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Original Article

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Abstract

The New Zealand green-lipped mussel (*Perna canaliculus*) (NZGLM), a well-known source of n-3 PUFAs, has been shown to have a highly-potent anti-inflammatory action. The dietary fatty acid supplement used in this study was obtained from the NZGLM by a patented supercritical carbon dioxide (CO_2) extraction method. Effects of the dietary PUFA supplement on healthy Beagle dogs on hematology, blood chemistry and blood coagulation profiles were evaluated. A randomized complete blocked design was assigned to 40 healthy Beagle dogs aged between 1 to 3 years old. All dogs were separated into 4 groups, each of which had 5 males and 5 females. Adaptation and test periods were of 2 and 8 weeks duration, respectively. Four dietary treatments were composed of basal diet plus 20 empty capsules as placebo (negative control), basal diet plus 2 (the recommended dose), 6 (3 times the recommended dose) and 20 capsules (10 times the recommended dose) of n-3 PUFAs (PCSO-524®: Antinol®). Clinical examination, body weights, CBC, blood chemistry analyses and coagulation profile were performed prior to and after providing the supplement. Bodyweight of the dogs in all treatment groups were about the same when compared between before and after receiving the dietary supplement. General physical examination did not find abnormalities of the ocular, nervous, musculoskeletal, or integumentary systems during the 8 weeks of the treatment period. For clinical observations, no signs of illness or behavioral change were observed. Parameters that indicated kidney and liver functions appeared to be normal after the administration of n-3 PUFA supplement at all various doses for 8 weeks. After the administration of dietary polyunsaturated fatty acids to the healthy Beagle dogs for 8 weeks, there was no observable change in feed intake, behavior, CBC, blood chemistry values and coagulation profile. Therefore, this supplement did not have any side effects.

Keywords: anti-inflammation, dog, New Zealand green-lipped mussel, polyunsaturated fatty acids

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Introduction

For decades, specific dietary fatty acid supplements, mainly omega-3 polyunsaturated fatty acids (n-3 PUFAs), e.g. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been known for their cardioprotective effect (Adkins & Kelley, 2010), anti-inflammatory effect and immunomodulatory activity (Anderson and Ma, 2009; Fritsche, 2006). As well as fish oils, the New Zealand green-lipped mussel (*Perna canaliculus*) (NZGLM), a well-known source of n-3 PUFAs, has been shown to have a more potent anti-inflammatory action than fish oil or drugs (Whitehouse et al., 1997).

Lipids extracted from the New Zealand green-lipped mussel contain a complex mixture of mainly phospholipids (PL, 57-79%), triglycerides (TG, 10-25%), free-fatty acids (FFA, 7-12%) and sterols (ST, 12-18%) (Murphy et al., 2002). The total content of fatty acids (FA) contained in the NZGLM lipid extract was 0.664 g/mL. The n-3 and n-6 polyunsaturated fatty acids (PUFAs) found in this lipid extract were composed of, in descending order, EPA (20:5n-3), DHA (22:6n-3), the two main n-3 PUFAs at low concentrations were stearidonic acid (SA; 18:4n-3), arachidonic acid (AA; 20:4n-6), a-linolenic acid (ALA; 18:3n-3), docosapentaenoic acid (DPA; 22:5n-3), heneicosapentaenoic acid (HPA; 21:5n-3) and some others in levels less than 0.50% (Wolyniak et al., 2005). Many green-lipped mussel powder products are available on the market now. Many methods used to open the mussel shell, heat and oxidation can destroy the essential lipid fractions and other active compounds. To preserve the anti-inflammatory action of the extracted lipids used in the current study, no heat was used in this process. The n-3 PUFAs were obtained from the NZGLM by centrifugation after the mussels had been crushed and stabilized. The mussels were freeze-dried and extracted by a patented supercritical carbon dioxide (CO_2) method (Wolyniak et al., 2005). This PUFA supplement has been used to treat canine allergic skin diseases (Mueller et al., 2004) and osteoarthritis (Roush et al., 2010). The safety of n-3 PUFAs used was determined as maintenance therapy for children who had moderate asthma and was found to be a safe nutritional supplement, however information regarding the safety of n-3 PUFAs used for dogs is limited. Therefore, this current study aimed to evaluate the side effects of various doses of the dietary polyunsaturated fatty acids supplement in healthy Beagle dogs and to compare haematology, blood chemistry and blood coagulation profile of dogs that received various doses of n-3 PUFAs.

