DOI:10.1021/jf903384c

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Acute and Repeated Dose (28 Days) Oral Safety Studies of an Alkoxyglycerol Extract from Shark Liver Oil in Rats

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Shark liver oil has been used for over 50 years as both a therapeutic and preventive agent. The active ingredients in shark liver oil have been found to be a group of ether-linked glycerols known as alkoxyglycerols. Despite its popularity, there is little published toxicology data on alkoxyglycerols. The toxicity of a supercritical fluid extract of shark liver oil (AKG-1 extract) has been evaluated in acute and repeated dose (28 days) oral toxicity studies in rats at doses of 200 and 100 times the maximum recommended dose by supplement manufacturers in humans, respectively. The AKG-1 extract administered in a single oral gavage dose of 2000 mg kg^{-1} of body weight resulted in no adverse events or mortality. The AKG-1 extract administered as a daily dose of 1000 mg kg^{-1} of body weight for 28 days by gavage resulted in no adverse effects or mortality. For both studies, no abnormal clinical signs, behavioral changes, body weight changes, or change in food and water consumption occurred. There were no changes in hematological and serum chemistry values, organ weights, or gross or histological characteristics. It is concluded that the AKG-1 extract is well tolerated in rats at an acute dose of 2000 mg kg^{-1} and at a subchronic (28 days) dose of 1000 mg kg^{-1}.

KEYWORDS: Alkoxyglycerols; toxicity; rats

INTRODUCTION

Shark liver oil is used in traditional medicine in Europe for multifunctional properties such as stimulation of hematopoiesis and wound healing (*1*). Shark liver oil has been found to contain a group of ether-linked glycerols known as 1-*O*-alkylglycerols (*2*). Shark liver oil is a natural source of diesterified alkoxyglycerols, together with squalene and triacylglycerols. **Figure 1** compares the chemical structure of alkoxyglycerols and acylglycerols. Comparatively, a high content of nonsubstituted glycerol ethers along with traces of methoxy-substituted compounds are found in the human bone marrow. These natural etherlipids or alkoxyglycerols have been the subject of much attention because of their multiple biological activities (*3*−*6*). Anticarcinogenic and immune stimulator properties have been attributed to dietary ingestion of these substances (*1*, *7*−*10*). They are located in the human body mainly in the cells of the immune system and in higher doses in human breast milk. In some circumstances, the endogenous synthesis is reduced and the oral administration of alkoxyglycerols is recommended. An improvement in offspring health status by shark liver oil supplementation to the mother has been observed (*11*, *12*); therefore, it could be of interest for optimization of the lipid diet during and after pregnancy (*12*). In the field of fertility, beneficial effects *in vitro* of alkoxyglycerols on boar sperm motility and fertility have been observed (*13*, *14*). As an adjunct therapy to traditional cancer treatment and for prophylactic and therapeutic measures in immune deficiency, the approximate maximum dosage would be 600 mg of alkoxyglycerols per day (i.e., 10 mg kg^{-1}) (*1*).

In another context, the inadequate drug delivery across the blood-brain barrier is a major factor that explains the poor response rates of chemosensitive brain tumors. The blood-brain barrier represents the major impediment to successful delivery of therapeutic agents to target tissue within the central nervous system. The transfer of a variety of chemotherapeutic drugs across the blood-brain barrier was shown to be increased dramatically by intracarotid drug administration in the presence of alkylglycerols (*15*−*17*). Alkylglycerols are thought to be a very promising principle to facilitate the transport of therapeutics across the blood-brain barrier. Alkylglycerols increase delivery of compounds to normal brain and brain tumors, and this effect is mediated at least in part by enhanced permeability of the tight junctions (*18*).

Shark liver oil is available as a dietary supplement in capsule and liquid forms. Currently, there is variation in the purity and content of alkoxyglycerols in preparations labeled as shark oil. It is important to check the purity as well as the standardization of the contents of alkylglycerols. The usual dosage forms are capsules with 250−500 mg of shark liver oil containing 20% of alkylglycerols, which means there is 50−100 mg of available alkylglycerols. In human, dosages of 100 mg three times a day have not shown to cause adverse events (*1*). Shark liver oil extracts

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 Published on Web 12/18/2009

