

Original

Hematologic and immunological indicators are altered by chronic intake of flaxseed in Wistar rats

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Abstract

This work sought to evaluate the effects of chronic intake of flaxseed upon hematologic parameters and immunological findings on body development of Wistar rats. Female Wistar rats were used after gestation. They were randomly assigned into two groups during lactation period: Control group (CG), fed with casein based diet, made up of 17% protein and flaxseed group (FG), fed with casein based diet with the addition of 25% flaxseed. At weaning, 12 male pups of each group continued to receive the experimental diets of their mothers (with only 10% of protein) until adult age, when they were killed at 250 days of life aiming at blood collection. At 250 days old FG presented significant reduction in body mass ($p < 0.000$) and higher levels of hemoglobin ($p = 0.019$) and albumin ($p = 0.030$) than CG. It was observed smaller percentage of segmented lymphocytes ($p = 0.016$) in rats from FG and bigger percentage of segmented leucocytes ($p = 0.023$) when compared to CG. The chronic consumption of flaxseed altered hematologic and immunological indicators in adult Wistar rats. Supplementation with flaxseed seems to be beneficial to maintenance or reduction of body mass.

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Key words: Body weight. Flaxseed. Hematologic indicators. Leukocytes. Rats.

INDICADORES HEMATOLÓGICOS E INMUNOLÓGICOS ALTERADOS POR EL CONSUMO CRÓNICO DE LINAZA EN RATAS WISTAR

Resumen

Este trabajo pretendía evaluar el efecto de la ingestión crónica de linaza sobre parámetros hematológicos y hallazgos inmunológicos del desarrollo corporal de ratas Wistar. Se emplearon ratas hembra Wistar tras la gestación. Se las distribuyó al azar en dos grupos durante el período de lactancia: grupo control (GC), alimentado con una dieta basada en caseína, con un 17% de proteína y el grupo linaza (GF), alimentado con una dieta basada en caseína con la adición de un 25% de linaza. En el destete, 12 ratas macho continuaron recibiendo las dietas experimentales consumidas por sus madres (con sólo el 10% de proteína) hasta la edad adulta, en que fueron sacrificados a los 250 días de vida para la recogida de las muestras. A los 250 días de edad, el GF presentaba una reducción significativa de la masa corporal ($p < 0,000$) y mayores concentraciones de hemoglobina ($p = 0,019$) y albúmina ($p = 0,030$) que el GC. Se observó un menor porcentaje de linfocitos segmentados ($p = 0,016$) en las ratas del GF y un mayor porcentaje de leucocitos segmentados ($p = 0,023$) en comparación con el GC. El consumo crónico de linaza alteró los indicadores hematológicos e inmunológicos en las ratas Wistar adultas. La suplementación con linaza parece ser beneficiosa en el mantenimiento o la reducción de la masa corporal.

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Palabras clave: Peso corporal. Linaza. Indicadores hematológicos. Leucocitos. Ratas.

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Abbreviations

AA: Arachidonic acid.
CG: Control group.
DHA: Docosahexaenoic acid.
EPA: Eicosapentaenoic acid.
FEC: Food Efficiency Coefficient.
FG: Flaxseed group.
GI: Growth index.
LabNE: Experimental Nutrition Laboratory.
PER: Protein Efficiency Ratio.
PUFAs: Polyunsaturated fatty acids.

Introduction

Functional food, such as flaxseed, has attracted great attention due to beneficial effects in preventing diseases.¹ Previous studies justify its utilization to ameliorate lipid profile,^{2,3} reduce glycemia,⁴ diminish tumor growth⁵ and autoimmune diseases.⁶

This seed is made up of 41% lipids, 28% fibers, 21% protein, 4% minerals and 6% carbohydrates.⁷ Benefits to health, mediated by flaxseed, are mainly attributed to its main components: high content of linolenic acid (50-55%) and the secoisolariciresinol diglucoside, which is a lignan present in flaxseed 100 times more than in other food source.^{8,9}