Materials and Methods

Animals: Forty healthy Beagle dogs (20 males and 20 females) aged between 1 to 3 years old, which had body weights between 10 and 15 kilograms, were used in the study. The dogs were sourced from commercial breeders in Thailand. Before the study began, all these healthy dogs were wormed and vaccinated against rabies, hepatitis, viral enteritis and distemper. Selamectin (Revolution®) was used to prevent heartworm and external parasites. The dogs were kept individually in stainless steel cages (0.90 x 1.0 x 1.05 m^3) in a conventional kennel that had natural light and was kept at an ambient temperature. The cages were cleaned twice a day. All dogs were separated into 4 groups, each of which had 5 males and 5 females. Duration of adaptation and test periods were 2 and 8 weeks, respectively. Every day, after the morning meal, all dogs were free to exercise for at least 30 minutes per day.

Feed and feeding: Premium commercial dog food (Science Diet® for adult) was used as a basal diet throughout the study. This diet contained 24% crude protein (min), 8.9% crude fat (min) and 12.4% crude fiber (max) on a dry-matter basis. Amounts of feed for each individual dog were calculated by the use of the following formula: maintenance energy requirement (kcal/day) = 132 x body weight (kg) 0.75. The dogs were fed two meals per day, at 8:00 am and 4:00 pm. Fresh water was supplied ad libitum.

The dietary PUFA supplement was given once each day with the morning meal. The recommended dose of this PUFA supplement was 1 to 2 capsules (248 mg) per 20 kg body weight/day. For weight greater than 20 kg the dose was 2 to 4 capsules/day. Therefore, treatments used during the current study were (VICH, 2009):

Group 1: basal diet plus 20 empty capsules as placebo
Group 2: basal diet plus 2 capsules (1x normal dose)
Group 3: basal diet plus 6 capsules (3x normal dose)
Group 4: basal diet plus 20 capsules (10x as maximum dose)

Tables 1 and 2 show the active ingredients and nutrient content of n-3 PUFAs used (Antinol®). The manufacturer, Pharmalink International Co. Ltd., provided laboratory analyses which stated that each capsule contained less than 0.1 ppm of heavy metals (lead, arsenic, cadmium and mercury); less than 0.02 pm of pesticide residue (organochlorine and organophosphorus pesticides); less than 0.002 ppm of dioxins and furans; and less than 6.0 mEq/kg of peroxides. Microbial determination met the standard allowance for a dietary supplement (Table 3).

Table 1 Composition of dietary polyunsaturated fatty acids supplement

n-3 PUFAs	mg/capsule
PCSO-524® mussel oil (*Perna canaliculus*)	**50**
Olive oil (food grade)	**100**
□-tocopherol	**0.225**

Sample collection and determination: Clinical examination that included animal behavior and any abnormal signs was performed every day. Body weights of all the dogs were measured and recorded, then used for the calculation once per week of the amount of feed.

Before the beginning of the study and at intervals of 2 weeks during the study, a total of approximately 3 ml (1 ml with EDTA and 2 ml with no anticoagulant) of blood samples were collected from either cephalic or saphenous veins for determination of haematology, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate transaminase (AST), alkalinephosphatase (ALP), total

protein, albumin, glucose, cholesterol, and blood parasites. Every 4 weeks, 2 ml of blood was collected and analyzed to measure trisodium citrate for thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (aPTT).