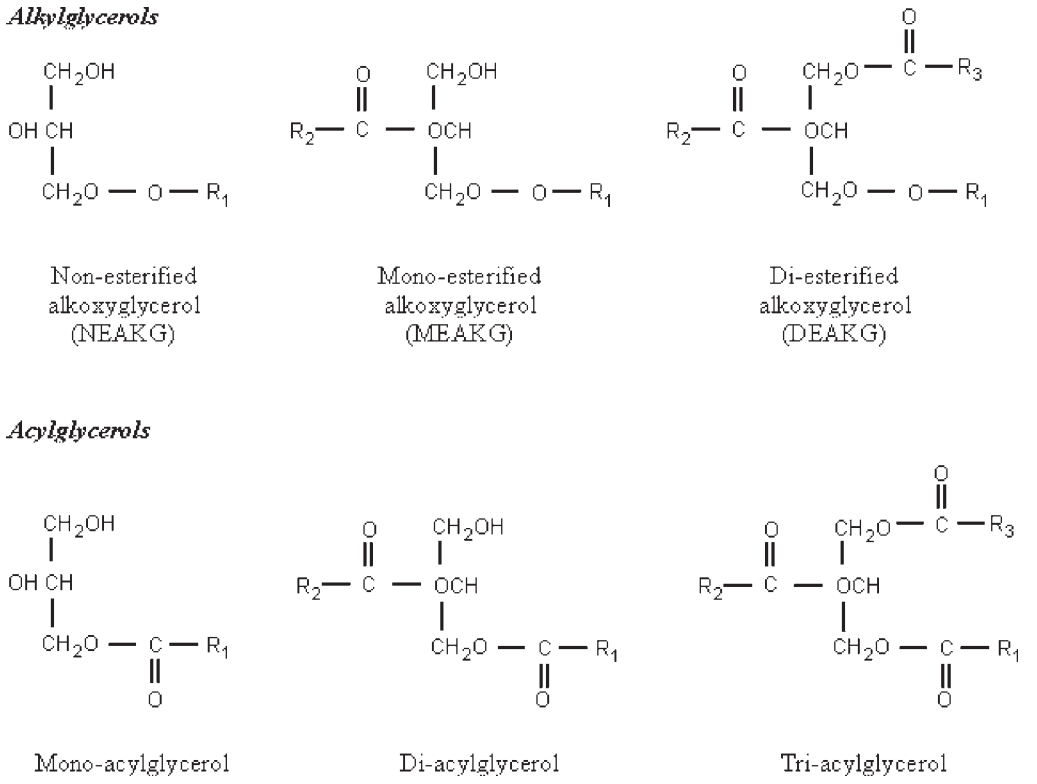

Figure 1. Chemical structures of alkoxyglycerol and acylglycerol compounds.

may be legally marketed as dietary supplements if claims about diagnosis, cure, mitigation, treatment, or prevention are removed from the promotional materials. Although many people have taken shark liver oil, the issue of potential toxicity at the usual doses has not been well studied.

To date, there have been no conventional safety studies published for shark liver oil extracts. Spurred by the growing interest in the use of alkoxyglycerol extracts as a dietary supplement for potential chemopreventive effects and to establish safety data, the objective of this study was to evaluate the potential acute and subchronic (repeated doses, 28 days) oral toxicity of a supercritical fluid extract of shark liver oil, a mixture of nonesterified alkoxyglycerol (NEAKG) and monoesterified alkoxyglycerol (MEAKG) in the rat. The dosages were administered by gavage. Doses of 2000 mg kg^{-1} of body weight (acute) and 1000 mg kg^{-1} of body weight (subchronic) were selected, since 2000 mg kg^{-1} represents the limit dose used for acute (limit test) (*19*) and in the subchronic study the dose is 100 times the maximum recommended daily dose in humans of 10 mg kg^{-1} (*1*).

MATERIALS AND METHODS

Test Compound and Reagents. Shark liver oil (squalene free) was purchased from Lysi (Reykjavik, Iceland). Carbon dioxide (99.98%) was purchased from AL Air Liquide España S. A. (Madrid, Spain). All solvents used were HPLC grade from Lab-Scan (Dublin, Ireland).

The product utilized in the present study has been previously characterized (*20*). In brief, the production process can be described as follows: shark liver oil (squalene free) was trans-esterified with ethanol to produce a mixture that contains mainly fatty acid ethyl esters (FAEE), nonesterified alkoxyglycerols (NEAKG), and monoesterified alkoxyglycerols (MEAKG). Then countercurrent supercritical fluid extraction (CC-SFE) was employed to eliminate FAEE from the mixture and thus concentrate NEAKG and MEAKG in the raffinate product (AKG-1 extract).

Ethanolysis of Shark Liver Oil. Shark liver oil was mixed with sodium ethoxide (5.25% w/v) in absolute ethanol at a ratio of 4:1 (v:v). The mixture was allowed to stand for 30 min at 60 °C while being stirred. Then two washings were effected: first with a sodium chloride solution (0.5 M) at 50 °C and second with distillate water. The volume utilized in these two washings was half of the volume of shark liver oil utilized. After the second washing, the mixture was centrifuged at 585*g*. Finally, the product of the ethanolysis reaction was dried with sodium sulfate and vacuum filtrated (*20*, *21*).

