- und Ostsibirien sowie im Fernen Osten lagen im Jahre 1993 die durchschnittlichen Morbiditätswerte der Lungentuberkulose bei Menschen über dem Landesdurchschnitt von 39,5 Fällen auf 100 000 Einwohner (Moskva, 1994). So zum Beispiel betrug die Tb-Morbidity in Tuva 83,1 Fälle pro 100 000 Einwohner, in Primorje (Vladivostok) 71,3/100 000 E., im Chabarover Gebiet 63,3/100 000 E. und im Novosibirsker Gebiet 59,0 Fälle auf 100 000 Einwohner (Abbildung 1). Günstiger war die Situation im Murmansk, Archangelaker, Vologodaker und Stavropoler Gebiet sowie in Baskonostan. Hier lagen die Morbiditätswerte unter 30 Fällen/100 000 E. Eine hohe Erkrankungshäufigkeit wurde in einigen nördlichen Landesteilen der Jakutischen Republik beobachtet. Die Regionen des Hohen Nordens gehören hinsichtlich des vorherrschenden Klimas zu Extremgebieten. Typisch sind lange, raue Winter, kurze, kalte Sommer, ein verändertes Lichtregime (Polarnächte, Polartage), kräftige Winde, intensive magnetische Einflüsse, unbewohnte und eintönige Landschaften sowie eine artenarme Pflanzen- und Tierwelt (Avcyn u. Kenig, 1970). Neben geographischen und klimatischen Faktoren beeinflussen soziale Gegebenheiten das Krankheitsgeschehen der Tuberkulose. Mazina (1970) erwähnte, daß in der Jakutischen Republik schlechte Wohn- und Lebensbedingungen (die Unterbringung mehrerer Familien auf kleinem Raum), gesundheitswidrige Verhaltensweisen (Unterkühlungen) oder die ungenügende Gesundheitsbetreuung eine Verschlechterung der Immunitätslage besonders bei Kindern bewirkten und die Verbreitung der Tuberkulose förderten.

Literatur bei der Autorin.

Anschrift der Verfasserin:
Dr. Silke Mitro, Otto-Behagel-Str.
10/D, 35394 Gießen.

Tierärztl. Umschau 52, 728 - 733 (1997)

p.d. 1997

p. 728-733

(6)

Aus dem Veterinär-Physiologisch-Chemischen Institut der Universität Leipzig, der Hoffmann-La Roche AG Grenzach-Wyhlen und der Effem GmbH Verden

Zum Bedarf an Vitaminen und an Ascorbinsäure beim Hund, mit Bemerkungen zur Publikation von M. Torel, TU 51, 785-790, 1996

von E. Kolb, J. Seehawer und K. Wiegand

(3 Tabellen, 33 Literaturangaben)

XP-000978781

Kurztitel: Vitaminbedarf beim Hund

Schlagworte: Vitamin A - Vitamin D₃ - Vitamin E - Vitamin K - B-Vitamine - Ascorbinsäure - Vitaminbedarf - Vitaminanwendung - Vitamin-Intoxikation

Zusammenfassung

Für den Hund ist ein Zusatz an Vitamin A von 8 000-12 000 I.E. je kg Futter-TM empfehlenswert, der eine gute Funktionsfähigkeit des Immunsystems sichert. Wegen des Fehlens von 7-Dehydrocholesterol in den Zellen der Epidermis können Hunde und Katzen bei UV-Bestrahlung kein Vitamin D₃ bilden. Der Bedarf wird durch einen Zusatz von 800-1 200 I.E./kg Futter-TM gedeckt. Eine gute Versorgung mit

Vitamin E (40-70 mg/kg) und mit Se (0,1 mg/kg) hemmt das Altern, insbesondere die Entwicklung von Arteriosklerose. B-Vitaminpräparate sind bei Anorexie über 10-14 Tage zu verabreichen. Bei vielen Erkrankungen ist der Verbrauch an Ascorbinsäure erhöht, ohne daß die Biosynthese zunimmt. Zur Aktivierung des Immunsystems werden Präparate mit den Vitaminen A, D₃ und E sowie mit Ascorbinsäure angewendet.

Abstract

Vitamin and ascorbic acid requirements of dogs
The provision of 8 000-12 000 IU vitamin A/kg food, dry matter (dm), is recommended to ensure an appropriate activity of the immune system of the dog. The absence of 7-dehydrocholesterol in the cells of the epidermis of dogs and cats means that vitamin D₃ is not produced by the influence of UV-light. This demand is provided by 800-1 200 IU/kg dm. An adequate supply of vitamin E (40-70 mg/kg) and with Se (0,1 mg/kg) inhibits ageing, notably the development of arteriosclerosis. Preparation of B-vitamins should be provided after periods of anorexia for 10 to 14 days. In many diseases the utilization of ascorbic acid increases, but the biosynthesis is not increased. Preparations containing vitamin A, D₃ and E together with ascorbic acid are indicated for the stimulation of the immune system.

1 Einleitung

Kürzlich hat Torel (1996) die Frage der Entstehung und Verhütung der Hüftgelenkdysplasie beim Hund diskutiert und dabei u.a. eine erhöhte Aufnahme der Vitamine A und D₃ als möglichen Hilfsfaktor für ihre Entstehung angesehen. Von diesem Autor werden verschiedene neuere ernährungsphysiologische Erkenntnisse zum Bedarf an Vitaminen beim Hund nicht berücksichtigt und überdies überholte Vorstellungen (z.B. die Eigensynthese von Vitamin D₃ in der Haut bei UV-Bestrahlung) vertreten, so daß eine Darstellung der tatsächlichen Verhältnisse des Bedarfs und der Bedeutung der Sicherung der Versorgung für die Erhaltung der Gesundheit und Leistungsfähigkeit von Hunden zweckmäßig ist.

2 Zum Bedarf an Vitamin A

Von Torel (1996) wird »kritisch« bemerkt, daß auf dem Markt kaum noch Produkte vorkommen, die weniger als

15 000 I.E. Vitamin A je kg Futter enthalten. Als »schlechtes« Beispiel wird die Zusammensetzung eines Fertigfutters angeführt, das 18 000 I.E./kg enthält. Nach Meinung von Torel (1996) ist ein Gehalt an Vitamin A von 9 000 I.E./kg Futter ausreichend. Von der Arbeitsgemeinschaft für Wirkstoffe in der Tierernährung (Behm et al., 1991) wird für Hundefutter ein Zusatz an Vitamin A in Höhe von 8 000 - 12 000 I.E./kg TM bzw. von Meyer (1990) - je nach Leistung - ein Gehalt von 8 000-24 000 I.E./kg TM empfohlen.

Bei der Bemessung des Bedarfs an Vitamin A ist zu berücksichtigen, daß dieser zum Erreichen einer optimalen Funktionsfähigkeit des Immunsystems höher als der für gutes Wachstum liegt. Das Vitamin A ist für alle Zelltypen dieses Systems notwendig (Lit. s. Kolb, 1995). Als Modell zum Nachweis der Bedeutung des Vitamins A für das Immunsystem wird besonders häufig das Küken verwendet. Von Sklan et al. (1994) wurde der Einfluß eines unterschiedlichen Gehalts an Vitamin A

(0,0833, 1,50, 3,33, 6,66 und 13,2 mg/kg) im Futter von Küken vom Schlupf an auf die Antikörperbildung untersucht. Am 21. Tag des Versuchs wurde als Antigen β -Casein (1 mg) mit Zusatz von zerkleinerten Zellen von *Mycobacterium tuberculosis* (2 mg/ml) in einem Volumen von 1 ml an mehreren Stellen verabreicht. Mit zunehmendem Gehalt an Vitamin A im Futter nahm die Antikörperbildung zu und erreichte bei einem solchen von 22 200 I.E./kg den höchsten Wert, bei dieser Zulage war auch die Proliferation von T-Lymphozyten bei Züchtung unter Zusatz von β -Casein oder von Antigen aus *M. tuberculosis* am größten.

Bei einem anderen Versuch an Küken wurde der Einfluß einer ähnlichen Abstufung im Gehalt an Vitamin A im Futter (s. Tab. 1) auf die Proliferationsfähigkeit von T-Lymphozyten bei Inkubation mit β -Casein über 96 Stunden geprüft. Die größte Zellvermehrung lag bei einem Gehalt an Vitamin A im Futter von 22 000 I.E./kg (6,6 mg/kg) vor (Halevy et al., 1994). Aus diesem

Grund erscheint die Empfehlung für einen Zusatz von 8 000-12 000 I.E./kg Hundefutter-TM durchaus berechtigt. Aber auch gegen einen Zusatz von 18 000 I.E. an Vitamin A je kg TM zum Hundefutter bestehen unter Berücksichtigung der genannten Ergebnisse keine Bedenken. Die Meinung von Torel (1996), daß »durch einen Gehalt von 15 000 bis 18 000 I.E. je kg Futter eine Intoxikation ausgelöst werden könnte«, ist nämlich völlig unbegründet. Beim Hund und bei anderen Karnivoren findet bekanntlich der Transport von Vitamin A im Blutplasma in Form von Vitamin-A-Estern - von Vitamin-A-stearat und -palmitat - in Bindung in Lipoproteinen statt, ohne daß ein mäßig erhöhter Gehalt zu einer Schädigung führt (Schweigert, 1990; Schweigert u. Thomann, 1991). Hunde können bei Fütterung von Leber von Fischen, Wilderkäfern und Schweinen in Leber und Nieren große Mengen an Vitamin A und an Vitamin-A-Estern ohne Beeinflussung der Funktion dieser Organe anreichern. Nach Kronfeld (1989) wird

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Tabelle 1: Gehalt an Vitamin A und an Retinsäure in T-Lymphozyten von 3 Wochen alten Hündchen sowie Größe des Einbaues von ³H-Thymidin (in Bq) nach Stimulierung mit Antigen (β-Casein) im Verlaufe von 96 Stunden bei unterschiedlichem Gehalt des Futters an Vitamin A vom Schlupf an (Haley et al., 1994).

