

**EFFECT OF DIET SUPPLEMENTED BY
EARTHWORM (*EISENIA ANDREI*) FLOUR ON GROWTH
AND HEPATIC PARAMETERS IN MICE**

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Abstract. We studied the effect of diets, supplemented with different percentages of earthworm (*Eisenia andrei*) flour, on growth, hepatic profile and blood cholesterol levels in mice. Three diets were used: one control diet and two experimental diets with a 25%:75% and 50%:50% earthworm flour:fish flour ratio. Mice were fed the diets for 28 days, and diet materials were analyzed to verify their nutritional content. Size and weight were determined at 0, 7, 14, and 28 days. On day 28, a blood sample was taken to measure the transaminase ALT enzyme and liver biopsies were analyzed using the nonalcoholic fatty liver disease (NAFLD) score. Results showed that both experimental diets were high in protein, an indispensable nutrient for growth in mice. There were no significant differences in size or ALT transaminase. Weight and serum cholesterol showed significant differences. Changes in the NAFLD score of the animals were not observed. The use of earthworm flour in these diets did not alter growth or hepatic tissue in mice. *Received: 23 November 2007, accepted: 12 March 2008.*

Key words. Earthworm, *Eisenia andrei*, animal nutrition, liver, body weight, Alanine-aminotransferase.

EFFECTO DE DIETAS SUPLEMENTADAS CON HARINA DE LOMBRIZ (*EISENIA ANDREI*) EN EL CRECIMIENTO Y LOS PARÁMETROS HEPÁTICOS DE RATONES

Resumen. Este estudio evaluó el efecto de dos dietas, suplementadas con diferentes porcentajes de harina de lombriz (*Eisenia Andrei*), en el

crecimiento, colesterol plasmático y la función hepática de ratones. Una dieta control y dos experimentales con una relación harina de lombriz:harina de pescado de 25%:75% y 50%:50%, respectivamente fueron usadas para alimentar ratones durante 28 días. Se le realizó un análisis a las dietas para conocer su contenido nutricional. El tamaño y el peso de los animales fueron medidos a los días 0, 7, 14 y 28 del estudio. El día 28, se tomaron muestras de sangre para determinar los niveles de la transaminasa ALT, así como biopsia hepática que fue analizada usando la escala para la enfermedad grasa hepática no alcohólica (NAFLD). Los resultados muestran que las dietas experimentales tienen un alto contenido protéico, necesario para el crecimiento de los ratones. No se encontraron diferencias estadísticamente significativas en el tamaño ni en los niveles de la enzima ALT; en tanto que, el peso y los niveles séricos de colesterol mostraron diferencias significativas. No hubo cambios en la escala NAFLD en los grupos. El uso de la harina de lombriz no alteró el crecimiento ni el tejido hepático en estos ratones. *Recibido: 23 noviembre 2007, aceptado: 12 marzo 2008.*

Palabras clave. Lombriz, *Eisenia andrei*, nutrición animal, hígado, peso corporal, Alanina-aminotransferasa.

INTRODUCTION

In Venezuela, the design of new animal feed formulas is greatly affected by the availability of different ingredients such as imported fish flour. New formulations are necessary to limit dependency on these imported products, replacing them with unconventional, economical and safe animal feed materials. Live earthworm (*Eisenia andrei*) flour has been used as animal food for trout (Isea *et al.* 2007), due to its mixing capacity without altering food flavor. *Eisenia andrei* has also been used as a partial substitute for fish flour, soy, and meat, in feeding fish and pigs (Salazar and Rojas 1992).

Previous studies using earthworm flour to feed pigs, chickens, shrimp, and young trout, have obtained growth rates similar to those observed in animals fed with reference diets (López-Aliaga 1986, Carmona 1989, Ortega *et al.* 1996). However, the effect of diet on fatty liver deposition and blood cholesterol levels has not been studied in these animals.

As a result of increasing research and availability of supplies in the field of raw material substitution in animal feed, food production companies are constantly widening production lines, launching novel products, and changing to more technical formulas for feed (Salazar and Rojas 1992, Vielma *et al.* 2003a, Vielma *et al.* 2003b). However, before these new formulas can be

commercially produced, their effects must be studied to allow introduction of these new foods to animals.

In this study, we evaluate the quality of diets using earthworm flour as a partial substitute for the traditional source of protein, by determining the effect on the following variables in mice: weight, size, serum cholesterol concentrations, and liver alanine-aminotransferase (ALT) enzyme concentrations.