Contercurrent Supercritical Fluid Extraction. The extraction of CC-SFE pilot plant employed in this work is a homemade device and comprises a countercurrent extraction column (316 stainless steel), two cascade separator cells (270 mL capacity), and a cryogenic trap at atmospheric pressure. The extraction column is 300 cm in height and 17 mm in internal diameter, and is packed with Fenske rings (3 × 0.5 mm). At 140 bar and 50 °C, the raffinate product obtained was composed of ca. 11.9% fatty acid ethyl esters, 53.6% nonesterified alkoxyglycerols, 18.8% monoesterified alkoxyglycerols, 6.7% triacylglycerols, and 9.0% free fatty acids. By this methodology, ca. 500 g of raffinate product (AKG-1 extract: a mixture of NEAKG and MEAKG) was obtained.

HPLC Analysis. The analysis of supercritical fluid extracts and raffinate product (AKG-1 extract: a mixture of NEAKG and MEAKG) from shark liver oil was carried out in a HPLC system composed of a kromasil silica 60 column (250 mm by 4.6 mm, Análisis Vinicos, Tomelloso, Spain) coupled to a CTO 10A VP 2 oven, a LC-10AD VP pump, a gradient module FCV-10AL VP, a DGU-14A degasser, and an evaporative light scattering detector ELSD-LT from Shimadzu (IZASA, Spain). The ELSD conditions were 2.2 bar, 35 °C, and gain 3. The flow rate was 2 mL min^{-1}. A splitter valve was used after the column and only 50% of the mobile phase was directed through the detector. The column temperature was maintained at 35 °C. The mobile phase utilized has been previously reported by Torres et al. (*22*). This methodology permits one to analyze up to 18 different classes of neutral lipids including NEAKG, MEAKG, DEAKG, sterols, tocopherols, free fatty acids, mono-, di-, and triacylglycerols. Quantification was based on calibration curves effected with appropriate standards. Although the present methodology is also able to identify and quantify fatty acid ethyl esters (as a single lipid class), these compounds were much more precisely and accurately analyzed via gas chromatography.

Acute and Repeated Dose (28 Days) Oral Toxicity Studies. Wistar male and female rats (Charles River Inc., Marget, Kent, U.K.) were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with

sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50% ± 10% relative humidity) with a 12 h light-dark cycle (light from 08.00 to 20.00 h). Food (A03 rodent diet, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France) and water were available *ad libitum*. The rats were 56 days old at initiation of treatment. Acute (limit test) and repeated dose (28 days) studies were conducted according to the guidelines recommended by the European Union (*19*, *23*). Both studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University.

In the acute (limit test) study, 24 rats (12 males, 12 females) were distributed into two groups of 6 males and 6 females each. After an overnight fast, each rat received distillate water orally (control group or Group 1) or a single oral dose of 2000 mg kg^{-1} of body weight of the AKG-1 extract at 30 °C (treated group or Group 2). Doses of the test and control articles were administered by oral gavage at a volume of 2.5 mL kg^{-1} body weight based on the individual animal body weights obtained on the day dosing. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m). At the end of a 14 day observation period, the rats were weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied.

The repeated dose (28 days) safety study was conducted in 40 rats (20 males, 20 females) divided in four groups of 5 males and 5 females each (control group or Group 3; treated group or Group 4; satellite control group or Group 5; and satellite treated group or Group 6). Rats received a daily dose of either distilled water (Groups 3 and 5) or 1000 mg kg^{-1} of body weight of the AKG-1 extract (Groups 4 and 6) orally once a day over 28 days. Doses of the test and control articles were administered by gavage at a volume of 1.25 mL kg^{-1} body weight based on the individual animal body weights obtained on the day dosing. Animals were dosed at approximately the same time each day (approximately 4−6 h into light cycle). Food but not water was withheld from 4 h before until 2 h after control and test article administration. During dose administration, AKG-1 extract preparation was maintained in a water bath set to maintain approximately 30 °C. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). All rats of the Groups 3 and 4 were deprived of food for 16 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on day 29. All animals of the satellite groups (Groups 5 and 6) were kept a further 14 days without treatment to detect delayed occurrence of, persistence of, or recovery from toxic effects. All rats of the Groups 5 and 6 were deprived of food for 16 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on day 42.

Observations. All animals were observed twice daily for general appearance, behavior, and signs of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin and fur, eyes, nose, oral cavity, abdomen, and external genitalia, evaluated for respiration rate, and palpated for masses. Body weight and food and water consumption were measured daily, and at the end of the observation periods the rats were examined by necropsy and the weights of the organs recorded.

Clinical Test Parameters. Blood samples for hematology and clinical chemistry examination were collected from the retro-orbital plexus from animals under light anesthesia induced by CO_2 inhalation after the 14 day observation period in the acute oral study and after 28 days of treatment and 14 days of recovery for the repeated dose 28 days safety study. EDTA was used as an anticoagulant for hematology samples and sodium citrate was used as an anticoagulant for clinical chemistry. Food was withheld for approximately 18 h before blood collection, and samples were collected early in the working day to reduce biological variation; water was provided *ad libitum*. Clinical pathology parameters (hematological and clinical biochemical) were evaluated (**Table 1**). Most hematology variables were measured with a Coulter/CELL-DYN 3500 whole blood automated analyzer (Abbott, Chicago, ILL). Blood cell smears were observed with an Olympus Microscopy BX41 instrument (Olympus, Tokyo, Japan). Clinical chemistry parameters were evaluated with a spectrophotometer Konelab PRIME 30 (Thermofisher Scientific Inc. Waltham, MA), and special biochemistry parameters with a clinical chemistry analyzer AU640 (Olympus, Tokyo, Japan). Coagulation parameters were analyzed with a coagulation analyzer Coatron M1 (Teco Medical Instruments, GMBH, Neufahrn, Germany).