Statistical analysis: Results are expressed as means. Data analyses were carried out as a randomized complete blocked design. A general linear model (GLM) was used to analyze effects of treatments. Significant differences of data between the treatment means were tested by Duncan's multiple-range test at α=0.05. A Student's test or Wilcoxon's rank-sum test was used to analyze differences between baseline (week 0) and week eight values. A P value of less than 0.05 was considered to be significantly different.

Results

Body weight and blood samples: The bodyweights of all the dogs at the beginning and at the end of the test period were about the same. The feed intake was not significantly different between any of the treatment groups. Evaluation of fecal excretion found that all the dogs defecated normally during the eight-week test. None of them had diarrhea or constipation.

General physical examination did not find abnormalities of the ocular, nervous, musculoskeletal, or integumentary systems during the 8 weeks of the safety test. For clinical observations, no sign of illness or behavioral change (which included anorexia and depression) was identified during the test period. All 40 dogs appeared to have regular feed intake and activity.

Table 2 Nutritional analysis of dietary polyunsaturated fatty acids supplement (Pharmalink International Co. Ltd)

Nutrient Contents	Amount per 100 g	Analysis Method
Energy	3,702 kJ or 884 kcal	By calculation
Crude protein	< 0.1 g	AOAC 18th Edition 981.10 mod
Carbohydrate	< 0.1 g	By calculation
Total fat	100 g	AOAC 18th Edition 948.15 mod
Saturated fat	21 g	AOAC 18th Edition 963.22
Cholesterol	186 mg	JAOAC 76, 4, 1993
Monounsaturated fat	54 g	AOAC 18th Edition 963.22
Polyunsaturated fat	22 g	AOAC 18th Edition 963.22
Omega-3 fatty acids	14 g	AOAC 18th Edition 963.22
Vitamin A (as retinol)	1 mg	COST 91 p23-32 (mod)
Vitamin E	188 mg	COST 91 p97-106 (mod)
Salt (chlorine as NaCl)	< 0.1 g	AOAC 18th Edition 937.09
Ash	< 0.1 g	AOAC 18th Edition 938.08 mod
Moisture at 70°C	< 0.5 g	AOAC 18th Edition 926.12

Table 3 Microbial determination of dietary polyunsaturated fatty acids supplement (Pharmalink International Co. Ltd)

Microbial Type	Result	Unit	Determination Method
Total aerobic plate count	< 100	Gram	Compendium 4th Edn 2001
Yeast and mold count	< 100	Gram	Compendium 4th Edn 2001
Escherichia coli	Not detected	Gram	Compendium 4th Edn 2001 mod
Salmonella spp.	Not detected	10 Grams	ISO 6579:2002 (E)

Table 4 Haematology values of the dogs at the beginning and the end of this safety study

Parameter	Normal value	Control		1X		3X		10X		
		Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	SE
RBC ($x10^6$)/ul	5.5-8.5	6.10	6.54	5.78	6.38	6.24	6.48	6.17	5.86	0.08
Haemoglobin, g/dl	12.0-18.0	13.2	15.6	13.0	14.9	13.9	14.9	13.6	13.6	0.19
Haematocrit, %	37.0-55.0	38.2	40.0	37.9	39.6	37.7	39.2	36.5	36.0	0.43
Indices -MCV	60.0-77.0	62.8	61.0	62.1	61.7	61.0	62.5	61.8	61.6	0.27
-MCH	19.5-25.5	22.8	23.4	23.4	23.5	22.8	22.8	22.8	23.3	0.08
-MCHC	32.0-36.0	36.4	36.1	36.3	36.0	36.3	35.6	36.1	36.4	0.11
Platelet ($x10^3$)/ul	200-500	340	298	287	311	340	355	295	247	6.88
WBC ($x10^3$)/ul	6.0-17	12.8	11.0	9.07	8.75	10.8	8.4	10.6	9.12	0.23
Neutrophils, %	60-77	66.8	66.8	66.5	70.2	77.2	76.4	68.3	68.6	0.68
Bands, %	0-3	0.20	0.00	0.20	0.00	0.00	0.00	0.20	0.00	0.03
Eosinophils, %	2.0-10.0	3.10	3.90	3.90	4.10	2.6	4.0	2.50	3.80	0.20
Lymphocytes, %	12.0-30.0	25.2	24.3	24.7	20.3	16.0	15.7	24.5	24.2	0.66
Monocytes, %	3.0-10.0	4.50	5.00	4.70	5.40	4.00	3.90	4.50	3.40	0.21