Little is known about chronic use of flaxseed upon hematologic indicators, once it has got good amino acids profile despite its low biologic value when compared to animal derived protein.¹⁰ Furthermore, in its composition there are anti-nutritional factors that can cause adverse effects. Linatin can interfere with B6 vitamin absorption, causing deficiency; cyanogenic compound and phytic acid which can chelate minerals such as zinc, iron and calcium.^{11,12}

It is important to determine protein concentrations into blood so as to evaluate if flaxseed intake can provoke hematologic disorders. In humans, it has been studied the short term effects of flaxseed intake upon hematopoietic system, albumin and serum proteins, demonstrating that flaxseed do not provoke deleterious effects when consumed during four weeks.¹³ In rodents, a different result was reported when 10% flaxseed diet was offered to rats during 56 days. Authors report increase in hematocrit and unchanged values of hemoglobin.¹⁴ Supplementation with a lignan complex from flaxseed in humans for 2 months showed no adverse effects upon hematopoietic system. However, a reduction of leukocytes at the end of the study was found.¹⁵ In this way, it is known that diet is important to maintain adequate body lipid composition. There is increasing interest in utilization of polyunsaturated fatty acids (PUFAs) as natural anti-inflammatory agents against inflammatory response and destructive auto-immunity. Greater interest is drawn to n-3 family PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whose precursor is alpha-linolenic acid, which

is also in flaxseed composition. These PUFAs are widely considered to suppress lymphocyte proliferation and activate macrophages, limiting arachidonic acid (AA).¹⁶ A cohort study revealed the presence of abnormal leukocytes counting, which when increased is associated to higher mortality in successive generations.¹⁷ This counting seems to be an independent risk factor, being a cheap and handy indicator to evaluate inflammatory process related to countless diseases.¹⁸

No study on possible effects of chronic intake of diet containing 25% of flaxseed was found in the literature. Therefore, the present work aimed to evaluate whether chronic flaxseed intake yields alterations upon Wistar rats hematologic and immunological indicators, besides effects upon body development.

Material and methods

Experimental protocol

The research project was submitted to Ethics Committee in Animal Research of Fluminense Federal University (UFF), N° 00105-09. All procedures were carried out according to Brazilian Scientific Society of Laboratory Animals (SBCAL).

Female Wistar rats were obtained from colonies kept at Experimental Nutrition Laboratory (LabNE) at UFF, nulliparous, 90 days old, matched in a proportion of 3 females to 1 male, receiving commercial chow (23% protein, Nuvilab®, Nuvital Ltda, Paraná, Brazil). After deliver, mothers were randomly assigned to 2 groups during lactation period: Control group (CG), with casein based diet, containing 17% of protein and Flaxseed group (FG), with casein based diet, containing 17% protein with the addition of 25% of flaxseed. At weaning, 12 male pups from each group continued receiving the same experimental diets of their original groups (with only 10% of protein, AIN-93M) until adult age, when they were sacrificed at 250 days old. Body mass and diet intake were evaluated 3 times a week. All animals were kept under controlled temperature (21-23°C) and dark/light cycle (12/12 h), receiving water and diet *ad libitum*. Rats were anesthetized with intraperitoneal injection of Thiopentax (sodic thiopental 1 g, Cristália Produtos Químicos Farmacêuticos Ltda, Brazil) at a dose of 5% (0.15 mL/100 g of body mass) so as to obtain blood sample through cardiac puncture, being part of the sample placed into tubes containing ethylenediamine tetraacetic acid (EDTA) in order to determine hemoglobin and hematocrit concentrations.