Anatomical Pathology. All rats were euthanized by CO_2 inhalation and necropsied. The necropsy included a macroscopic examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal, and pelvic cavities and viscera. Descriptions of all macroscopic abnormalities were recorded. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate-buffered 4% formaldehyde solution: adrenal glands, brain, heart, ileum, jejunum, cecum, colon, duodenum, rectum, stomach, esophagus, trachea, kidneys, liver, lungs, pancreas, spleen, skin, testicles, ovaries with oviducts, bone marrow, thymus, thyroid and parathyroid glands, seminal vesicles, urinary bladder, and uterus. The organ/body weight ratios were calculated. All organ and tissue samples for histopathological examination were processed, embedded in paraffin, cut at an approximate thickness of 2−4 μm, and stained with hematoxylin and eosin. Slides of all organs and tissues listed above were collected from all animals of the control and treated groups.

Table 1. Clinical Pathology Parameters

hematological parameters
red blood cell count (RBC)
hemoglobin
hematocrit
mean corpuscular volume (MCV)
mean corpuscular hemoglobin (MCH)
mean corpuscular hemoglobin concentration (MCHC)
platelet count
white blood cell count (WBC)
nucleated red blood cell count (RDW)
segmented neutrophils count
band neutrophils count
lymphocytes count
monocytes count
eosinophils count
basophils count
mean platelet volume (MPV)
clinical biochemistry parameters
glucose
urea
creatinine
total protein
total bilirubin
calcium
sodium
potassium
aspartate aminotransferase (ASAT)
alanine aminotransferase (ALAT)
alkaline phosphatase
albumine (28 days)
triglyceride (28 days)
cholesterol (28 days)
HDL (28 days)
LDL (28 days)
lipoprotein A
prothrombin time (28 days)
thromboplastin partial time (28 days)
fibrinogen (28 days)
prothrombin time (28 days)

Statistical Analyses. For the acute oral study, data are expressed as means ± SEM of 12 determinations (i.e., 12 samples each from six male and six female rats). For the repeated dose (28 days) safety study, data are expressed as means ± SEM of 10 determinations (i.e., 10 samples each from five male and five female rats). Differences between control and treated groups were evaluated with a one-way analysis of variance (ANOVA) followed by Dunnett's test (*24*), and differences were considered significant at $P < 0.05$.

RESULTS

Characterization of AKG-1 Extract. The product utilized in the present study has been previously characterized (*20*). Compositions

Table 2. Composition of Original Shark Liver Oil, after the Transesterification and Raffinate Obtained via CC-SFE[a]

%(w/w)	shark liver oil	transesterified	raffinate CC-SFE
squalene	0.5	0.3	0
cholesteryl esters	1.5	0.4	0
FFA	0	1	9
FAEE	0	71.8	11.9
DEAKG	44.6	0	0
TAG	53.4	1	6.7
cholesterol	0	0.3	0
MEAKG	0	4.3	18.8
NEAKG	0	20.9	53.6

[a] FAEE, fatty acid ethyl esters; DEAKG, diesterified alkoxyglycerols; TAG, triacylglycerols; MEAKG, monoesterified alkoxyglycerols; NEAKG, nonesterified alkoxyglycerols; FFA, free fatty acids.

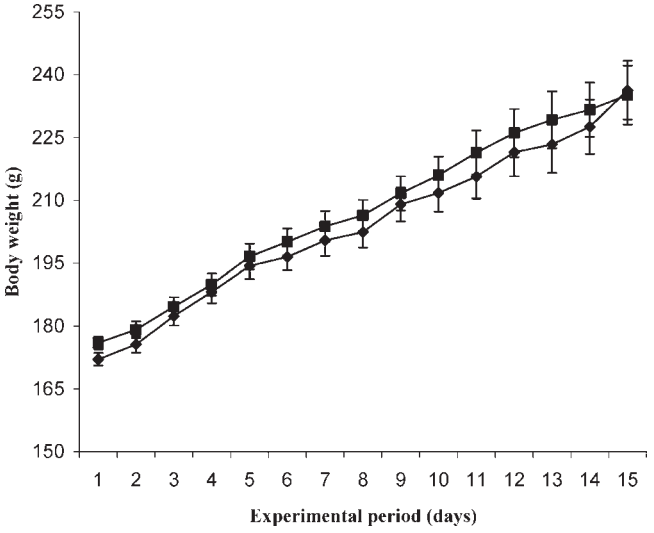

Figure 2. Changes in body weight of rats during the 2 week observation period after treatment with AKG-1 extract. Group 1, control (◆); Group 2, treated with single oral dose of AKG-1 extract at 2000 mg kg^{-1} of body weight (■). Data represent the mean ± SEM for 12 rats (6 male and 6 female rats).

of the original, transesterified, and raffinate attained after CC-SFE from shark liver oil are given in **Table 2**.