Table 5 Blood chemistry values of the dogs at the beginning and the end of this safety study

Parameter	Normal value	Control		1X		3X		10X		
		Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	SE
AST (SGOT), units	5.0-80	41.8	35.5	40.0	53.8	48.7	39.9	34.7	38.7	0.90
ALT (SGPT), units	5.0-50	37.3	46.2	30.8	42.6	44.4	47.6	32.7	37.8	0.94
Alk.P/tase, IU/Ls	20.0-120.0	76.9	53.5	78.0	85.5	58.9	53.3	74.6	53.3	2.40
BUN, mg%	10.0-22.0	10.2	13.3	10.3	13.5	10.2	13.4	9.3	14.2	0.39
Creatinine, mg%	0.4-1.5	1.07	1.03	1.02	0.97	1.03	0.94	0.95	0.99	0.06
Glucose, mg%	50.0-120.0	81.0	59.5	68.3	57.3	82.7	59.9	80.5	55.3	1.11
Total protein, g%	5.4-7.8	7.30	7.01	6.12	6.10	8.41	7.56	7.45	6.91	0.11
Albumin, g%	2.2-3.4	2.90	2.81	2.79	2.80	3.11	3.25	2.71	2.64	0.05
Cholesterol, mg%	125-300	149	106	144	117	146	127	160	130	2.38
Sodium, mEq/L	144-154	149	151	146	150	148	152	150	152	0.24
Potassium, mEq/L	3.8-5.8	5.51	5.20	5.22	5.27	5.42	5.37	5.47	5.31	0.03
Choride, mEq/L	93-121	113	113	112	113	111	114	112	113	0.22

Table 6 Coagulation profile of the dogs at the beginning and the end of this safety study

Parameter	Control		1X		3X		5X			
	Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	Wk0	Wk8	P-value	SD
Trombin time, sec	11.6	6.9	12.6	6.8	10.2	4.6	9.7	5.7	0.75	2.91
Prothrombin time, sec	7.1	9.9	6.8	10.7	6.1	8.2	7.3	8.6	0.23	1.56
Activated partial thromboplastin time, sec	18.3	23.5	18.8	23.6	16.9	17.6	17.0	17.3	0.06	2.79

Blood analyses for haematology and blood chemistry values are shown in Tables 4 and 5. Most haematology values (which included RBC, haemoglobin, haematocrit, MCV, MCH, MCHC, platelet, WBC, neutrophils, bands, eosinophils, lymphocytes and monocytes of all treatment groups and the times of collection) fell in the normal range (Merck Veterinary Manual, 2011).

These results were also the same for blood chemistry values, e.g. AST, ALT, ALP, BUN, creatinine, total protein, albumin, glucose, cholesterol and electrolytes (Na+, K+ and Cl-). No blood parasites were found in any dogs during the test period. No significant differences were observed for such parameters. Comparison of coagulation profile between before and after receiving the dietary n-3 PUFA supplement including TT, PT and aPTT (Table 6) were not significantly different.