Gehalt im Futter in mg/kg	Gehalt in 10 ⁶ T-Zellen, in nmol Vitamin A	Retinsäure	Vermehrungs- größe, in Bq
0,10	0	0,80 ± 0,13	394 ± 48
0,83	0	0,90 ± 0,10	720 ± 94
1,50	0	0,93 ± 0,06	660 ± 81
3,30	0	1,41 ± 0,16	1310 ± 248
6,60	0,03 ± 0,01	1,82 ± 0,13	2036 ± 489
13,20	0,20 ± 0,04	2,21 ± 0,23	1309 ± 189

Tabelle 2: Gehalt an B-Vitaminen in einem Fertigfutter (Torel, 1996), empfohlener Zusatz zu Hundefutter (Behn et al., 1991) sowie Bedarf (Lewis et al., 1990), in mg/kg.

Gehalt im Futter	Empfohlener Zusatz	Bedarf
Vitamin B ₁	7,6	2 - 4
Vitamin B ₂	6,4	3 - 6
Vitamin B ₃	6,4	2 - 5
Niacinamid	61	20 - 25
Pantothensäure	31,9	4 - 6
Biotin	0,47	0,1 - 0,25
Folsäure	1,4	0,2 - 0,5
Vitamin B ₁₂	0,065	0,02 - 0,04

Tabelle 3: Gehalt an Vitaminen und an Ascorbinsäure im Blutplasma von Hunden unter physiologischen Verhältnissen, bei Mangel sowie bei einer Intoxikation.

	Physiologischer Gehalt	Bei Mangel	Bei Intoxikation
Vitamin A plus Ester, in mg/l	2 - 3	< 0,8	20 und mehr
25-Hydroxyvitamin D ₃ , in µg/l	28 - 38	< 10	700 - 600
1,25-Dihydroxyvitamin D ₃ , in ng/l	20 - 53	< 20	30 - 64
Vitamin E, in mg/l	5 - 24	< 3	
Vitamin B ₁ , in µg/l	50 - 110	< 50	
Vitamin B ₁₂ , in µg/l	200 - 400	< 40	
Ascorbinsäure, in mg/l	5 - 15	< 2	

ein Gehalt an Vitamin A im Hundefutter bis zu 36 600 I.E./kg TM toleriert; als optimal wird ein Gehalt von 5 800-10 800 I.E./kg TM beschrieben. Bei täglicher Aufnahme von 15 000-30 000 I.E./kg KM über 3 Monate tritt keine Störung auf (Meyer, 1990). Erst bei stark erhöhter Zufuhr von 90 mg Vitamin A je kg KM und Tag (= 300 000 I.E. je kg KM und Tag) über einen längeren Zeitraum werden Störungen der Funktion des Gehirns (in Form von Übererregbarkeit), Abnahme des Futterverzehrs, Haarausfall, Entkalkung der Knochen (mit Frakturen) sowie Abnahme des Gehalts an Thyroxin im Blutplasma festgestellt (Dunghue et al., 1987). Unter physiologischen Verhältnissen enthält das Blutplasma 0,3-1 mg Retinol, 0,8-1 mg Retinylstearat und 0,07 mg Retinylpalmitat je l. Bei Intoxikation liegen stark erhöhte Werte auf einen Gesamtgehalt an Vitamin A und an -Ethern von 20 mg/l und

mehr vor (Pulz, 1994). Mit steigendem Gehalt an Vitamin A im Futter nimmt in den Leukozyten die Bildung der biologisch aktiven Formen - der Retinsäuren - zu; bei dem genannten Versuch von Haley et al. (1994) wurde bei einem solchen von 22 000 I.E./kg eine Menge von 1,82 ± 0,13 nmol Retinsäure je 10⁶ T-Zellen erreicht. Bei einer Zulage an Vitamin A von 44 000 I.E./kg nahm der Gehalt an Retinsäure in den T-Zellen weiter zu, die Vermehrungsfähigkeit verminderte sich jedoch beträchtlich (Tab. 1). Eine Zulage an Vitamin A von mehr als 22 000 I.E./kg ist daher nicht empfehlenswert. Der Bedarf für wachsende Hunde wird vom Nationalen Forschungsrat der USA (NRC) mit 202 und für adulte Hunde mit 75 I.E./kg KM angegeben (NRC, 1983). Bei Analysen von Fertigfutter für Hunde in Dosen wurde bei 8% der Produkte ein geringerer Gehalt an Vitamin A - als vom

NRC gefordert - festgestellt (Heuner, 1990). Bei der Produktion und Lagerung von Trockenfuttermitteln kann der Gehalt um bis zu 35% abnehmen (Hiltna, 1989).

Die Sicherung der Versorgung mit Vitaminen erfordert besonders bei kranken Hunden Aufmerksamkeit, bei denen der Verbrauch erhöht und die Aufnahme mit der Nahrung häufig vermindert ist. Durch Verabreichung von Kombinationspräparaten mit den Vitaminen A, D₃ und E sowie mit Ascorbinsäure wird das Immunsystem aktiviert und die Heilung gefördert.

3 Zum Bedarf an Vitamin D

Zur Frage des Bedarfs vertritt Torel (1996) die Meinung, »das Vitamin D₃ wird beim Hund bei UV-Bestrahlung aus Ergosterin (?) und Cholesterol in den äußersten Schichten der Haut selbst synthetisiert«. Tatsächlich können Hunde und Katzen in der Haut kein Vitamin D₃ bilden und entwickeln bei vitamin-D-freier Ernährung und UV-Bestrahlung Rachitis. Die UV-Bestrahlung von Hunden - bei der Torel (1996) Zurückhaltung empfiehlt - ist daher völlig zwecklos! Bei Untersuchung der Haut von Ratten belief sich der Gehalt an 7-Dehydrocholesterol auf 1900 ng je cm²; in der Haut von Hunden und Katzen war die Verbindung nicht nachweisbar (How et al., 1994). Auf die fehlende Bildung von Vitamin D₃ beim Hund weist auch Meyer (1990) hin. Übrigens hatten bereits Wheelley und Sher (1961) das Fehlen von 7-Dehydrocholesterol in den Lipiden der Haut von Hunden festgestellt. Bei Jungkatzen hatte der Verzehr eines vitamin-D-freien Futters bei UV-Bestrahlung über 6 Stunden je Tag eine ebenso schnelle Abnahme im Gehalt des Blutplasmas an 25-Hydroxyvitamin D₃ - des ersten Aktivierungsmoleküls des Vitamins D₃ - wie bei Katzen ohne Bestrahlung zur Folge. Bei Verabreichung eines Hemmstoffs der 7-Dehydrocholesterol-Reduktase erfolgte die Abnahme des Gehalts langsamer. Der fehlende Gehalt an 7-Dehydrocholesterol in der Haut von Hunden und Katzen ist daher die Ursache der nicht vorhandenen Eigensynthese von Vitamin D₃ (Morris et al., 1994). Einen Gehalt des Hundefutters von 1800 I.E. an Vitamin D₃ hält Torel

(1996) für erhöht. Von der AG für Wirkstoffe in der Tierernährung (Rehm et al., 1991) wird ein Zusatz von 800-1200 I.E./kg TM Hundefutter bzw. von Meyer (1990) ein Gehalt von 800-1000 I.E./kg TM empfohlen. Torrel (1996) hält einen Gehalt von 1000 I.E./kg Futter als ausreichend. Gegen eine Zulage von 1800 I.E./kg TM bestehen aber nach dem Ergebnis des nachfolgend aufgeführten Versuchs keinerlei Einwände. Bei diesem Versuch führte eine Zulage von Vitamin D₃ in einer Menge von 2420 I.E./kg (60,5 µg/kg) bei Fütterung von Deutschen Schäferhunden sowie von Pointern mit einer üblichen Ration aus Fleisch, Soja, Reis und Knochenmehl – bei einem Gehalt an Ca von 1,4 und an P von 1% – zu keiner Beeinflussung des Wachstums, der Skelettentwicklung und der Futteraufnahme; Im Vergleich zu den Kontrollhunden ohne Zulage waren der Gehalt an Ca und an Phosphat im Blutplasma, die Aktivität der alkalischen Phosphatase des Blutplasmas sowie der Gehalt an Hydroxyprolin im Harn

gleich groß. Mit dem Grundfutter wurde dabei offenbar genügend Vitamin D₃ aufgenommen. Diese und andere Befunde zeigen, daß der Bedarf des Hundes an Vitamin D bei ausreichendem Gehalt des Futters an Ca und an Phosphat und bei einem physiologischen Ca:P-Verhältnis für das Wachstum relativ niedrig liegt (Kealy, 1991). Da der Gehalt an Ca und an Phosphat im Futter gewissen Schwankungen unterworfen ist, so ist ein Zusatz von 800-1200 I.E./kg Hundefutter in jedem Falle empfehlenswert. Aus Versuchen an Menschen und an Versuchstieren ist übrigens ersichtlich, daß das Vitamin D in der aktivierten Form – als 1,25-Dihydroxyvitamin D – für die Funktionsfähigkeit des Immunsystems (Lit. s. Kolb, 1995) und der Fortpflanzungsorgane (Lit. s. Kolb, 1997) notwendig ist. Mangelnde Bildung von 1,25-Dihydroxyvitamin fördert z.B. das Angeden und die Ausbreitung einer Tuberkuloseinfektion; die Spermienbildung vermindert sich. Als gefährlich für den Hund sieht Torrel

(1996) die »extrem vitaminisierte Welpenmilch« an, und er schreibt »cave Welpenmilch«, in Anlehnung an die Warnung an Häusern im alten Rom »cave canem«, wenn ein Wachhund im Gelände war. Da die Welpen mit sehr niedrigem Gehalt an den Vitaminen A, D₃ und E geboren werden, so ist ein hoher Gehalt im Kolostrum und in der Milch bzw. in Milchersatzpräparaten notwendig! In zweckmäßiger Weise enthalten z.B. die Milchersatzpräparate für Welpen der Firma Tiermahrung, Bausfelden, Mengen an Vitamin A von 15000 I.E., an Vitamin D₃ von 1200-1500 I.E. und an 150 mg Vitamin E je kg TM! Der Bedarf an Vitamin D beim wachsenden Hund wird mit 22 I.E. (0,55 µg) je KM und Tag angegeben. Für therapeutische Zwecke wird die Applikation von 2000 I.E./kg KM empfohlen. Bei Welpen tritt auch nach Verabreichung von 2000 I.E./kg KM keine Schädigung auf. Eine Intoxikation wurde bei Welpen bei Aufnahme eines Milchersatzpräparats mit einem Gehalt an Vit-

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amin D₃ von $3,45 \times 10^4$ I.E./kg ermittelt; sie ging mit Störungen der Entwicklung des Skeletts und der Nierenfunktion (durch Verkalkung) einher (Kumphues et al., 1990). Bei Aufnahme von etwa 60000 bis 120000 I.E. an Vitamin D₃ je kg KM tritt bei Hunden eine akute Vergiftung mit Erbrechen und Anorexie auf (Garlock et al., 1991).