Acute Oral Toxicity in Rats. No abnormal clinical signs, behavioral changes, body weight changes, macroscopic findings, or organ weight changes were observed. All animals survived the 2 week observation period. Body weight data are depicted in **Figure 2**. There were no statistical differences in body weights among groups. Similarly, no statistically significant differences in food and water consumption were noted. Body weight and food and water consumption thus were unaffected ($P < 0.05$) by the treatment (single oral dose of 2000 mg kg^{-1} of the AKG-1 extract).

The hematological and clinical chemistry parameters assessed 2 weeks after administration of the AKG-1 extract as a single oral dose of 2000 mg kg^{-1} of body weight were not significantly different compared with those of controls (**Tables 3** and **4**). Individual values and group mean values were within the physiological range. No treatment-related changes were noted.

There were no statistical differences in organ weight or tissue/body weight ratios related to the test material. AKG-1 extract was not associated with any incidence of macroscopic and microscopic changes. No treatment-related histopathological changes were observed 2 weeks after administration of AKG-1 extract as a single oral dose of 2000 mg kg^{-1} of body weight, and histological correlates for the organ weight changes were found. Therefore, it

Table 3. Hematological Parameters in Rats after the 2 Week Observation Period Following a Single Oral Dose of AKG-1 Extract at 2000 mg kg^{-1} of Body Weight[a]

	acute oral dose	
parameter	Group 1 (control)	Group 2 (treated with AKG-1 extract)
RBC ($\times 10^6/\mu L$)	7.79 ± 0.09	8.42 ± 0.30
hemoglobin (g/dL)	14.18 ± 0.23	14.79 ± 0.21
hematocrit (%)	43.48 ± 0.66	46.33 ± 1.23
MCV (fL)	55.83 ± 0.87	55.08 ± 0.60
MCH (pg)	18.21 ± 0.27	17.58 ± 0.21
MCHC (g/dL)	32.60 ± 0.28	31.93 ± 0.29
RDW (%)	15.86 ± 0.16	15.59 ± 0.16
WBC ($\times 10^3/\mu L$)	6.14 ± 0.35	7.05 ± 0.49
banded neutrophils ($\times 10^3/\mu L$)	0.00 ± 0.00	0.00 ± 0.00
neutrophils ($\times 10^3/\mu L$)	1.05 ± 0.11	0.86 ± 0.12
eosinophils ($\times 10^3/\mu L$)	0.06 ± 0.02	0.04 ± 0.01
lymphocytes ($\times 10^3/\mu L$)	4.86 ± 0.37	5.99 ± 0.46
monocytes ($\times 10^3/\mu L$)	0.18 ± 0.03	0.17 ± 0.03
basophils ($\times 10^3/\mu L$)	0.00 ± 0.00	0.00 ± 0.00
platelets ($\times 10^3/\mu L$)	756.42 ± 67.29	846.25 ± 69.25
MPV (fL)	6.21 ± 0.10	5.84 ± 0.16

[a] Data are expressed as mean ± SEM ($n = 12$; 6 male and 6 female rats) in each group. Differences between the treated group and the control group were not significant.

Table 4. Clinical Chemistry Parameters in Rats after the 2 Week Observation Period Following a Single Oral Dose of AKG-1 Extract at 2000 mg kg^{-1} of Body Weight[a]

	acute oral dose	
parameter	Group 1 (control)	Group 2 (treated with AKG-1 extract)
glucose (mg/dL)	116.92 ± 2.73	113.75 ± 4.15
urea nitrogen (mg/dL)	34.50 ± 1.47	38.08 ± 1.91
creatinine (mg/dL)	0.44 ± 0.01	0.54 ± 0.05
total protein (g/dL)	7.46 ± 0.31	7.24 ± 0.27
total bilirubin (mg/dL)	1.26 ± 0.25	1.09 ± 0.21
calcium (mg/dL)	10.93 ± 0.20	10.94 ± 0.22
sodium (mequiv/L)	149.83 ± 1.46	141.50 ± 3.76
potassium (mequiv/L)	6.44 ± 0.44	5.97 ± 0.26
ASAT (u/L)	277.83 ± 22.69	205.83 ± 27.79
ALAT (u/L)	99.17 ± 8.10	75.83 ± 8.69
alkaline phosphatase (u/L)	508.17 ± 78.33	409.33 ± 67.03

[a] Data are expressed as mean ± SEM ($n = 12$; 6 male and 6 female rats) in each group. Differences between the treated group and the control group were not significant.

is concluded that AKG-1 extract has a low order of acute toxicity and that the oral lethal dose (LD_{50}) for male and female rats is higher than 2000 mg kg^{-1} of body weight.