Experimental diets

Flaxseed was ground into a blender to obtain a flour, which afterwards was weighted and immediately used to manufacture diet. Experimental isocaloric diets were

Table 1
Composition of 100 g of each experimental diet given during lactation (17% of protein; AIN93-G) and after lactation phase (10% protein; AIN93-M)

| Ingredient | Control 17% (g) | Flaxseed 17% (g) | Control 10% (g) | Flaxseed 10% (g) |
|--------------------------|-----------------|------------------|-----------------|------------------|
| Casein | 20 | 14.11 | 11.8 | 5.9 |
| Flaxseed | 0 | 25 | 0 | 25 |
| Cornstarch | 52.95 | 45.84 | 61.2 | 54.1 |
| Sucrose | 10 | 10 | 10 | 10 |
| Mineral mix ^a | 3.50 | 3.50 | 3.50 | 3.50 |
| Vitamin mix ^a | 1 | 1 | 1 | 1 |
| Soybean oil | 7 | 0 | 7 | 0 |
| Cellulose | 5 | 0 | 5 | 0 |
| Choline bitartrate | 0.25 | 0.25 | 0.30 | 0.30 |
| L-Cystine 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| Tert-Butylhydroquinone | 0.0014 | 0.0014 | 0.0008 | 0.0008 |
| Total | 100 | 100 | 100 | 100 |

^aReeves et al.¹⁹

prepared at LabNE, with 17% of protein and the addition of recommended amounts of vitamins and minerals, following patterns established by *Committee on Laboratory Animal Diets*, 1979, modified by *American Institute of Nutrition-93* (AIN-93G) so as to guarantee that each nutrient exerts its specific function during nursing.¹⁹ Diet offered to FG had a concentration of 25% flaxseed, aiming at reaching the recommendation of fiber. This amount of flaxseed had been previously used in other study.²⁰ Ingredients of experimental diets (table I) were weighted and homogenized in industrial mixer Hobart® (São Paulo, SP, Brazil) with boiling water to allow amid gelatinization. The resulting mass was transformed into pellets and dried into ventilated oven (Fabbe-Primar® n°171, São Paulo, SP, Brazil) at 60°C for 24h, and after identification, diet was kept under refrigeration until be used. After lactation phase, rats received diets containing 10% of protein, following AIN-93M (table I).

Evaluation of biological value of diets

In order to evaluate biological value of diets, the following indexes were used: Protein Efficiency Ratio (PER), this method is defined as PER = variation of weight gain (g)/protein intake (g). Evaluation of PER was made into a group of animals being fed with the studied protein during 28 days. Considering total variation of total body protein stemmed from differences in the quality of dietary proteins, it is common to measure variation of body mass as a reflection of global actuation of protein intake.²¹ Afterwards, growth index (GI) was used, which is represented by the application of the same formula of PER, but using data concerning 28 post weaning days.²² The analysis of Food Efficiency Coefficient (FEC) is determined by the relationship between weight variation

of animals and dietary intake during 28 days following weaning and demonstrates to which extent one gram of diet promotes increase of body weight.²³

In order to calculate the above mentioned indexes, body weight and diet intake were collected each 2 days during the whole experiment. For body mass determination, an electronic digital scale with precision of 0.05 g, Gehara®, was used.

Biochemical determination

Blood collection and sample preparation

Rats were anesthetized with an intraperitoneal injection of Thiopentax (sodic thiopental 1 g, Cristália Produtos Químicos Farmacêuticos Ltda, Brazil) at a dose of 5% (0.15 mL/100 g of body mass) so as to obtain blood sample through cardiac puncture. Blood sample placed into EDTA containing tube was used to determination of hemoglobin and hematocrit; blood sample without reagents was used to determination of albumin and total protein. Blood was centrifuged (Sigma centrifugal) at 3,500 rpm during 15 minutes to obtain serum, which was stored at -20°C. Analyses of hemoglobin, albumin and total proteins were carried out using BIOCLIN kits (Quibasa industry-Química Básica Ltda/Belo Horizonte-MG). On the other hand, hematocrit was determined with total blood sample (with EDTA), using microhematocrit technique through disposable microcapillary.