MATERIALS AND METHODS

DIET PREPARATION

We used two experimental diets (G2 and G3) prepared from a mixture of home-made fish flour (HPA) and earthworm (*Eisenia andrei*) flour (HL). The G2 diet was prepared using 25% HL and 75% HPA, while the G3 diet was comprised of 50% HL and 50% HPA. Once prepared, diet material was bottled and kept at 4 °C until used.

Fish flour was prepared at La Mucuy Trout Station-INIA (Mérida, Venezuela) using adult trout, which were grounded in a meat grinder and cooked. Fish paste was placed in thin layers on trays and dried in an oven heated to 60 °C. Earthworm flour was obtained from a local supplier and other ingredients were obtained at the local market. The HL, together with the rest of the elements, was mixed by adding water until a dough was obtained. Pellets were made using a meat grinder and put into an oven at 60 °C, for 42 h.

DIET ANALYSIS

Characterization of proteins, humidity, ash, lipids and carbohydrates in the diets prepared for both control and experimental groups was made using the official 1999 Association of Official Analytical Chemists (AOAC 1999) analysis, of the Department of Food Science, Faculty of Pharmacy, University of the Andes, Venezuela.

ANIMALS

Twelve female, 3-week-old NMRI mice were distributed in three groups, of four mice each: a control group (C) fed with the standard diet (Rataharina, National Product), group 2 (G2) fed with the G2 diet (75% HPA and 25% HL), and group 3 (G3) fed with the G3 diet (50% HPA and 50% HL). Diet food and water were supplied *ad libitum* for 28 days. Mice were housed in

stainless steel cages in a room maintained at $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and with a 12-h light-dark cycle. Size and weight measurements were performed at 0, 7, 14, and 28 days. A scale (Mettler PJ400, Germany) was used to weigh mice. Mass was expressed in grams (g), and size was determined by measuring the total length (from tip of nose to end of tail) of the animal in cm.

At the end of the study a 5 mL blood sample was taken by cardiac puncture to measure ALT and total cholesterol. Blood samples were centrifuged to $2,000 \times g$ for 2 min and kept at -20°C until used. Animals were then euthanized and a liver biopsy was taken. Protocol and use of mice was approved by the Local Ethics Committee.

DETERMINATION OF PLASMA ALANINE-AMINOTRANSFERASE ENZYME

To determine ALT, a reaction was used whereby this enzyme, present in the mouse serum, catalyzed amino group transfers from the L-alanine to δ -ketoglutarate resulting in pyruvate and L-glutamate formation. In the next step, lactate dehydrogenase catalyzed the pyruvate reduction and simultaneous oxidation of NADH to NAD⁺. Decrease in absorbance was directly proportional to the ALT. Then, 100 μL of the sample was added to 1 mL of the reaction solution (acid- δ -ketoglutarate 13 mM, D-alanine 400 mM, NADH 0.2 mM, LDH 1,200 U/L in tris Buffer, pH 7.5) and incubated for 3 min at 37°C . The absorbance value was determined every minute for 3 min with a spectrophotometer (Abbot SPEC-310, USA) at a wavelength of 340 nm. Finally, the average absorbance change per minute ($\Delta\text{Abs}/\text{min}$) was transformed to IU/L when multiplied by the constant 1768 (Henry 1968).

DETERMINATION OF TOTAL PLASMA CHOLESTEROL

Total serum cholesterol measurement was performed using an enzymatic method, following the manufacturer's instructions (Cholesterol MR, Linear chemicals, España). In this test, the cholesterol esters are transformed into quinoneimine by action of the cholesterol esterase enzymes, cholesterol oxidase and peroxidase. The amount of this product produced is proportional to the cholesterol concentration in the sample. Ten μL of the sample were added to 1 mL of the enzymatic solution (PIPES 200 mmol/L pH 7, sodium cholate 1 mmol/L, cholesterol esterase 250 U/L, cholesterol oxidase 250 U/L, peroxidase 1KU/L, 4-aminoantipyrine 0.33 mmol/L, ADPS 0.4 mmol/L, non-ionic tensioactives 2 g/L) and incubated for 10 min at room temperature. The samples were analyzed using a spectrophotometer (Abbot SPEC-310, USA) at

a 550 nm wavelength. A cholesterol standard (20.0 mg/dL) was used to calculate sample concentration.

LIVER BIOPSY

Liver tissues obtained from mice after 28 days were weighed using an electronic scale (UME-NJW300, USA) and measured in centimeters. Tissues were later embedded in paraffin, cut to a thickness of 0.3–0.4 μm , mounted on slides, and colored with hematoxiline and eosin (H & E), according to Lee's (1968) protocol. Tissues were observed in an optical microscope (Nikon Labofhot 2, Germany) using 20x and 40x zooms. Hepatic alterations were classified according to the scale used for nonalcoholic fatty liver disease (NAFLD) (Brunt *et al.* 1999, Angulo 2002) (Table 1). A zero grade was used, if no hepatocellular damage was observed. In each case, tissues of mice fed with earthworm flour were compared to the control group.