Repeat Dose (28 Days) Oral Toxicity in Rats. No mortality was observed. No treatment-related changes in the general condition and external appearance were observed in male and female rats treated with the AKG-1 extract at the 1000 mg kg^{-1} of body weight daily dose. The development of the animals during the experimental period corresponded to their species and age. There was no significant difference in body weight or daily body weight gain (**Figure 3**) among groups treated with AKG-1 extract in comparison to the control groups at any time point of the experimental period. All AKG-1 extract treated groups consumed similar amounts of food and water to that of the corresponding control groups.

All hematology data were within normal limits, and differences between groups were not observed (**Table 5**). Clinical chemistry data showed no treatment-related alterations at the

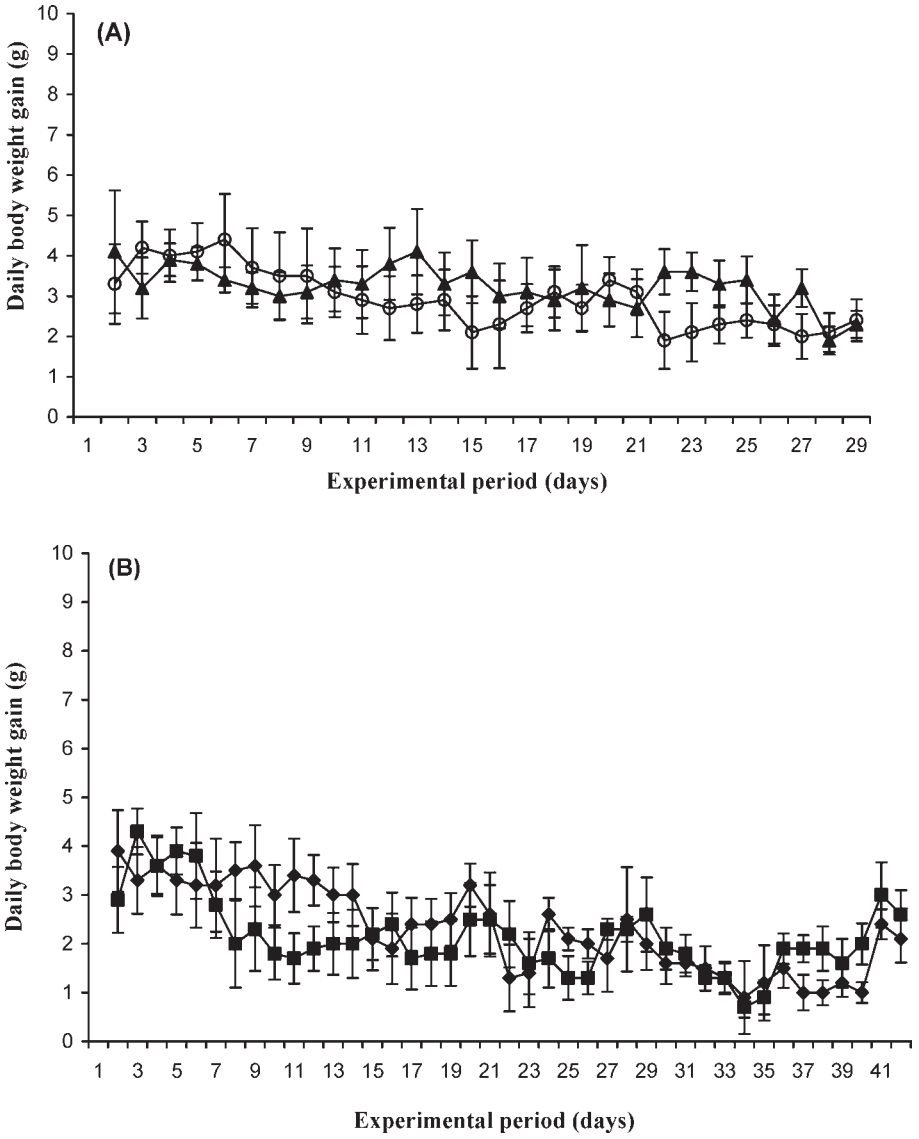

Figure 3. Daily body weight gain of rats exposed to repeated (28 days) oral doses of the AKG-1 extract. (**A**) Group 3 (○), control; Group 4 (▲), treated (28 days) with AKG-1 extract at 1000 mg kg^{-1} of body weight. (**B**) Group 5 (◆), satellite control; Group 6 (■), satellite treated group, treated (28 days) with AKG-1 extract at 1000 mg kg^{-1} of body weight and moreover observed 14 days after treatment. Data represent the mean ± SEM for 10 rats (5 male and 5 female rats).

end of the 28 day treatment period (**Table 6**). Individual values and group mean values were within the physiological range. Only the responses after treatment (28 days) of male and female rats differ significantly for ALAT, ASAT, triglyceride, and LDL parameters, but these changes were within the physiological range and not related to the treatment. Therefore, male and female data were combined. After 14 days without treatment to detect delayed occurrence of potential toxic effects, there were no treatment-related changes in hematological and clinical test parameters (data shown in **Tables 5** and **6**, satellite treated group).