Zum Nachweis der Versorgungslage mit Vitamin D₃ wird der Gehalt an 25-Hydroxyvitamin D₃ im Blutplasma verwendet. Werte von weniger als 10 ng/ml zeigen einen Mangel, von 28-38 ng/ml eine ausreichende Versorgung und solche von 300-640 ng/ml eine Intoxikation an (Puls, 1994). Der Gehalt an dem biologisch besonders stark wirksamen 1,25-Dihydroxyvitamin D₃ im Blutplasma ist bei Mangel kleiner als 20 pg/ml (Hawewinkel u. Schoenmakers, 1995). Vom nationalen Forschungsrat der USA wird der Bedarf an Vitamin D für wachsende Hunde mit 22 und für adulte Hunde mit 8 I.E./kg KM angegeben; der Minimalbedarf im Futter für wachsende Hunde beläuft sich auf 400 I.E./kg TM (Kronfeld, 1989).

4 Zum Bedarf an Vitamin E

In dem von Torel (1996) angeführten »schlechten« Beispiel der Zusammensetzung eines Hundefutters beläuft sich der Gehalt an Vitamin E auf 150 mg/kg; dazu wird bemerkt, daß das Vitamin E in Hundefuttermitteln »eigentlich unterschwellig dosiert ist«. Von der AG für Wirkstoffe in der Tierernährung (Behm et al., 1991) wird ein Zusatz von 40-70 mg Vitamin E je kg Hundefutter-TM empfohlen. Von Lewis et al. (1990) wird der Bedarf mit 50 mg/kg Futter und von Meyer (1990) mit 80-100 mg/kg Trockenfutter angegeben. Bei Verzehr von Fleisch und von Fleischprodukten ohne Zulage ist die Versorgung mittelmäßig; Rindfleisch enthält etwa 4 bis 5 und Rinderleber 6-8 mg/kg. Größere Mengen sind in grünem Gemüse enthalten. Die Sicherung der Versorgung mit antioxidativ wirksamen Vitaminen und mit Se ist für die Verhütung von Altersvorgängen von Bedeutung. Das Se ist für die Bildung der Glutathionperoxidase (GPx) und anderer Se-haltiger Proteine notwendig. Vitamin E und GPx hem-

men in Lipoproteinen und im Bereich arteriosklerotischer Herde in den Arterien die Bildung von Peroxidverbindungen aus ungesättigten Fettsäuren und damit Entwicklung und das Fortschreiten der degenerativen Veränderungen. Für einen Hund mit etwa 15 kg KM wird die tägliche Aufnahme von 4,5 mg β -Carotin, von 80 I.E. Vitamin E und von 50 mg Ascorbinsäure empfohlen (Moser, 1994).

Eine gute Versorgung mit Vitamin E ist für die Funktionsfähigkeit der Herz- und Skelettmuskulatur, des Immunsystems und der Fortpflanzungsorgane von Bedeutung. Es hemmt die Ausbildung der Arteriosklerose, die durch fettreiche Fütterung gefördert wird. Bei ausreichender Versorgung beläuft sich der Gehalt im Blutplasma auf 5 bis 24 mg/l. Werte von weniger als 4 mg/l zeigen einen Mangel an und werden besonders bei Störungen der Verdauung und der Resorption ermittelt (Puls, 1994).

Bei Verabreichung eines Futters mit einem Gehalt an Vitamin E von nur 1 mg/kg und an Se von 0,01 mg/kg an 5 bis 8 Wochen alte Hunde bildete sich nach etwa 6 Wochen eine Muskeldystrophie aus. Weitere Symptome waren degenerative Veränderungen an den glatten Muskelfasern, an Nervenzellen und an der Retina (Vleer, 1975).

5 Zum Bedarf an Vitamin K

In dem von Torel (1996) angeführten Beispiel der Zusammensetzung eines industriell hergestellten Hundefutters ist eine Zusatz an Vitamin K₁ (Mena-dionbisulfit) von 2 mg/kg angegeben. Von der AG für Wirkstoffe in der Tierernährung wird eine Zugabe von 0,5-1 mg/kg empfohlen (Behm et al., 1991). Mangel an Vitamin K tritt beim Hund bei Störung der Verdauung (mangelnde Sekretion von Galle, Pankreatitis, Pankreasatrophie) und der Resorption (bei Enteropathie) auf. Gute Quellen für Vitamin K sind Leber, Fischmehl und Grüngemüse (Meyer, 1990). Bei Welpen beläuft sich der Bedarf auf 5-15 und bei adulten Hunden auf 0,5 μ g/kg KM und Tag (Quick, 1962).

6 Zum Bedarf an B-Vitaminen

Die B-Vitamine sind nach Auffassung von Torel (1996) im Hundefertigfutter »unterschwellig« dosiert. Der von dem

Autor für ein Fertigfutter angegebene Gehalt, der von der AG für Wirkstoffe in der Tierernährung (Behm et al., 1991) empfohlene Zusatz sowie der von Lewis et al. (1990) angegebene Bedarf sind in Tabelle 2 aufgeführt. Aus Tabelle 2 ist ersichtlich, daß das von Torel (1996) in seiner Zusammensetzung angeführte Hundefutter weit über den Bedarf liegende Mengen an B-Vitaminen enthält. Dies wirkt sich günstig auf die körperliche Leistungsfähigkeit, auf die Stoffwechselregulation und auf die Anpassungsfähigkeit aus.

Bei mit Unterbrechung der Futteraufnahme einhergehenden Krankheiten (Infektionskrankheiten) sowie bei Störungen der Verdauung und Resorption reichen die Speicher an B-Vitaminen nur zur Deckung des Bedarfs für etwa 10 bis 14 Tage aus. In solchen Fällen ist die Anwendung eines Injektionspräparates mit solchen empfehlenswert. Bei einer Infektion findet unter dem Einfluß der verstärkten Bildung verschiedener Interleukine vorübergehend eine starke Zunahme der Bildung von Monozyten und Makrophagen, von Granulozyten und von T- und B-Lymphozyten statt, für die eine ausreichende Bereitstellung von vitamin-B-haltigen Koenzymen notwendig ist. Bei Mangel an B-Vitaminen ist die Reaktionsfähigkeit des Immunsystems bzw. die Bildung von Antikörpern eingeschränkt (Lit. s. Kolb, 1996). Bei Hunden mit Leberkrankheiten empfiehlt sich die Zufuhr der doppelten Menge des Bedarfs an B-Vitaminen von gesunden Hunden.

7 Zum Bedarf an Ascorbinsäure

Mit Recht stellt Torel (1996) fest, daß die Ascorbinsäure für den Stoffwechsel des Hundes von immenser Bedeutung ist. Neuere Arbeiten zur Verwertung (Kolb et al., 1993) und zum Gehalt (Kuba et al., 1995) fehlen allerdings im Literaturverzeichnis.

Gesunde Hunde ohne besondere Belastung und ohne Gendefekt haben in der Regel eine ausreichend große Eigensynthese an Ascorbinsäure in der Leber. Die Ausbildung der Enzyme zur Synthese und zum Abbau der Ascorbinsäure ist genetisch festgelegt. Zahlreiche Berichte im Schrifttum deuten darauf hin, daß beim Hund Gendefekte vorkommen, die zu einer verminderten

Bildung von Ascorbinsäure und zu skorbutähnlichen Symptomen führen; der Gehalt an Ascorbinsäure im Blutplasma ist dann kleiner als 1 mg/l. Bei der Untersuchung von Serum liegt der Gehalt an Ascorbinsäure niedriger als der bei der Analyse von Blutplasma. Beim Stehenlassen des Blutes bei der Gerinnung wird Ascorbinsäure durch die Leukozyten in beträchtlichem Umfang verbraucht; die Werte des Gehalts schwanken unter physiologischen Verhältnissen zwischen 5 und 15 mg/l (Tab. 3). Durch Verabreichung von 2,5 oder 3 g je Tag wurde in solchen Fällen von Skorbut ein günstiger Einfluß erzielt (Väänänen u. Wikman, 1979).

Bei zahlreichen Erkrankungen des Hundes ist der Verbrauch an Ascorbinsäure erhöht und der Gehalt im Blutplasma vermindert. So wies Brehm (1964) bei Hunden mit eitriger Ohr-entzündung, mit Pyometra und mit Tumorentwicklung eine verminderte Konzentration im Blutplasma nach. Bei Infektion durch Parvovirus, bei Nephropathie sowie bei Störungen der Leberfunktion ist sie gleichfalls herabgesetzt (Souza, 1985). Von mehreren Autoren wurde ein günstiger Einfluß der i.v. Verabreichung von 1,5 bis 2,5 g je Tag über mehrere Tage bei der Behandlung der Staupe festgestellt (Lit. s. Kolb, 1994).

Bei Infektionskrankheiten, nach Operationen und Verletzungen, bei Leberkrankheiten sowie bei besonderen Belastungen wirkt sich ein täglicher Zusatz an Ascorbinsäure bei Hunden kleiner Rassen von 50 mg und bei solchen großer Rassen von 200 mg zum Futter fördernd auf die Heilung bzw. Anpassungsfähigkeit aus. Als Futterzusatz ist besonders ROVIMIX® Stay C geeignet, das stabile Ascorbylphosphatester enthält. Diese werden an der Oberfläche der Darmepithelzellen zerlegt, die Ascorbinsäure wird dann in die Zellen aufgenommen. Die Ascorbinsäure wird

im Wundbereich angereichert und fördert die Bildung von Bindegewebe durch die Fibrinblasten. Bei Störung der Verdauung wird die Ascorbinsäure in einer Dosis von 20 mg/kg KM i.v. verabreicht.