Table 1. Grading and staging the histopathological lesions of nonalcoholic fatty liver disease.

Grading for Steatosis
Grade 1: < 33% of hepatocytes affected
Grade 2: 33% to 66% of hepatocytes affected
Grade 3: > 66% of hepatocytes affected
Grading for Steatohepatitis
Grade 1, mild: steatosis (66% of lobules), ballooning (occasionally observed), lobular inflammation (mild), portal inflammation (none or mild).
Grade 2, moderate: steatosis (macrovesicular and microvesicular), ballooning (obvious), lobular inflammation (pericellular fibrosis), portal inflammation (mild to moderate).
Grade 3, severe: steatosis (> 66% of lobules), ballooning (predominantly zone 3), lobular inflammation (perisinusoidal fibrosis), portal inflammation (mild to moderate).
Staging for Fibrosis
Stage 1: zone 3 perivenular, perisinusoidal or pericellular fibrosis.
Stage 2: as above, with focal or extensive periportal fibrosis
Stage 3: bridging fibrosis, focal or extensive
Stage 4: cirrhosis

*from Brunt *et al.* (1999).

STATISTICAL ANALYSIS

Body weight, size, ALT and total cholesterol are shown as mean \pm standard error for each group. For total cholesterol and ALT enzyme analyses,

Kruskal-Wallis and Mann-Whitney non-parametric tests were performed. An ANOVA test was used for weight and size.

RESULTS

FLOUR ANALYSIS

Results showed that the three diets exhibited high protein levels. Protein is an important nutrient in the growth of mice. The composition of the experimental diets is shown in Table 2.

Table 2. Percent composition of the experimental diets fed to mice.

Analysis	Control Diet (C)	Diet G2	Diet G3
Humidity (%)	8.70	7.20	15.28
Ash (%)	7.81	10.95	9.21
Fat (%)	1.66	23.57	18.39
Protein (%)	30.73	53.13	48.21
Carbohydrate by difference	51.10	5.15	8.91

WEIGHT AND SIZE

There was no significant difference in weight of mice at the beginning of the study, with an average of 9.3 ± 0.28 g. On day 7, a slight weight gain was observed in mice fed with the G2 diet, which increased through days 14 and 28 (Fig. 1). In the case of the G3 diet, mice had similar weight at the beginning of the study, but at the end there was an increase, in comparison to the control group. On day 28, mean weight was 17.2 g in group C, 23.4 g in group G2 and 19.6 g in group G3. Mice in G2 had a significantly greater weight gain from day 14 to the end of the study, when they were compared to C and G3 ($P < 0.05$). There was no significant difference between C and G3, and weight in the control group only increased slightly during the study.

The three groups exhibited an increase in growth throughout the study (Fig. 2). At the end, mean total lengths were 16.4 cm in the control group, 16.3 cm in G2, and 15.6 cm in G3. There were no significant differences in total length among the three types of diet ($P > 0.05$).

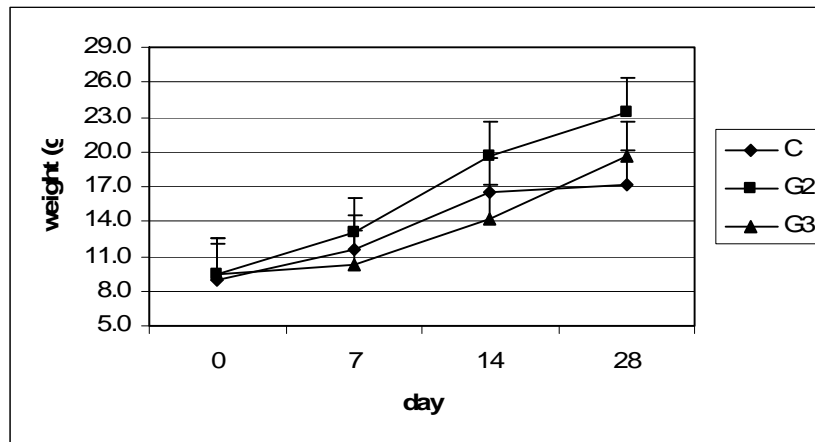


Figure 1. Average weight of mice fed with three diet regimens: C.- Control group, G2.- Diet substituted with 25% earthworm flour, G3.- Diet substituted with 50% earthworm flour.