Discussion

Nutrient contents: The Veterinary International Conference on Harmonization (VICH) (2009) released the latest version of Target Animal Safety for Veterinary Pharmaceutical Products in July, 2009. This guideline indicates that the plan for margin of safety used during a study should include a negative control, the highest recommended dose level and 2 multiples of this dose (in most cases three times and 5 times for a period of time in excess of the recommended maximum duration of use). The dietary treatments of the current safety study were designed to comply with this VICH guide. The test period was only eight weeks, because the largest test dose was 5X of the recommended dose.

In general, proteins are substances, which mainly cause an allergic reaction. The n-3 PUFAs are not considered as regular allergen for this reaction. Nonetheless, classes of polyunsaturated fatty acids are highly prone to lipid peroxidation, which creates compounds that are toxic to the liver. They may also inhibit the higher homologous synthesis of essential fatty acids, alteration of membrane structures and impairment of immune function (Halliwell and Chirico, 1993; Martinez and Ballabriga, 1987).

Blood Analyses: Parameters that indicated kidney and liver functions appeared to be normal after the administration of n-3 PUFA at various doses for eight weeks. The cyclooxygenase-mediated generation of thromboxane A2 from ARA in platelets has an important role in blood coagulation (Lien, 2009). Feeding diets containing high levels of fish or fish oils can possibly reduce platelet aggregation through reduction in platelet ARA concentrations (Mori et al., 1997) and through cyclooxygenase-mediated generation of thromboxane A3 (not a potent platelet aggregator) from EPA (Bays, 2007). DHA supplement at doses as large as 6 g/day does not have any effects on platelet aggregation or other clotting parameters in normal ones (Lien, 2009). In general, the DHA-induced increase in LDL-C was compensated for increases in LDL particle size, HDL-C and HDL2/HDL3 ratio. DHA does not appear to have an overall negative effect on the lipid profiles of humans, even though it is hard to separate all the variables (Lien, 2009). Supplementation of DHA does not increase lipid peroxidation (Lien, 2009). Regarding the DHA-mediated inhibition of such immune cell functions with increased DHA intake levels, persons may be at potentially greater risk for incidence of infections when they consume advanced levels of DHA (Lien, 2009). These previous reports were in agreement with the results found in the current study that there was no significant difference in the blood platelet level, or coagulation time. Moreover, there are no reliable reports on literature concerning disease or allergenic responses to this dietary PUFA. No production of such abnormal signs were observed, in line with the absence of literature reports of toxicity and with the results of toxicological tests.

So far, the Association of American Feed Control Officials (AAFCO) (2013) does not have a recommendation for the requirement of PUFAs in dogs. No statistically significant differences in such parameters were observed with the dose of n-3 PUFA used as supplement for infants ranging from 0.1% to 1.0%, with DHA as the outstanding n-3 LC-PUFA in many studies (Makrides et al., 2005). The concentration of n-3 PUFA used in this safety study was 22 mg/100 g Antinol® or about 0.022%. This concentration seems to be quite low if compared with the therapeutic dose required for humans. In addition, the US Food and Drug Administration has make firmed the general safety of both DHA and EPA. These n-3 LC-PUFAs are mainly recognized as safe at levels of up to 3 g/day (Food and Drug Administration (FDA, 1997).

In conclusion, the dietary polyunsaturated fatty acid supplement PCSO-524® in healthy Beagle dogs demonstrated no change in feed intake, behavior, CBC and blood chemistry values, which suggested that this supplement did not have a harmful effect. In addition, there was no sign of an adverse effect after the administration of 20 capsules of dietary supplement (10 times of the recommended dose) for eight weeks.