The necropsy performed on day 29 after the last dose of AKG-1 extract (Group 4) and on day 42 after 14 days without any treatment (Group 6) did not reveal any gross pathological changes or any differences in organ weights in comparison to the corresponding control groups. After 28 days of treatment, there were no histopathological findings in the organs examined considered as being treatment-related in male and female rats (data not shown). There were also no treatment-related histopathological findings in the recovery group or satellite treated group (Group 6) (data not shown).

DISCUSSION

There are no published conventional safety studies on alkoxyglycerol extract from shark liver oil despite widespread intake. This study represents the first standard toxicology data on an alkoxyglycerol extract from shark liver oil (AKG-1 extract: 11.9% fatty acid ethyl esters, 53.6% nonesterified alkoxyglycerols, 18.8% monoesterified alkoxyglycerols, 6.7% triacylglycerols, and 9.0% free fatty acids). Acute and subchronic (4 weeks) oral toxicity studies in rats indicate that AKG-1 extract from shark liver oil is not associated with any untoward toxicologic effects at doses of 2000 and 1000 mg kg^{-1} of body weight, respectively.

In the present study, a single oral limit dose of 2000 mg kg^{-1} of body weight of the AKG-1 extract was well tolerated by both male and female rats (limit test). A single dose of 2000 mg kg^{-1} of body weight did not result in any observable adverse effects or mortality. Daily oral administration of 1000 mg AKG-1 extract kg^{-1} of body weight for 28 consecutive days did not cause mortality or changes in body weight, body weight gain, or food consumption. No hematological or clinical pathologic alterations were noted. Both gross and histopathological examinations did not reveal any treatment-related changes. The no-observed-adverse-effect

Table 5. Hematological Parameters in Rats after Repeated (28 Days) Oral Doses of the AKG-1 Extract at 1000 mg kg^{-1} of Body Weight[a]

	28 day repeated dose			
Parameters	Group 3 (control)	Group 4 (treated with AKG-1 extract)	Group 5 (satellite control)	Group 6 (satellite treated with AKG-1 extract)
RBC ($\times 10^6/\mu$L)	8.85 ± 0.09	8.45 ± 0.17	9.03 ± 0.12	8.89 ± 0.15
hemoglobin (g/dL)	15.28 ± 0.15	15.38 ± 0.26	15.69 ± 0.17	15.34 ± 0.22
hematocrit (%)	49.32 ± 0.43	47.27 ± 0.92	49.28 ± 0.48	49.70 ± 0.56
MCV (fL)	56.00 ± 0.58	56.00 ± 0.72	54.60 ± 0.73	55.60 ± 0.56
MCH (pg)	17.50 ± 0.36	18.26 ± 0.39	17.30 ± 0.29	17.15 ± 0.21
MCHC (g/dL)	31.50 ± 0.23	32.54 ± 0.35	31.24 ± 0.25	30.70 ± 0.14
RDW (%)	15.87 ± 0.16	15.77 ± 0.11	15.73 ± 0.16	16.02 ± 0.17
WBC ($\times 10^3/\mu$L)	9.47 ± 0.35	9.01 ± 0.63	9.13 ± 0.64	10.23 ± 0.73
banded neutrophils ($\times 10^3/\mu$L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
neutrophils ($\times 10^3/\mu$L)	1.34 ± 0.12	1.43 ± 0.17	1.33 ± 0.19	1.36 ± 0.26
eosinophils ($\times 10^3/\mu$L)	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.11 ± 0.03
lymphocytes ($\times 10^3/\mu$L)	8.20 ± 0.46	7.36 ± 0.54	7.59 ± 0.54	8.68 ± 0.53
monocytes ($\times 10^3/\mu$L)	0.20 ± 0.03	0.20 ± 0.06	0.17 ± 0.03	0.21 ± 0.03
basophils ($\times 10^3/\mu$L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
platelets ($\times 10^3/\mu$L)	848.00 ± 27.43	819.50 ± 46.73	850.00 ± 35.12	881.80 ± 40.70
MPV (fL)	6.31 ± 0.16	5.99 ± 0.11	6.31 ± 0.13	6.66 ± 0.14

[a] Data are expressed as mean ± SEM (n = 10; 5 male and 5 female rats) in each group. Groups 3 and 4 were sacrificed after treatment on day 29. Differences between the treated group (Group 4) and the control group (Group 3) were not significant. Groups 5 and 6 were sacrificed on day 42 after an additional observation period of 14 days without treatment. Differences between the satellite treated group (Group 6) and the satellite control group (Group 5) were not significant.