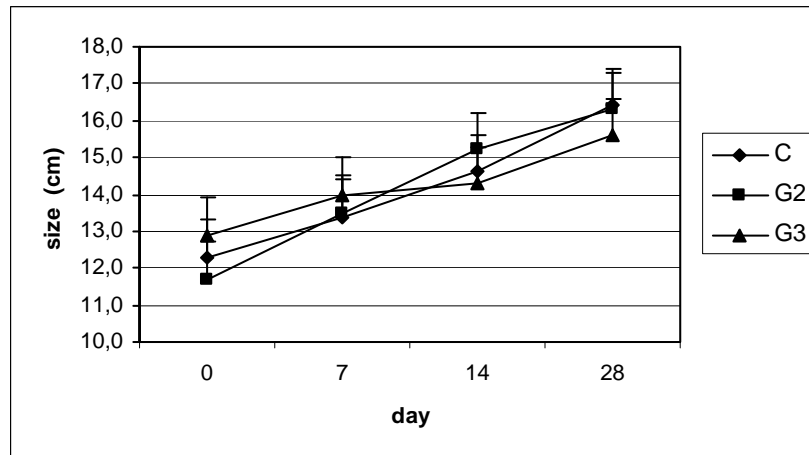


Figure 2. Average size of mice fed with the three diet regimens: C.- Control group, G2.- Diet substituted with 25% earthworm flour, G3.- Diet substituted with 50% earthworm flour.

ALANINE-AMINOTRANSFERASE ENZYME

The ALT enzyme has been used to determine the presence of liver tissue inflammation and destruction in many studies that evaluate drug toxicity. Mean ALT's averaged 13.4 IU/L for C, 14.1 IU/L for G2, and 10.5 IU/L for G3. Enzymatic levels did not show significant differences among groups fed

with the diets ($P > 0.05$) (Fig. 3). According to these results, the introduction of a diet comprising 25% to 50% *Eisenia andrei* earthworm flour does not induce hepatic necroinflammatory enzymatic alterations.

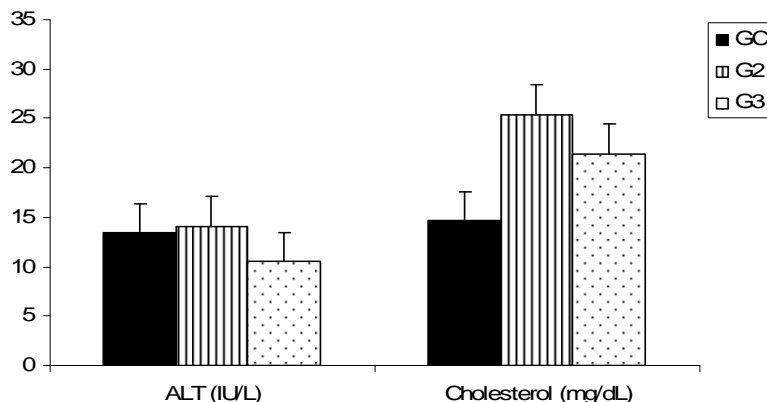


Figure 3. Average ALT and total cholesterol level of mice fed with the three diet regimens: GC.- Control group, G2.- Diet substituted with 25% earthworm flour, G3.- Diet substituted with 50% earthworm flour; ALT: Alanine-aminotransferase.

TOTAL CHOLESTEROL

The impact of formulated diets on cholesterol blood levels is noteworthy, because there is a relationship between blood cholesterol levels and fatal cardiovascular events. Cholesterol levels were different for each group, showing values of 14.6, 25.4 and 21.4 mg/dL for groups C, G2 and G3, respectively (Fig. 3). In this study, mice fed with the control diet had significantly lower cholesterol levels when compared to G2 and G3 ($P < 0.05$). There were no significant differences in cholesterol levels among groups fed with the earthworm flour substitute.

HEPATIC MICROSCOPIC STUDY

Normal hepatic parenchyma and histological architecture were observed in all groups. The lobules were comprised of hepatocytes with normal trabecular architecture. Cells were polygonal with eosinophilic cytoplasm and a central nucleus with chromatin distributed in fine groups (Fig. 4). No inflammatory infiltrates, ballooning or fibrotic zones were observed. The NAFLD score was grade zero and stage zero in all samples.