Differential counting of leukocytes

For differential counting of leukocytes, blood smear technique was used, being staining of slides made by

Table II
Effect of diets upon protein efficiency ratio, food efficiency coefficient, accumulated intake of diet and protein, growth index and final body mass of animals

| | FG (n = 12) | CG (n = 12) | P value |
|--|--------------------------------|--------------------------------|-----------|
| Protein Efficiency Ratio | 3.02 ± 0.33 ^a | 3.84 ± 0.14 ^b | p < 0.000 |
| Food efficiency coefficient | 0.30 ± 0.034 ^a | 0.38 ± 0.014 ^b | p < 0.000 |
| Accumulated intake of diet (g) | 3,318.06 ± 205.90 ^a | 3,929.76 ± 268.74 ^b | p < 0.000 |
| Accumulated intake of diet protein (g) | 331.81 ± 20.59 ^a | 392.98 ± 26.87 ^b | p < 0.000 |
| Growth index | 1.56 ± 0.21 | 1.65 ± 0.14 | p = 0.276 |
| Final body mass (g) | 522.36 ± 54.29 ^a | 654.18 ± 77.24 ^b | P < 0.000 |

FG = flaxseed group; CG = control group.
Different letters in the same row indicate significantly different at p < 0.05.

Table III
Effect of diets upon biochemical analyses of animals at the end of experiment

| | FG (n = 12) | CG (n = 12) | P value |
|----------------------|---------------------------|---------------------------|-----------|
| Hemoglobin (g/dL) | 13.20 ± 1.24 ^a | 11.89 ± 1.23 ^b | p = 0.019 |
| Hematocrit (%) | 42.27 ± 4.34 | 38.60 ± 4.78 | p = 0.081 |
| Albumin (g/dL) | 3.94 ± 0.26 ^a | 3.42 ± 0.69 ^b | p = 0.030 |
| Total protein (g/dL) | 6.85 ± 0.78 | 6.64 ± 0.35 | p = 0.425 |

FG = flaxseed group; CG = control group.
Different letters in the same row indicate significantly different at p < 0.05.

Instant-Prov kit (Newprov). Different types of cells were evaluated by manual counter ELO'S, with Keys correspondent to each sort of cell.²⁴

Statistical analysis

Data is presented as average and standard deviation. The normal distribution of the values found was tested through Kolmogorov-Smirnov test. Once the normality of data was verified, it was submitted to comparison between groups using Student T test to independent data. In the results that did not follow normal distribution, non-parametric Wilcoxon test was chosen. The established significance level was p ≤ 0,05. All these analysis were made by S-Plus 8.0.

Results

Evaluation of biological value of diets

It was verified that FG had inferior values of PER (p < 0.000) and CEA (p < 0.000) when compared to CG (table II). Diet intake (p < 0.000) and protein intake (p < 0.000) during this period were significantly inferior in FG, being both evaluations related to consumption if the animal was maintained until 250 days of life (table II). Considering GI, both groups behaved in a similar way (table II). At 250 days old, FG presented body smaller mass than CG (table II).

Biochemical methods

In table III, FG showed the highest values of hemoglobin (p = 0.019). It was not observed alterations regarding hematocrit percentage and total proteins at 250 days old animals. Higher albumin values (p = 0.030) were found in the group fed onto a diet containing 25% flaxseed.

Table IV shows results of differential counting of leukocytes at the end of the experiment. It was observed smaller percentage of lymphocytes (p = 0.016) in FG and higher percentage of segmented leukocytes (p = 0.023) when compared to CG. It was not verified differences between means of any following parameter: band neutrophils, monocytes, basophils and myelocytes.

Discussion

Taking PER and FEC results into account, it can be perceived that flaxseed based diet resulted in reduced growth during 28 days after weaning. Lenzi-Almeida et al.²⁵ concluded that flaxseed had inferior impact upon growth than casein diet, indicating that flaxseed cannot be used as exclusive protein source in human diet.