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บทคัดย่อ

ผลของอาหารเสริมกรดไขมันไม่อิ่มตัวในสุนัขพันธุ์บีเกิ้ลสุขภาพดี

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หอยแมลงภู่นิวซีแลนด์ *(Perna canaliculus)* (NZGLM) เป็นแหล่งของกรดไขมันไม่อิ่มตัวกลุ่มโอเมกา 3 ที่รู้จักกันอย่างแพร่หลายว่ามีฤทธิ์ต้านการอักเสบ อาหารเสริมกรดไขมันที่ใช้ในการศึกษาครั้งนี้เป็นสารสกัดจากหอยแมลงภู่นิวซีแลนด์ด้วยกรรมวิธี supercritical carbon dioxide (CO_2) ซึ่งได้รับการคุ้มครองสิทธิบัตร ทำการประเมินผลของอาหารเสริมกรดไขมันไม่อิ่มตัวในสุนัขพันธุ์บีเกิ้ลสุขภาพดีโดยการตรวจค่าทางโลหิตวิทยา องค์ประกอบทางเคมี และการแข็งตัวของเลือด วางแผนการทดลองแบบสุ่มบล็อกสมบูรณ์ แบ่งสุนัขทดสอบจำนวน 40 ตัว อายุระหว่าง 1-3 ปี ออกเป็น 4 กลุ่ม แต่ละกลุ่มประกอบด้วยสุนัขเพศผู้จำนวน 5 ตัว และเพศเมียจำนวน 5 ตัว ระยะปรับตัวและระยะทดสอบนาน 2 และ 8 สัปดาห์ ตามลำดับ กลุ่มทดสอบทั้ง 4 ประกอบด้วย กลุ่มควบคุมซึ่งได้รับอาหารพื้นฐานเสริมแคปซูลเปล่า 20 แคปซูล กลุ่มทดสอบที่ได้รับอาหารพื้นฐานเสริมกรดไขมันไม่อิ่มตัวกลุ่มโอเมกา 3 (PCSO® -524 : Antinol®) จำนวน 2 แคปซูล (1 เท่าของขนาดแนะนำ) 6 แคปซูล (3 เท่าของขนาดแนะนำ) หรือ 20 แคปซูล (10 เท่าของขนาดแนะนำ) ทำการตรวจร่างกายทางคลินิก ชั่งน้ำหนักตัวสัตว์ ตรวจค่าทางโลหิตวิทยา องค์ประกอบทางเคมีและรูปแบบการแข็งตัวของเลือดก่อนและหลังทดสอบ พบว่าน้ำหนักตัวสุนัขทุกกลุ่มทดสอบไม่แตกต่างกันเมื่อเปรียบเทียบระหว่างก่อนและหลังการให้อาหารเสริม การตรวจทางกายภาพทั่วไปตลอดระยะทดสอบนาน 8 สัปดาห์ไม่พบความผิดปกติของระบบตา ประสาท กล้ามเนื้อหรือผิวหนัง การสังเกตอาการทางคลินิกไม่พบอาการผิดปกติหรือการเปลี่ยนแปลงใดทางพฤติกรรมหลังจากสุนัขได้รับอาหารเสริมกรดไขมันไม่อิ่มตัวกลุ่มโอเมกา 3 ติดต่อกันนาน 8 สัปดาห์ที่ทุกระดับการทดสอบตัวชี้วัดการทำงานของไตและตับมีค่าปรากฏอยู่ในช่วงปกติ โดยสรุป การศึกษาครั้งนี้ไม่พบการเปลี่ยนแปลงหรือความผิดปกติของปริมาณอาหารที่สัตว์ได้รับ พฤติกรรม ค่าทางโลหิตวิทยา องค์ประกอบทางเคมีและรูปแบบการแข็งตัวของเลือด แสดงว่าการใช้อาหารเสริมกรดไขมันไม่อิ่มตัวกลุ่มโอเมกา 3 ติดต่อกันนาน 8 สัปดาห์ไม่มีผลข้างเคียงใดในสุนัขพันธุ์บีเกิ้ลสุขภาพดีที่ใช้ในการทดสอบทั้งหมด

คำสำคัญ: ฤทธิ์ต้านการอักเสบ สุนัข หอยแมลงภู่นิวซีแลนด์ กรดไขมันไม่อิ่มตัว

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