Table 6. Clinical Chemistry Parameters in Rats after Repeated (28 Days) Oral Doses of the AKG-1 Extract at 1000 mg kg^{-1} of Body Weight[a]

	28 day repeated dose			
parameters	Group 3 (control)	Group 4 (treated with AKG-1 extract)	Group 5 (satellite control)	Group 6 (satellite treated with AKG-1 extract)
glucose (mg/dL)	119.10 ± 3.43	108.90 ± 3.44	120.90 ± 4.04	108.20 ± 5.90
urea nitrogen (mg/dL)	43.50 ± 1.38	40.40 ± 0.94	44.30 ± 2.11	45.80 ± 1.84
creatinine (mg/dL)	0.50 ± 0.02	0.46 ± 0.02	0.51 ± 0.02	0.48 ± 0.02
albumin (g/dL)	5.21 ± 0.06	5.38 ± 0.13	5.22 ± 0.07	5.26 ± 0.19
total protein (g/dL)	7.71 ± 0.35	7.31 ± 0.16	7.56 ± 0.27	7.77 ± 0.33
total bilirubin (mg/dL)	0.80 ± 0.16	0.50 ± 0.03	0.75 ± 0.17	1.17 ± 0.22
calcium (mg/dL)	11.53 ± 0.20	10.90 ± 0.22	11.99 ± 0.28	11.18 ± 0.49
sodium (mequiv/L)	143.40 ± 0.67	142.40 ± 0.67	143.80 ± 0.42	147.60 ± 3.34
potassium (mequiv/L)	7.42 ± 0.52	6.36 ± 0.24	7.31 ± 0.55	7.80 ± 0.49
ASAT (u/L)	213.40 ± 14.28	178.00 ± 13.60	254.70 ± 35.08	267.80 ± 23.27
ALAT (u/L)	79.30 ± 4.58	63.80 ± 5.83	103.20 ± 14.25	144.10 ± 15.87
alkaline phosphatase (u/L)	282.10 ± 32.19	276.00 ± 51.92	268.60 ± 31.18	306.20 ± 45.30
triglyceride (mg/dL)	230.20 ± 13.23	199.00 ± 16.47	218.50 ± 16.70	220.90 ± 21.31
cholesterol (mg/dL)	71.1 ± 5.35	62.70 ± 5.40	69.30 ± 3.83	57.20 ± 5.16
HDL (mg/dL)	43.17 ± 3.73	41.90 ± 3.21	41.33 ± 2.84	35.69 ± 3.41
LDL (mg/dL)	22.06 ± 2.02	19.14 ± 3.46	31.45 ± 2.68	26.97 ± 11.68
lipoprotein A (mg/dL)	<2	<2	<2	<2
prothrombin time (s)	18.78 ± 1.05	17.81 ± 0.59	21.83 ± 4.09	16.83 ± 0.24
thromboplastin partial time (s)	33.69 ± 3.81	36.30 ± 1.97	32.15 ± 3.83	30.43 ± 2.77
fibrinogen (mg/dL)	249.60 ± 22.76	248.65 ± 22.40	279.60 ± 29.15	295.30 ± 36.38

[a] Data are expressed as mean ± SEM (n = 10; 5 male and 5 female rats) in each group. Groups 3 and 4 were sacrificed after treatment on day 29. Differences between the treated group (Group 4) and the control group (Group 3) were not significant. Groups 5 and 6 were sacrificed on day 42 after an additional observation period of 14 days without treatment. Differences between the satellite treated group (Group 6) and the satellite control group (Group 5) were not significant.

level in this subchronic toxicity study was the dose tested, that is, 1000 mg kg^{-1}. This finding provides the basis for the selection of doses for use in long-term toxicity studies.

On the basis of the findings from this subchronic (28-day) oral study, there were no indications of toxicological effects in rats treated orally at 1000 mg AKG-1 kg^{-1} of body weight. Therefore, a logical conclusion from this study is that the AKG-1 extract was well tolerated as a daily dose at 1000 mg kg^{-1} of body weight for 28 days in the rat and there was no evidence for systemic toxicity. The 1000 mg kg^{-1} dose used in the rat represents 100 times that of the 600 mg daily supplement in a 60 kg human (*1*). This statement only implies achievement of an applied dosage exaggeration over a typical supplement level in humans, and it is realized that metabolic and physiologic differences between humans and rats exist that would influence tissue utilization or responses to AKG-1 extract from shark liver oil. Further safety studies (at least a subchronic study, 91 days of daily oral gavage treatment) of AKG-1 extract from shark liver oil in rats should be evaluated before its use as a dietary supplement in humans.

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Received for review September 25, 2009. Revised manuscript received November 11, 2009. Accepted November 30, 2009. This work was supported by the *Comunidad de Madrid* and the *Ministerio de Educación y Ciencia*, Projects ref. S-0505/AGR/0153 and ref. AGL2006-02031/ALI, and Consolider-Ingenio 2010 ref. CSD/2007/00063 (FUN-C-FOOD), Madrid, Spain.