8 Schlußbetrachtung

Bei Deckung des Bedarfs an allen essentiellen Nahrungsfaktoren ist die Größe des Wachstums hauptsächlich von der genetischen Konstruktion und von der Höhe der Aufnahme von Nährstoffen (von umsetzbarer Energie) und von Eiweiß abhängig. Durch Verminderung des Gehalts an Vitamin A und D, im Futter aus dem Bereich der opti-

malen Deckung des Bedarfs in den der Deckung des Minimalbedarfs für das Wachstum ist nicht mit einer Hemmung des Wachstums bzw. der Ausbildung der Hüftgelenkdysplasie zu rechnen.

Das Schrifttum kann bei den Verfassern angefordert werden.

Anschrift der Verfasser:

Prof. Dr. Dr. Dr. h.c. E. Kolb, Semmelweisstr. 4, 04103 Leipzig; Dr. J. Sechawert, Hoffmann-La Roche AG, 79630 Grenzach-Wyhlen; Dr. K. Wiegand, Effem GrubH, 27283 Verden.

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Aus dem Institut für Epidemiologie und WHO Collaborating Centre for Rabies Surveillance and Research, Bundesforschungsanstalt für Viruserkrankungen der Tiere

Stellungnahme zum Artikel »Darstellung der Tollwutsituation in Nordrhein-Westfalen 1985 - 1995; Auftreten und Bekämpfungsstrategie« von A. vom Schloß, TU 52, 540-564, 1997

von H. Schlüter

Ohne auf durchaus bemerkenswerte und diskussionswürdige Definitionen oder Einschätzungen (z.B. Verwendung des Begriffs »epidemiologische Einheit« in Wildbeständen, »Tollwutfreiheit« eines Gebietes oder die im genannten Beitrag vorgenommene qualitative Bewertung der Markerdiagnostik und Alternativimpfstrategien) einzuge-

hen, erscheint es uns wichtig, auf zwei wesentliche Aspekte hinzuweisen:

1. Die Teilflächenbeköderung eines Gebietes kann nur dann als erfolgreich gewertet werden, wenn es gelingt, die Ausbreitung der Tollwut auch auf benachbarte Gebiete zu verhindern. Ein Rückgang der Tollwutfälle im Impfgebiet ist nur ein

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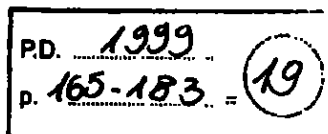
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Effect of age, breed and dietary omega-6 (n-6) : omega-3 (n-3) fatty acid ratio on immune function, eicosanoid production, and lipid peroxidation in young and aged dogs

Robert J. Kearns^{a,*}, Michael G. Hayek^b, John J. Turek^c,
Mohsen Meydani^d, John R. Burr^b, Robert J. Greene^b,
Craig A. Marshall^b, Scott M. Adams^b, Robert C. Borgert^b,
Gregory A. Reinhart^b

^aUniversity of Dayton, Dayton, OH 45469, USA

^bThe Iowa Company, Lewisburg, OH 45338, USA

^cPurdue University, Lafayette, IN 47907, USA

^dTufts University, Boston, MA 02111, USA

Abstract

The focus of this study was to examine the influence of age and diet on various parameters of immune function in young and old Fox Terriers and Labrador Retrievers. Eighteen young and old dogs were utilized for this study. Young and old dogs were fed a basal diet containing an (n-6) : (n-3) ratio of 25 : 1 for sixty days (Phase I). Half of the dogs were then switched to a diet with an (n-6) : (n-3) ratio of 5 : 1, and all were maintained on their respective diets for an additional sixty days (Phase II). Results from these studies revealed an age-associated decline in several immune parameters measured. Both these breeds demonstrated a reduction in sheep red blood cell titers, as well as in their ability to respond to different mitogens. Interestingly, this decline was greater in Fox Terriers, suggesting a decrease in cellular proliferative capacity in lymphocytes isolated from the larger breed. Neither cytokine production or DTH response was affected by age. Diet and breed interactions resulted in a significant increase in T- and B-cell mitogen responsiveness. In contrast, supplementation with n-3 fatty acids did not affect IL-1, IL-6 or TNF- α production. Supplementation with n-3 fatty acids resulted in increased PGE₃ production from peritoneal macrophages but had no effect on PGE₂ production from peripheral blood mononuclear cells or peritoneal macrophages. The n-3 fatty acid supplementation did not influence α -tocopherol status although older dogs had significantly lower serum α -tocopherol concentrations. Oxidative status

* Corresponding author. Tel.: +1-937-229-2510; fax: +1-937-229-2021.
E-mail address: kearns@nedix.dayton.edu (R.J. Kearns).

of these dogs was assessed by serum levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Feeding an n-3-enriched diet did not affect 4-HNE levels but significantly decreased MDA levels in old dogs. In summary, this study indicates that feeding a diet containing an (n-6):(n-3) fatty acid ratio of 5:1 had a positive, rather than a negative, effect on the immune response of young or geriatric dogs © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Canine; Omega-3; Omega-6; Prostaglandin; Age; Immune response; Vitamin E; Lipid peroxidation; Eicosanoids; Cytokine

1. Introduction

The interaction between dietary fat and the immune response has been an area of growing interest. Omega-3 (n-3) fatty acids have been used in veterinary medicine as supplements for pruritic patients to control the inflammatory response that is associated with the disease (Miller et al., 1993; Paradis et al., 1991; Scott and Bueger, 1988). The influence of n-3 fatty acids on inflammation has been suggested to be through the incorporation of these fatty acids into cellular membranes and serving as substrates for eicosanoid metabolism, resulting in the production of eicosanoids with lower inflammatory potential than those produced from omega-6 (n-6) fatty acids (Reinhart, 1996). Recent studies have demonstrated that eicosanoid metabolism can be altered in the dog with dietary adjustments of n-6 to n-3 fatty acids (Vaughn et al., 1994). These studies have led to widespread incorporation of n-3 fatty acids in commercial pet foods.

Increased utilization of n-3 fatty acids has heightened the interest in the interaction between these fatty acids and the immune response. Studies examining this interaction have reported mixed results. While some studies have demonstrated an increase in T-cell mitogen response due to n-3 feeding (Levy et al., 1982; Payan et al., 1986), others have reported a decline in T-cell mediated immunity as well as in the production of interleukin (IL)-1, IL-2, IL-6 and tumor necrosis factor (TNF)B- α (Meydani M. et al., 1991a; Santoli and Zurier, 1989). In dogs, it has been demonstrated that feeding diets containing an (n-6):(n-3) ratio of 1.4:1 results in a decline in delayed type hypersensitivity responsiveness (DTH) which is an *in vivo* measure of T-cell immunity (Wander et al., 1997). Feeding diets containing n-3 fatty acids has been reported to result in decreased serum vitamin E concentrations and increased serum lipid peroxidation markers (Meydani et al., 1991; Wander et al., 1997). These changes have been suggested to contribute to the decline in T-cell responsiveness in the presence of n-3 fatty acids. Studies have suggested that the negative aspects of n-3 fatty acid supplementation may be negated by vitamin E supplementation (Kramer et al., 1991).

A negative interaction between n-3 fatty acids and the immune response would be of particular concern for the geriatric population. The negative influence on the immune response may be more pronounced in the elderly (Levy et al., 1982). The aging process has been associated with a decline in immune responsiveness in a variety of species. The age-associated changes in the immune response have mainly been attributed to a decrease in responsiveness to T-cells (Miller, 1989; Makinidan, 1981). A decline in immunological vigor has been demonstrated in the dog. Laboratory studies have observed a decrease in

mitogen stimulation (Sheffy et al., 1985; Greeley et al., 1996), chemotaxis and phagocytosis (Tizard and Warner, 1987). A recent longitudinal study has reported age-related changes in clinical immunological parameters, such as decreased numbers of white blood cells and immature neutrophils and increased numbers of mature neutrophils and concentration of immunoglobulin G (Strasser et al., 1993). Together, these data demonstrate an age-related decline in immunity in dogs that may increase the susceptibility of the geriatric animal to infection (Mosier, 1989).

Few studies have been conducted to examine the influence of various (n-6) : (n-3) ratios on the immune response of young and old dogs (Wander et al., 1997). The purpose of this study was to examine the influence of diets formulated to contain (n-6) : (n-3) ratios of 25 : 1 or 5 : 1 on the immune response and oxidative status of young and old Fox Terriers and Labrador Retrievers. A secondary objective of this study was to determine if the effect of age on the immune response differs between different breeds.

2. Materials and methods

2.1. Diet

The formulation of the diet is presented in Table 1. The (n-6) : (n-3) fatty acid ratio of the diets was 25 : 1 for the control diet and 5 : 1 for the test diet. The choice of the 5 : 1 (n-6) : (n-3) ratio was influenced by the fact that this ratio was demonstrated to decrease LTB₄ synthesis while increasing LTB₅ synthesis by neutrophils and skin of the dogs (Vaughn et al., 1994).

Table 1
Diet composition

Ingredient	(n-6) : (n-3) fatty acid ratio	
	25 : 1 ^a	5 : 1 ^b
Corn	27.00	27.00
Rice	26.00	26.00
Chicken protein	26.13	26.13
Chicken fat	8.21	6.50
Beet pulp	4.00	4.00
Egg	2.50	2.50
Chicken digest	2.00	2.00
Fish Oil	—	1.65
Yeast	1.00	1.00
Mineral mix ^a	2.91	2.71
Vitamin mix ^b	0.27	0.27
Ground flax seed	—	0.24

^a Diet was formulated to contain a final concentration of 11 000 ppm Ca, 8000 ppm P, 2400 ppm choline, 5500 ppm Na, 8000 ppm Mg, 200 ppm Zn, 400 ppm Fe, 25 ppm Cu, 3.5 ppm I, 800 ppm Mn, 0.2 ppm S, and 0.4 ppm Se.

^b Diet was formulated to contain a final concentration of 15 kIU/kg Vitamin A, 100 IU/kg, Vitamin E, 1200 IU/kg Vitamin D, 13 mg/kg thiamine, 50 mg/kg niacin, 25 mg/kg pantothenate, 5 mg/kg pyridoxine, 0.3 mg/kg biotin, and 1.0 mg/kg folic acid.