Despite having inferior diet and protein intake during all the experiment, FG presented GI similar to CG at 250 days. This result suggests that even if FG consumed smaller amount of protein and diet, it did not result in any difference concerning growth trajectory

Table IV
Dietary effects upon differential counting of leukocytes at the end of experiment

| | FG (n = 12) | CG (n = 12) | P value |
|----------------------|----------------------------|----------------------------|-----------|
| Lymphocytes (%) | 66.71 ± 8.98 ^a | 79.27 ± 10.37 ^b | p = 0.016 |
| Segmented (%) | 26.00 ± 10.38 ^a | 13.73 ± 8.05 ^b | p = 0.023 |
| Band neutrophils (%) | 2.00 ± 1.41 | 2.45 ± 1.86 | p = 0.744 |
| Monocytes (%) | 4.57 ± 2.23 | 3.91 ± 3.56 | p = 0.634 |
| Basophils (%) | 0.71 ± 1.88 | 0.27 ± 0.65 | P = 0.828 |
| Myelocytes (%) | 0 ± 0 | 0.36 ± 0.67 | P = 0.130 |

FG = flaxseed group; CG = control group.

Different letters in the same row indicate significantly different at $p < 0.05$.

after the initial period evaluated by PER. Similar result was found when a flaxseed based diet was offered for 180 days to rats immediately after weaning.²⁶

At the end of experimental period, FG showed a reduction in body mass. Countless authors state that this effect or body mass control can be accounted for intake of integral seed or its isolated components. A previous study showed that after the consumption of isocaloric diets, the group which did not receive flaxseed had greater body mass than the group whose diet contained flaxseed.²⁷ In comparison with control group, female Wistar rats presented smaller weight gain when supplemented with SDG (Secoisolariciresinol diglucoside) and SECO (secoisolariciresinol) for 4 weeks, both of which were obtained from flaxseed.²⁸ Less weight gain was also detected in rats fed with high fat diet made up with flaxseed oil.²⁹ Another study in humans, where three different groups received margarine supplemented with ALA, EPA or DHA for six weeks, demonstrated that effects upon body mass and body mass index seem to be related to duration of supplementation, given that authors did not found differences after treatment.³⁰ In this way, despite being inadequate during growth and development periods, flaxseed and/or its components are capable of reducing body mass gain, being an important tool to control risk factor associated with chronic degenerative diseases.

Serum proteins are cheap and accessible indicators, frequently used in clinical routine to evaluate nutritional status of patients. Furthermore, they are directly related with the intake of some nutrients.³¹ For instance, hypoalbuminemia is a predictor of bad prognostic in a wide range of situations, not only during the course of a disease but also in healthy population.³² Flaxseed intake seemed to improve these indicators once FG presented higher values of hemoglobin and albumin when compared to CG at 250 days. Alterations in hematocrit percentage and total protein were not observed. In a previous study from our group, pregnant rats were fed with flaxseed based diet during lactation period exclusively and the resulting offspring showed smaller values of hemoglobin at adult age when compared to CG.³³ Flaxseed flour when offered at a

concentration of 40% to pregnant rats also provoked reduction, but in serum protein.³⁴ In humans, daily supplementation of lignan complex from flaxseed for 12 weeks reduced hemoglobin values, without effect upon albumin, total protein and hematócrito.³⁵

Chronic intake of flaxseed yielded less percentage of lymphocytes together with less segmented leukocytes. Experimental researches revealed that inhibition of lymphocytes proliferation only when flaxseed was present at very high concentrations (40%) and this result was attributed to the presence of ALA, once the group that consumed the defatted seed did not show these differences.³⁶ Docosahexaenoic acid (DHA) is a fatty acid from ALA family, which has shown anti-inflammatory properties. A recent study determined the effect of DHA upon phagocytic and chemotactic action of peritoneal macrophages, being described a reduction of these activities in rats supplemented with DHA. These results demonstrate the effects of DHA upon immunological system modulation in rats.³⁷ Less deposition of lymphocytes was found in gut mucosa of Wistar rats that received flaxseed oil.³⁸ Supplementation of a complex containing SDG in humans did not alter total leukocytes.³⁵ Likewise, a study in vitro showed that lignans do not modulate leukocyte function in humans.³⁹

Conclusion

Our results suggest that chronic intake of 25% flaxseed into diet alters hematologic and immunological indicators in adult Wistar rats. Supplementation with flaxseed seems to be beneficial to maintenance or reduction of body mass. Further studies are required to elucidate the effects of this seed upon hematologic and immunological findings, considering that literature is scarce.

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