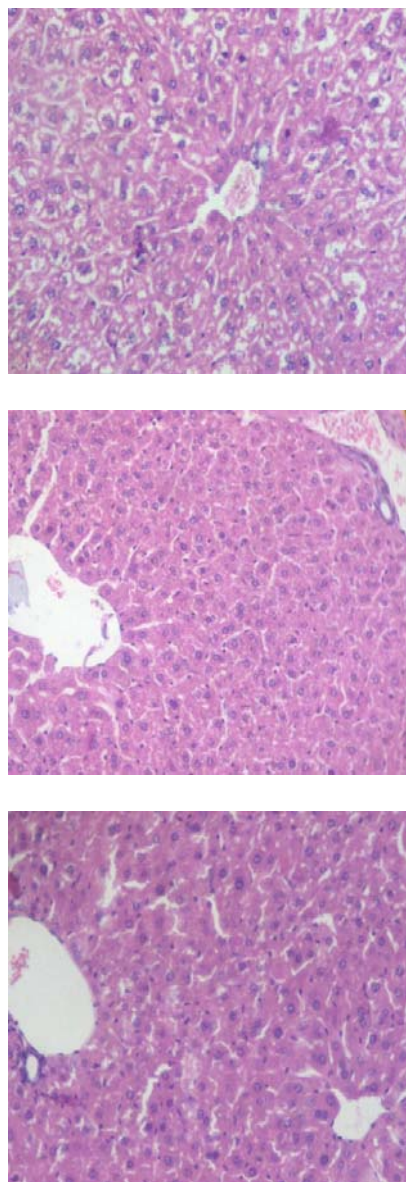


Figure 4. Histological examination of hepatic tissue from mice fed with the three different diet regimens: A.- control group (original magnification 400x), B.- mice fed with G2 (original magnification 400x), C.- mice fed with G3 (original magnification 400x). G2 and G3 sample groups showed normal tissue patterns compared to the control.

DISCUSSION

In this investigation, we studied the effect of two diets supplemented with earthworm flour, on growth, ALT enzyme level, serum cholesterol, nonalcoholic steatosis and steatohepatitis in mice, and compared the earthworm flour diets with the standard commercial diet. Results demonstrated that animals fed diets partially substituted with HL yielded similar weights and sizes as those fed with the control diet. Furthermore, a significant statistical increase in weight was found in the G2 diet group when compared with the control group, suggesting that animals fed with these new formulas will exhibit normal development. Other experimental nutritional supplements, such as pomegranate seed oil, do not induce changes in growth of mice (Yamasaki *et al.* 2006).

Plasma transaminase levels have been associated with hepatocellular damage. Transaminase enzymes include alanine-aminotransferase, present in the cytosol of the hepatocyte, and aspartate-aminotransferase (AST), found mainly in mitochondria. When hepatocytes suffer any membrane damage, ALT is released. If the damage continues, AST is released from mitochondria, and may be a sign of severe liver damage, as found in toxic and viral hepatitis (Herrera *et al.* 1993). Mice in experimental groups did not show ALT elevation when compared to the control group, suggesting that these diets do not alter hepatocyte homeostasis.

Use of new alternatives for animal feed could result in changes in cholesterol metabolism and excretion, thereby increasing plasma and hepatocyte fatty acids and inducing fatty liver or steatohepatitis (Duarte *et al.*, 2004, Park *et al.* 1997). In this study we observed a significant increase in cholesterol plasma levels in mice fed with diets G2 and G3. The high fat content in the experimental diets may explain the high animal cholesterol levels. However, histologic study showed no hepatic fatty acid deposition.

NAFLD may be the result of a decrease in fatty acid oxidation or an increase in fatty acid synthesis. NAFLD occurs when fat accumulation in the liver exceeds 5% to 10% of its weight and is correlated with the amount of fatty material found in the hepatocellular cytoplasm when a biopsy is performed. NAFLD begins with mild nonspecific inflammation without fibrosis but may progress to nonalcoholic steatohepatitis (NASH), which may ultimately result in fibrosis (Idrovo-Cubides *et al.* 2004). Some modified diets can induce alterations in lipid metabolism such as *Elaeis guineensis* plant oil, conjugated linoleic acid, and others, which may eventually lead to fatty liver

(Rasooly *et al.* 2007). The experimental diets used in this study did not produce such an effect.

Results indicate that animal feed, using earthworm flour instead of fish flour, appears to be safe and does not produce fatty changes in the liver, while at the same time, allowing normal development of animals that consume it. This locally designed alternative could decrease dependence on imported supplies, since it provides the nutritional requirements necessary for the first stages of animal development. Due to its high protein and amino acid content, as well as high reproductive rate, *Eisenia andrei* earthworms are an attractive substitute in the formulation of animal diets, including fish, rabbits, and pigs. Best results were obtained using a 25% earthworm flour substitute, which agrees with recent research performed on young trout (H. Bastardo, pers. commun. 2008).

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