2.2. Animals

Eighteen young (9 Labrador Retrievers, mean age 1.5 years; 9 Fox Terriers, mean age 1.8 years) and eighteen adult (9 Labrador Retrievers, mean age 9.6 years; 9 Fox Terriers, mean age 11.5 years) dogs were utilized for this study. During Phase I, all dogs were fed a basal diet containing an (n-6) : (n-3) ratio of 25 : 1 for eight weeks. During Phase II, half of the dogs were switched to a diet containing an (n-6) : (n-3) ratio of 5 : 1 and all dogs were fed their respective diets for the following eight weeks. Blood was drawn from each animal at the end of each respective feeding period.

2.3. Isolation of peripheral blood mononuclear cells

Venous blood was collected into heparinized tubes and diluted threefold with RPMI 1640 medium (GibcoBRL, Grand Island, NY). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque gradients (Pharmacia Biotech, Piscataway, NJ). The fraction containing mononuclear cells was isolated, washed twice, and re-suspended in the RPMI 1640 medium. These cells were then used either to measure mitogen-induced lymphoproliferative responsiveness or cytokine (IL-1, IL-6, TNF- α) levels. For cytokine induction, cells were cultured in RPMI-1640 medium in the presence of 10 μ g/ml of LPS (Sigma, St. Louis, MO) for 24 h. The culture supernatant fluids were collected and any non-adherent cells pelleted by centrifugation. The cell-free supernatants were frozen at -80°C until analysis and the non-adherent and adherent cells digested with 1N ammonium hydroxide and 0.2% Triton X-100 (Sigma, St. Louis, MO) for total cellular DNA quantification.

2.4. Isolation of peritoneal macrophages

Peritoneal cells were harvested by peritoneal lavage using buffered Hanks balanced salt solution. Peritoneal macrophages were re-suspended in RPMI 1640 medium containing 5 mg/ml bovine serum albumin (BSA)(Sigma), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1% L-glutamine (GibcoBRL, Grand Island, NY) and cultured in 25 ml tissue culture flasks (Corning, Corning, NY) for 2 h at 37°C and 5% CO₂. The non-adherent cells were washed off with medium, and the remaining adherent macrophages cultured for 24 h in a medium containing 10 μ g/ml of LPS. Culture supernatant fluids were collected and stored and the cells digested for total DNA as described above.

2.5. TNF bioassay

Assay for TNF-like activity was performed on L929 murine fibroblasts. L929 cells were grown in an RPMI 1640 medium supplemented with 1×10^5 U/l penicillin, 6.9×10^{-3} mol/l streptomycin, 2×10^{-3} mol/l L-glutamine and 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Dilutions of peritoneal macrophage or PBMC culture supernatant fluid (1 : 50 or 1 : 100) were added in octuplicate to 96-well culture plates containing L929 murine fibroblasts. Medium supplemented with actinomycin-D (3.98×10^6 mol/l) was added to each of the wells. After incubation for 20 h at 37°C.

the fluid in the wells was discarded, and 75 μ l of 7.24×10^{-7} mol/l (3-[4,5 dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) (MTT) (Sigma, St. Louis, MO) in Hanks balanced salt solution added to each well. After 3 h, 100 μ l of a solution of 6.9×10^{-1} mol/l SDS in 6.45 mol/l *N,N* dimethylformamide (pH 4.7) (Sigma, St. Louis, MO) were added to each well and incubated at 37°C overnight (Hansen et al., 1989). The absorbency at 550 nm was then determined using a microplate reader. To determine the amount of TNF-like activity, a standard curve was produced for each assay using human recombinant TNF- α (Genzyme, Boston, MA).

2.6. IL-6 Bioassay

The IL-6 bioassay was performed using B9-cells (Aarden et al., 1987). The cells were grown in Iscove's Modified Dulbecco's medium (GibcoBRL) supplemented with 10% (FCS), 40 pg/l of mouse recombinant IL-6, and 50 mM 2-mercaptoethanol (2-ME) (Sigma, St. Louis, MO). Dilutions of PBMC or peritoneal-macrophage culture supernatant fluid (1 : 100 or 1 : 200) were added in octuplicate to 96-well culture plates containing B9-cells. After 72 h of incubation, 20 μ l of 7.24×10^{-7} mol/l MTT in HBSS were added to each well and then, 6 h later, 100 μ l of SDS-DMF. The absorbency at 550 nm was recorded after an overnight incubation at 37°C. A standard curve using recombinant mouse IL-6 (Genzyme, Boston, MA) was used to determine the amount of IL-6 bioactivity.

2.7. IL-1 bioassay

A mouse plasmacytoma cell line (T1165.17) that proliferates in response to IL-1 was used to assay for IL-1 activity. The IL-1 receptor on the T1165.17 cell line was blocked using a monoclonal antibody (LA 15.6) to the IL-1 receptor. The cell line and antibody were generously supplied by Dr. Andrew Glasebrook (Eli Lilly, Indianapolis, IN). The cells were grown in minimal essential medium supplemented with 10% FCS, 50 mM 2-ME, and 200 pg/l of IL-1 β . Cells were seeded in 96-well plates (5×10^3 /ml), and samples assayed in quadruplicate. Four wells were blocked with antibody (1 μ g/ml) 30 min before addition of samples and four wells were unblocked. Samples diluted from 1 : 100 to 1 : 200 were added and incubated at 37°C for 18 h, and then MTT added as described for the IL-6 bioassay. Six hours later, SDS-DMF was added and the plates incubated and absorbency measured as described for the IL-6 bioassay. The difference in absorbency between blocked and unblocked wells, corrected for control levels, determined the amount of IL-1 activity in the culture medium. A standard curve was generated for each assay using mouse recombinant IL-1 β Genzyme, Boston, MA).

2.8. Mitogen-induced lymphocyte proliferation

Peripheral blood mononuclear cells (1×10^5) were dispensed in quadruplicate into individual wells of a 96-well microtiter plate (Costar, Corning, NY) for culture with three concentrations of either Concanavalin A (Con A), Phytohemagglutinin-P (PHA), or Pokeweed mitogen (PWM)(GibcoBRL). Cells were cultured at 37°C, 5% CO₂ for 72 h. Eighteen hours prior to termination, cells were pulsed with 1 μ Ci of tritiated thymidine (ICN

Radiochemical, Irvine, CA). After being harvested onto glass fiber filters, incorporation of tritiated thymidine was determined by liquid scintillation spectroscopy using a Beckman scintillation counter. Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures.

2.9. *In vivo* antibody production

On Day 60 following maintenance on the Phase II diet ((n-6) : (n-3) ratio of 5 : 1), dogs were intramuscularly injected with 5 ml of 10% suspension of sheep red blood cells (S-RBC)(Sigma). Blood samples were taken prior to injection as well as on days 7, 14 and 21 post-injection. Serum was separated by centrifugation and frozen at -20°C until analysis. Serum antibody titers to sheep red blood cells were determined according to the procedure of Wengman and Smithies (1966). Data was expressed as the log base 3 of the reciprocal of the titer dilution.

2.10. Oxidative stress markers

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations in the serum were determined by using a commercial spectrophotometric kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

2.11. Cholesterol and triglyceride analyses

Serum samples were analyzed by a Cobas Mira Analyzer (Roche Diagnostics Systems, Somerville, NJ).

2.12. Vitamin E analysis

Serum vitamin E was analyzed by the HPLC method described previously (Meydani et al., 1989). Tocol (gift from Hoffmann-LaRoche, Nutley, NJ) was used as an internal standard (Handelman et al., 1988), and eluted peaks were detected with a Perkin-Elmer 650-15 fluorescence spectrophotometer (Norwalk, CT).

2.13. Prostaglandin analysis

Concentration of PGE_2 and PGE_3 in supernatants were analyzed using a modified method of Reilly and Blair (Reilly et al., 1988; Blair et al., 1993). Unless otherwise stated, chemicals mentioned were obtained from Sigma. Briefly, 250–500 μl of supernatant were spiked with an internal standard ($\text{PGE}_2\text{-D}_4$; Cayman Chem). After 15 min, 200 μl of methoxyamine (0.5 g/ml) and 30 μl of 88% formic acid (Fisher Scientific, Pittsburgh) were added to the sample and allowed to stand for an additional 15 min. During this time a solid-phase extraction cartridge (Isolute C-18, Jones Chromatography, Lakewood, CO) was prepared by passing 2 ml of ethanol and 2 ml of distilled water through the cartridge. The sample was placed on the cartridge and washed with 2 ml of distilled water followed by 1 ml of hexane. The prostaglandin fraction was collected with 1 ml of methanol and the collected fraction

was dried completely under nitrogen. At the completion of drying, the sample is reconstituted with 100 μ l acetonitrile, 20 μ l *n,n*-diisopropylethylamine and 20 μ l pentafluorobenzylbromide, vortexed, and allowed to react for 15 min. The sample is dried under nitrogen, reconstituted with 30 μ l methanol and placed on a thin layer chromatography plate (Whatman LK6D silica gel, 5 x 20 cm 60A, 25 μ m; Whatman, Clifton, NJ) preconditioned by heating at 120°C for 1 h. Plates were developed in a tank with 50 ml 20% heptane/ethyl acetate. Regions of interest were scraped from each channel, placed in a centrifuge tube after identification by comparing migration patterns to a thin-layer chromatography plate run with 2 mg of derivitized compounds of interest and visualized by charring with 5% CuSO₄. Thirty microliters of distilled water and 1 ml of ethyl acetate were added to each sample tube, vortexed, and ethyl acetate was decanted to a second tube. Another 0.5 ml of ethyl acetate was added to the sample, vortexed, and decanted. The combined ethyl acetate was dried under N₂ and the sample was reconstituted with 20 μ l pyridine and 20 μ l bis-(trimethylsilyl) trifluoroacetamide. The sample was then vortexed and allowed to react for 15 min, then dried under N₂. The sample was reconstituted with 20 μ l of dodecane, vortexed, and transferred to an autosampler vial. Samples (1-4 μ l) were injected and separated on a HP 5890 Series II GC HP 5989B ms engine (Little Falls, DE) with a HP5MS column (30 m, 0.25 mm, 0.25 μ m) with an H₂ carrier gas flow rate of 3 ml/min. The mass spectrometer conditions were set at a source temperature of 150°C and quad at 100°C. PGE₂, PGE₃ and PGE₂-D4 peaks were identified under conditions established with known standards (PGE₂ 524 mass : charge ratio; *m/z*), 8.7 min run time; PGE₃ 522 *m/z*, 8.5 min run time; and PGE₂-D4 528 *m/z*, 8.6 min run time).

2.14. Delayed-type hypersensitivity test (DTH)

Hindquarters were shaved from each animal prior to injection with an area $\approx 2.5'' \times 5''$. Injection sites were marked with a felt pen and a paper card with the preset holes. Injection sites were swabbed with alcohol. One hundred microliters of Con A (0.05%), or saline were injected intramuscularly. Data was recorded as skinfold thickness at time intervals of 24, 48 and 72 h post-injection.

2.15. Statistical analysis

Statistical analysis was conducted using the SAS statistical program (Release 6.03, 1988, SAS Institute, Cary, NC). Data was analyzed by a 2 x 2 factorial two-tailed analysis of variance with individual differences analyzed by single degree-of-freedom comparison using Fischer's least significant difference procedure and are reported as mean $\pm$ standard error. Significance was set at $p < 0.05$.

3. Results

3.1. Age and breed interactions

Data presented in Fig. 1 illustrates that PBMCs obtained from Fox Terriers and Labrador Retrievers exhibit an age-associated decline in their ability to proliferate in

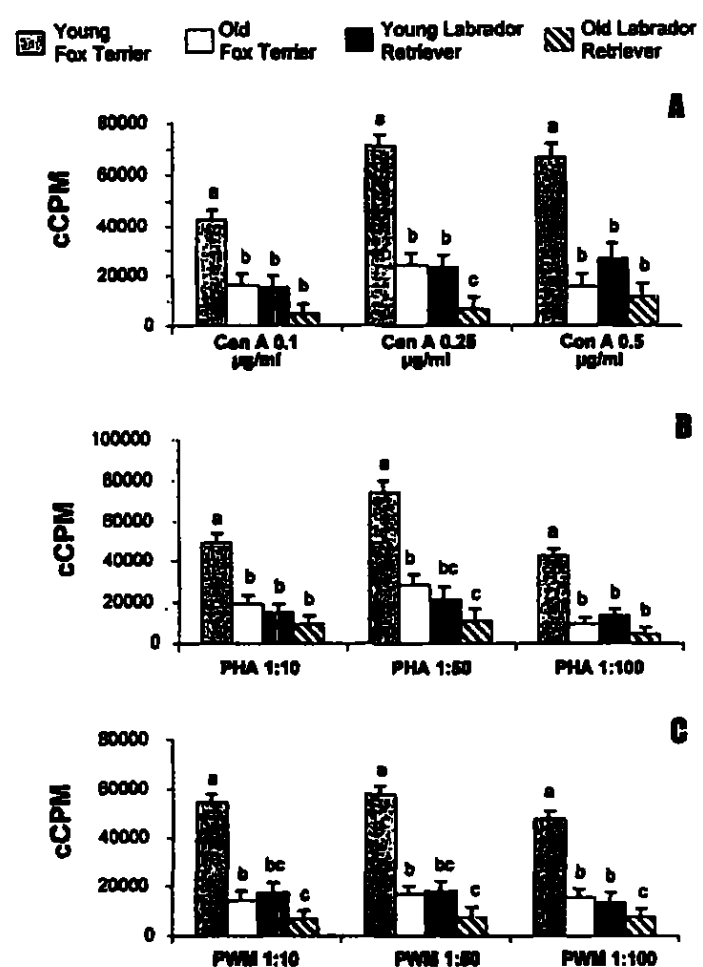


Fig. 1. Effect of breed and age on mitogen response of peripheral blood mononuclear cells from dogs. Peripheral blood mononuclear cells were isolated from heparinized venous blood via density gradient centrifugation over Ficoll-Hypaque gradients. 1×10^5 cells were cultured in quadruplicate in 96-well plates in the presence of media: (A) Con A (0.1; 0.25 and 0.5 µg/ml), (B) PHA (1 : 10, 1 : 50 or 1 : 100 dilution); or (C) PWM (1 : 10, 1 : 50 or 1 : 100 dilution). Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures. Data are expressed as the means $\pm$ SE. Means within the same mitogen concentration that do not share the same superscript are significantly different ($p < 0.05$).

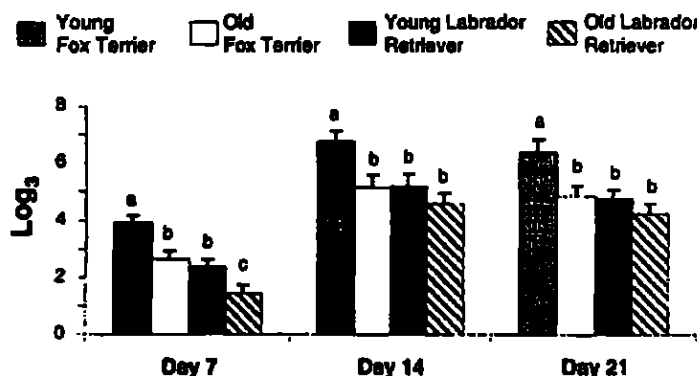


Fig. 2. Effects of breed and age on antibody production to sheep red blood cells. All dogs were administered a 5-ml intramuscular injection of a 10% solution of sheep red blood cells. Blood was collected from these dogs on days 0 (background) 7, 14 and 21 post-injection. Serum titers were determined via a hemagglutination assay. Data is expressed as mean $\pm$ SE of the log base 3 of the inverse of the titer. Means within the same day that do not share the same superscript are significantly different ($p < 0.05$).

response to Con A (Fig. 1(A)), PHA-P (Fig. 1(B)), and PWM (Fig. 1(C)). It is interesting to note that the degree of decline was greater for Fox Terriers than Labrador Retrievers. Also, thymidine incorporation was greater in lymphocytes isolated from Fox Terriers than Labrador Retrievers.

The humoral immune response, measured as antibody titer to S-RBCs, was also affected by age. There was a significant reduction in antibody titer in old vs. young Fox Terrier dogs at 7, 14 and 21 days post-immunization (Fig. 2). In contrast, although old Labrador Retrievers demonstrated lower antibody concentration at each time interval, significance was observed only on Day 7 (Fig. 2). The DTH skin response to PHA, used as an indicator of changes in cellular immunity, revealed no significant age differences (data not shown).

Pro-inflammatory cytokines, such as IL-1, IL-6, and TNF- α , mediate the response of the host to inflammation. Stimulation of either peripheral blood mononuclear cells or peritoneal macrophages obtained from young and old Fox Terriers and Labrador Retrievers with lipopolysaccharide (10 μ g/ml) resulted in no significant differences in TNF- α , IL-1 or IL-6 bioactivity (data not shown).

3.2. Diet and breed interactions

Data presented in Fig. 3 illustrates that switching the diet to an (n-6):(n-3) fatty acid ratio of 5:1 resulted in a significant increase in mitogenic response to Con A, PHA and PWM in young Fox Terriers and Labrador Retrievers. This was a consistent finding independent of mitogen concentration. Older animals, however, exhibited no significant change to any of the mitogens evaluated subsequent to switching these animals to the 5:1 diet. Interestingly, older dogs exhibited significantly lower mitogenic responses than

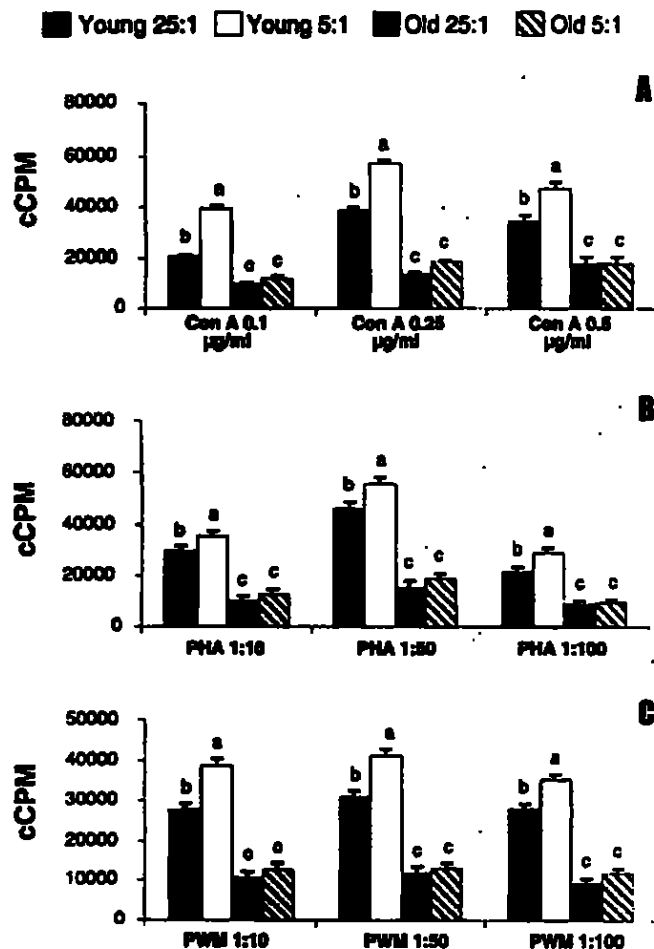


Fig. 3. Effect of n-6 : n-3 fatty acid ratio in the diet on mitogen response of peripheral blood mononuclear cells from dogs. Peripheral blood mononuclear cells were isolated from heparinized venous blood via density gradient centrifugation over Ficoll-Hypaque gradients. 1×10^5 cells were cultured in quadruplicate in 96-well plates in the presence of media: (A) Con A (0.1; 0.25 and 0.5 µg/ml); (B) PHA (1 : 10, 1 : 50 or 1 : 100 dilution); or (C) PWM (1 : 10, 1 : 50 or 1 : 100 dilution). Cells were cultured at 37°C, in 5% CO₂ for 54 h. Cells were then pulsed with 1 µCi of tritiated thymidine and incubated for an additional 18 h. PBMCs were then harvested on fiberglass filters and thymidine incorporation into the DNA was determined via scintillation spectroscopy. Results are presented as corrected CPMs (cCPM), which is the average counts per mitogen-stimulated cultures—average counts of unstimulated cultures. Data are expressed as the mean $\pm$ SE. Means within the same mitogen concentration that do not share the same superscript are significantly different ($p < 0.05$).

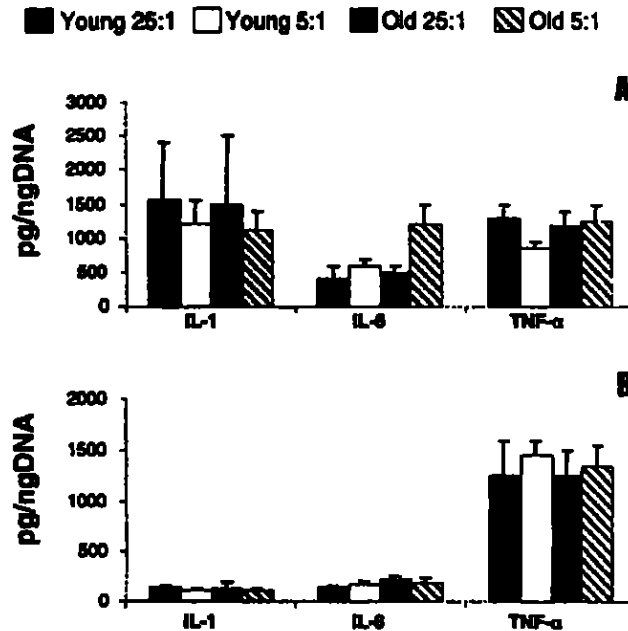


Fig. 4. Effect of n-6 : n-3 ratio in the diet on IL-1, IL-6 and TNF- α production from peritoneal cells (A) or PBMCs (B) from dogs. Two million adherent peritoneal cells or 5×10^6 PBMCs were cultured at 37°C, 5% CO₂ in RPMI 1640 containing 10 μ g/ml LPS. Cells were cultured for 24 h at which time supernatant was collected and stored at -80°C until analysis. Cells were digested with 1 N ammonia hydroxide and 0.2% Triton x-100 for DNA quantitation. IL-1, IL-6 and TNF- α activity in the supernatant was measured by bioassay. Data is expressed as mean $\pm$ SE.

young dogs to both, T- and B-cell mitogens, a fact that was not affected by switching from a low to a high n-3 diet.

The effect of n-3 fatty acid supplementation on the production of pro-inflammatory cytokines is shown in Fig. 4. Feeding a diet containing an (n-6) : (n-3) ratio of 5 : 1 had no significant effect on IL-1, IL-6 or TNF- α production by peritoneal macrophages (Fig. 4(A)) or peripheral blood mononuclear cells (Fig. 4(B)). In addition, omega-3 supplementation also did not influence sheep red blood cell antibody titer response (Fig. 5).

In order to determine if feeding a 5 : 1 ratio affects eicosanoid production, peritoneal macrophages and peripheral blood mononuclear cells were stimulated with LPS (10 μ g) to stimulate prostaglandin production. Neither age nor diet affected PGE₂ production from either macrophages or PBMC (Table 2). Interestingly, there was a significant difference observed in PGE₃ production in PBMCs of young and aged dogs fed the high n-3 diet (Table 2).

There was a significant increase in serum cholesterol, triglycerides, and total lipids observed in aged dogs fed a diet containing an (n-6) : (n-3) fatty acid ratio of 5 : 1

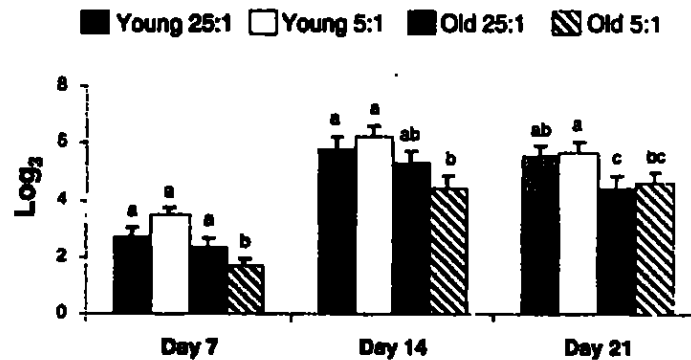


Fig. 5. Effect of n-6:n-3 fatty acid ratio in the diet on antibody production to sheep red blood cells. All dogs were administered a 5-ml intramuscular injection of a 10% solution of sheep red blood cells. Blood was collected from these dogs on days 0 (background) 7, 14 and 21 post-injection. Serum titers were determined via a hemagglutination assay. Data is expressed as mean $\pm$ SE of the log base 3 of the inverse of the titer. Means within the same day that do not share the same superscript are significantly different ($p < 0.05$).

Table 2

Effect of omega-6: omega-3 fatty acid ratio on cellular eicosanoid production, serum cholesterol, triglycerides, α - and γ -tocopherol, MDA and 4-HNE levels in the young and old dogs^{a,b}

Measurement	Young 25:1	Young 5:1	Old 25:1	Old 5:1
PBMC PGE ₂ (ng/mg DNA) ^c	160.09 $\pm$ 16.24a	159.85 $\pm$ 14.23a	85.55 $\pm$ 16.24b	118.31 $\pm$ 14.33b
PBMC PGE ₃ (pg/mg DNA)	94.53 $\pm$ 138.28a	547.29 $\pm$ 146.67b	59.04 $\pm$ 156.80a	782.75 $\pm$ 138.28b
M ϕ PGE ₂ (ng/mg DNA) ^d	131.96 $\pm$ 12.87	132.29 $\pm$ 11.51	114.47 $\pm$ 12.87	108.61 $\pm$ 12.13
M ϕ PGE ₃ (pg/mg DNA)	197.55 $\pm$ 78.77	205.35 $\pm$ 70.46	330.61 $\pm$ 78.77	241.04 $\pm$ 74.27
Cholesterol (mg/dl)	254.44 $\pm$ 4.74ab	242.11 $\pm$ 4.74a	266.12 $\pm$ 5.03b	294.00 $\pm$ 4.74c
Triglycerides (mg/dl)	38.11 $\pm$ 2.33a	40.17 $\pm$ 2.33a	57.51 $\pm$ 2.47b	76.72 $\pm$ 2.33c
Total lipid (mg/dl) ^e	292.56 $\pm$ 6.40a	282.28 $\pm$ 6.40a	323.63 $\pm$ 6.79b	370.72 $\pm$ 6.40c
α -Tocopherol (μ mol/mg total lipid)	8.27 $\pm$ 0.39ab	9.07 $\pm$ 0.39a	7.38 $\pm$ 0.42b	7.40 $\pm$ 0.40b
γ -Tocopherol (μ mol/mg total lipid)	0.27 $\pm$ 0.02a	0.29 $\pm$ 0.02a	0.32 $\pm$ 0.03a	0.21 $\pm$ 0.02b
MDA (μ mol/mg total lipid)	0.45 $\pm$ 0.02a	0.45 $\pm$ 0.02a	0.37 $\pm$ 0.02b	0.29 $\pm$ 0.02c
HNE (μ mol/mg total lipid)	0.35 $\pm$ 0.08	0.33 $\pm$ 0.07	0.37 $\pm$ 0.09	0.17 $\pm$ 0.09

^a Values are means $\pm$ SE. $n = 9$.

^b Means within the same row that do not share the same letters are significantly different ($p < 0.05$).

^c 1×10^6 PBMCs were cultured in RPMI-1640 in the presence of 10 μ g/ml LPS for 24 h at 37°C in 5% CO₂. Supernatants were collected and frozen at -70°C.

^d 2×10^6 Peritoneal cells were adhered to plastic Petri dishes for 90 min. Adherent cells were cultured in RPMI-1640 in the presence of 10 μ g/ml LPS for 24 h at 37°C in 5% CO₂. Supernatants were collected and frozen at -70°C.

^e Total lipid was calculated as the sum of cholesterol plus triglycerides.

(Table 2). The concentration of cholesterol was 10% higher than dogs who were fed an (n-6):(n-3) fatty acid ratio of 25:1; whereas triglyceride and total lipids were 33% and 15% higher, respectively, in aged dogs fed the high n-3 diet.

Feeding dietary n-3 fatty acids have been suggested to increase the oxidative stress of the animal, possibly by decreasing vitamin E levels in the blood and tissue (Tappel, 1972). In our study, oxidative stress was assessed by measuring MDA and HNE. Both of these compounds are by-products of arachidonic acid metabolism and serve as markers for oxidative status of animals (Yu, 1993). There was no difference in HNE levels in young or old animals fed a high n-3 diet, but there was a significant difference in MDA in old dogs maintained on the 5 : 1 diet (Table 2). No significant difference in α -tocopherol levels was seen in young or aged dogs fed the high n-3 diet (Table 2). However, there was a 34% decrease in α -tocopherol levels (0.32 ± 0.03 vs. 0.21 ± 0.02 ; $p < 0.05$) in aged dogs fed the high n-3 diet (Table 2). It should be noted that these parameters were corrected for total lipid concentrations (triglycerides + cholesterol) since total serum lipids were observed to be higher in older dogs.

4. Discussion

The mammalian immune system can be significantly impacted by both, the aging process and nutritional factors such as dietary lipids (Bradley and Xu, 1996; Chandra and Kumari, 1994; Hannigan, 1994; Miller, 1989; Wick and Grubeck-Loebenstein, 1997). Aging has been demonstrated to influence both, the humoral and cellular immune function, as well as cytokine production (Greeley et al., 1996; Hobbs et al., 1993; Makinidan, 1981). Consequently, the aging immune system may contribute to the increased incidence of infectious disease and cancer. Dietary lipids have been reported to modulate the immune system either through regulating cytokines or eicosanoids (Blok et al., 1996; Calder, 1996, 1997; Miles and Calder, 1998; Yaqoob and Calder, 1995). Interestingly, results of these studies are equivocal, depending on the form of fatty acid used (n-6 vs. n-3), concentration added to the diet, and the species studied. To date, most research addressing the impact of aging and/or dietary lipids on immune function has been done in humans and rodents (Meydani S.N. et al., 1991; Payan et al., 1986). Little information is available regarding the effects of either of these variables on the canine immune system (Wander et al., 1997). The purpose of the present study was twofold: first, to determine whether the effect of age on immune function differs between different breeds, and second, what impact does feeding a diet supplemented with different ratios of n-6 to n-3 fatty acids have on the immune function, eicosanoid production, and lipid peroxidation.

Our results indicate that the mitogenic response of PBMCs obtained from aged Fox Terriers and Labrador Retrievers was significantly suppressed following stimulation with Con A, PHA and PWM when compared with young dogs. These findings are consistent with those presented in previous studies using Beagle or Labrador Retriever dogs (Davila et al., 1992; Greeley et al., 1996). In Beagles, however, suppression of lymphoproliferative responses was observed only with PHA, and not Con A (Davila et al., 1992). A key difference noted in our study was that the magnitude of this decline was greater in Fox Terriers as compared to Labrador Retrievers. Moreover, increased thymidine incorporation was the greatest in lymphocytes isolated from the small breed dog. This observed age-associated decline in mitogen response could reflect either a change in the pattern of

cytokines produced by CD4⁺ T-cells (Hobbs et al., 1993), or a shift in the balance of naive cells to memory cells in aged animals (Nijhuis and Nagelkerken, 1992).

Cell-mediated immunity in Fox Terriers or Labrador Retrievers, detected as the DTH response to Con A, was not affected by age. Likewise, stimulation of peritoneal macrophages or adherent PBMCs with lipopolysaccharide resulted in no significant change in the levels of IL-1, IL-6 or TNF- α in these same dogs. There was, however, an age-associated decline in humoral immunity when expressed as a change in antibody titer to sheep red blood cells in young and aged dogs. This is in contrast to data presented in a previous study, in which no difference in antibody titer to keyhole limpet hemocyanin (KLH) was observed between young and old Labrador Retrievers (Greeley et al., 1996). Although the mean titer in old (9.4 years) dogs was less than that in young dogs, it did not represent a significant difference. These contradictory observations may simply reflect differences in the experimental protocols used, i.e. antigen dose, route of antigen administration, or sensitivity of the assay.

The results from our study indicate that supplementing the diet with an (n-6):(n-3) fatty acid ratio of 5:1 had a significant effect on several indexes of the immune function. Mitogen responses to Con A, PHA and PWM were enhanced significantly in young dogs maintained on the high n-3 diet for sixty days. Older dogs exhibit a significantly lower mitogen-induced proliferative response, and although there was a trend toward increased lymphoproliferative responses when these animals were switched to the high n-3 diet, these differences were not significant. Our data does not agree with the previous studies in which subjects maintained on a diet supplemented with n-3 exhibit suppressed blastogenesis of mitogen-stimulated PBMCs (Meydani S.N. et al., 1991). These differences may be attributed to reduced levels of α -tocopherol brought on by the long-term administration of n-3 fatty acids (Meydani et al., 1991). Increased intake of vitamin E has been reported to restore n-3-induced suppressed blastogenic responses (Kramer et al., 1991). It is important to note that in our study, the vitamin E content in the diet was high enough to maintain plasma levels of vitamin E.

The effect of diet and breed interactions on the humoral immune response was not as evident as the age $\times$ breed interactions. The antibody titer against SRBCs in young dogs, when switched to an (n-6):(n-3) ratio of 5:1 diet, increased on Day 7, 14 and 21 post-immunization. There were, however, no significant differences at any time point when compared with animals maintained on the (n-6):(n-3) ratio of 25:1. Aged dogs had reduced antibody titers compared to young dogs when maintained on different diets with varying ratios of n-6:n-3 fatty acids. In contrast to young dogs, aged animals exhibit a decrease in antibody titer when maintained on the high n-3 diet, with significance being observed on Day 7 post-immunization. The absence of any significant change in antibody titer in young animals maintained on a high n-3 diet might reflect a temporal change in the activity of antigen presenting cells. In a previous study, rats fed a high n-3 diet expressed diminished antigen presentation activity in the dendritic cell population (Sanderson et al., 1997). Dendritic cells are a principal cell type not only involved in T-cell activation, both in vivo and in vitro, but they are also required for T-cell-dependent antibody responses.

Pro-inflammatory cytokines, such as IL-1, TNF- α and IL-6, play a critical role in mediating the host response to infection and inflammation (Dinarello, 1992). It has been

proposed that supplementation of the diet with n-3 fatty acids may have therapeutic value in reducing inflammation associated with chronic debilitating illnesses, such as rheumatoid arthritis, colitis and psoriasis (Blok et al., 1996). The proposed mechanism for the mediation of this suppressive effect by dietary fatty acids is attributed to decreased production of pro-inflammatory cytokines. In our study, no differences were detected in IL-1, TNF- α , or IL-6 activity produced by either peritoneal macrophages or PBMC. These results contradict previously published reports evaluating the potential for diets rich in n-3 fatty acids to alter cytokine activity. For example, studies in humans have shown that the use of n-3 fatty acids in the diet decrease the production of pro-inflammatory cytokines (Endres et al., 1989; Meydani et al., 1991). In contrast, mice maintained on a diet high in n-3 fatty acids expressed increased cytokine activity (Hardardottir et al., 1992b; Lokesh et al., 1990). It is possible that these paradoxical responses observed in rodents and humans can, in part, be explained by the utilization of different experimental protocols as well as different cell types (Blok et al., 1996; Calder, 1997). In the canine model, it is possible that the dietary ratio of 5 : 1 is insufficient to alter cytokine activity. Many of the animal studies addressing the effects of dietary n-3 fatty acids on cytokines have used lower ratios of (n-6) : (n-3) to achieve the alteration in cytokine production (Hardardottir and Kinsella, 1991; Lokesh et al., 1990; Turek et al., 1991).

An alternative explanation for dietary lipids not altering cytokine activity in the canine model could be found in levels of eicosanoids produced by PBMC or peritoneal macrophages. It is known that eicosanoids regulate cytokine production by macrophages, and that supplementing the diet with fish oil results in decreased production of arachidonic acid-derived eicosanoids (Lokesh et al., 1986; Hardardottir and Kinsella, 1991, 1992a). In this study, there was no difference in PGE₂ levels detected in PBMCs or peritoneal macrophages. These data are consistent with those presented in a previous study in which no significant differences were detected in the concentration of PGE₂ in stimulated mononuclear cells obtained from geriatric beagles maintained on a low (33 : 1) or medium (5.4 : 1) fatty acid diet (Wander et al., 1997). There was a significant difference, however, in dogs fed a high (1.4 : 1) diet, as evidenced by a 52% reduction in PGE₂. This is in contrast to data on leukotriene production from neutrophil and skin in beagles in which feeding a diet with an (n-6) : (n-3) ratio of 10 : 1 or 5 : 1 resulted in decreased leukotriene B₄ production and increased LTB₄ production (Vaughn et al., 1994). This difference may be attributed to the examination of different eicosanoids or differences in tissues and/or cells examined. It should be noted that the reduction in PGE₂ synthesis from PBMCs of beagles fed a 1.4 : 1 fatty acid diet was associated with a decrease in DTH response signifying a depression in cell-mediated immune response.

Although there was no detectable difference in PGE₂ levels in young and old dogs maintained on the high n-3 diet, there was a significant difference in the PGE₃ levels observed in PBMCs of young and old dogs fed the high n-3 diets. This increase in PGE₃ production was not observed in the peritoneal macrophages isolated from dogs fed the high n-3 diet. This may be explained by the fact that PBMCs represent a heterogeneous population of T- and B-cells as well as macrophages. In this population, macrophages are the main source for prostaglandin production (Goldyne and Stobo, 1982; Goldyne, 1986). However, it has been demonstrated that arachidonic acid (AA) released from lymphocytes

can be used to synthesize cyclo-oxygenase products (Samuelson, 1995). Feeding a diet high in n-3 fatty acids increases membrane composition of eicosapentanoic acid (EPA) (Hwang et al., 1988) which promotes the generation of 3-series PG (Knapp, 1990). It is possible that the increase in PGE₂ synthesis observed in PBMCs is due to the release of EPA instead of AA and subsequent utilization by the macrophages.

The beneficial effects of dietary n-3 fatty acids in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, and colitis, as well as heart disease are well documented (Blok et al., 1996; Herold and Kinsella, 1986; Glomsaet, 1985). Additionally, the therapeutic benefits derived from these diets have been attributed to an impact on immune function. Ironically, a potential harmful effect of these diets rich in n-3 fatty acids may be increased levels of lipid peroxidation, the products of which may contribute to the onset and development of age-associated diseases. In this study, the level of lipid peroxidation in young dogs was unaffected by diet. In contrast, aged animals demonstrated a decrease in MDA levels after being switched to the high n-3 diet. These results are not consistent with previously reported data. In a study conducted with young and older women, it was shown that supplementation of the diet with n-3 fatty acids resulted in an increase in MDA levels, and this increase was greater in older women (Meydani et al., 1991). More recently, it was reported that aged beagle dogs, maintained on a n-3 fatty acid diet of 1.4:1, exhibited a significant increase in plasma and urine TBARS associated with a decrease in plasma α -tocopherol levels (Wander et al., 1997). We did not see a decrease in α -tocopherol in our study which may explain this discrepancy.

The main conclusion from this study was that feeding a diet containing an (n-6):(n-3) ratio of 5:1 did not have a negative effect on the immune response of young or geriatric dogs. If anything there was an increase in T- and B-cell activation in the cells isolated from the dogs fed an (n-6):(n-3) ratio of 5:1. Several interesting breed and age differences were observed in this study. First, the metabolism of n-3 fatty acids appears to be differentially regulated by breed and age in the dog. These breed-associated differences in fatty acid metabolism may explain why not all atopic dogs respond to fatty acid therapy. This suggests that consideration of specific dietary fatty-acid profiles may be warranted for different breeds and stages of life. Secondly, T- and B-cell proliferative ability appears to be lower in Labrador Retrievers than in Fox Terriers. How this effects the health of these two breeds will require further study.

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