IJBPAS, November, 2014, 3(11): 2372-2381

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

THE EFFECTS OF DIETARY n-6/n-3 RATIO OF POLYUNSATURATED FATTY ACIDS ON THE PHYSICAL PARAMETERS OF FEMUR AND BIOMARKERS OF BONE METABOLISM IN RATS

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ABSTRACT

The aim of this study was to investigate the effects of different n-6/n-3 fatty acid ratios in diets on body weight, physical characteristics of bones and plasma biomarkers in rats. Corn oil and flax oil were used as sources of n-6 and n-3 fatty acids, respectively, and by adding different ratios of corn and flax to a basal diet. Twenty Vistar male rats (25 days old) were randomly assigned to four dietary treatments included: treatment 1: basal diet + 5 g flax oil + 2 g corn oil; treatment 2: basaldiet + 5 g corn oil+ 2 g flax oil; and treatment 4: basal diet+ 7 g flax oil. Results showed non-significant effects of n-6 and n-3 or their combination on body weight of rats. The diameter of femur bones was significantly higher in treatment 1 than other treatments. The effect of treatments on length of femur bones was not significant ($p<0.05$). For calcium content of femur bones, the significant differences were found between treatments. While the dietary n-6 and n-3 fatty acid had no significant effects on the glucose and PGE2 concentration of plasma, their effects on IGF-I and Alkaline Phosphatase were significant. There were no significant differences among treatments for Calcitonin concentration, but parathormone was significantly higher in treatments with 7 g corn oil and treatment with 7 g flax oil than other treatments. Calciteriol was significantly higher in treatments 1 and 2. The concentration of plasma calcium was not significant among treatments, but Phosphorus concentration was significantly higher in treatment 3. The effects of n-6 and n-3 fatty acids was not significant on most of appropriate characteristics, in most cases a mixture of them (treatments 1 and 2) had better performance. Therefore, it was

suggested that to exploit of appropriate characteristics of n-6 and n-3 fatty acids, a mixture of them with an appropriate ratio should be used.

Keywords: Rat, n-6 and n-3 Fatty Acids, Femur Bone, Plasma Biomarker

INTRODUCTION

Humans require dietary intake of some certain polyunsaturated fatty acids (PUFAs) for the maintenance of health. The role of PUFAson the human health has been frequently investigated [**1**]. Among PUFAs, n-6 series especially linoleic acid (LA) and Arachidonic acid (AA) and the n-3 series;the most important of which are linolenic acid (LNA), Eicosapentaenoic acid (EPA) and Docohexaenoic acid (DHA), are essential for normal growth, and have a vital role in the prevention of coronary disease, osteoporosis, diabetes, arthritis, and some kinds of cancers.

Beside calcium, vitamin D, and protein whichare among the dietary essentials for skeletal health, certain families of PUFAs have been found to be effective in altering the activities of both osteoblasts and osteoclasts, thus affecting bone formation and bone resorption. n-6and n-3fatty acidsare involved in bone growth and development. The effects of n-6 and n-3 on the bone growth is mediated by PGE2, which is synthesized from arachidonic acid, and is a potent stimulator of bone resorption and, to date, is the primary prostaglandin affecting bone metabolism. Diets that contain various plant oils provide high levels of n-6 and can cause an overproduction of PGE2 and lead to reduced rate of bone formation [**2**].

Concerns about recent changes in the dietary balance of n-6 ton-3 fatty acids have raised interest in differential cellular effects of dietary fatty acids and in their potential skeletal effects. The ideal n-6/n-3 fatty acidratio is 1:1, but usually it is higher and in humans reaches 1:11 to 1:30 [**3**]. It is mainly because of the modification of dietary patterns over the last 100-150 years. A shift from oils with the animal origin to vegetable oils which has led to a change in fatty acid consumption, with an increase in the consumption of n-6 and a marked reduction in the consumption of n-3. In some varieties of sunflower, for example, the n-6/n-3 fatty acid ratio reaches 77:1. It is why, compared to the past, the occurrence of some diseases such as osteoporosis and cardiovascular diseases has increased significantly. The higher the ratio of n-6/n-3 fatty acids the higher is theosteoporosis cases and death rate from cardiovascular diseases.

Albeit, the role of n-3 fatty acid has been frequently investigated both in human and animals [**1,2, 4, 5, 6**] and research and discoveries regarding this fatty acid have expanded over time, little is known about the effects of different n-6/n-3 fatty acid ratio in diets on physical characteristics of bones and plasma biomarkers. This research, therefore, conducted to investigate the effect of differentn-6/n-3 fatty acid ratio on the body weight, physical parameters of bones and plasma biomarkers in rat.

MATERIALSAND METHODS

Animals and Diets

Twenty Vistar male rats (25 days old) were bought from the Shahid Chamran University, (Ahwaz, Iran) initially weighing 80-90 g. A standard diet was purchased from the Pars Company and used as the basal diet from which 4 treatments were formed by adding different ratios of corn and flax as the source of n-6 and n-3 fatty acids, respectively. The n-3 content of flax oil reaches more than 60% of total fatty acids of flax oil and the n-6 comprises more than 60% of corn oil. The nutrient composition of the experimental diets was given in **Table 1**. The rats were randomly assigned to one of the four dietary treatments. Treatment 1: basal diet + 5 g flax oil + 2 g corn oil, treatment 2: basal diet + 5 g corn oil + 2 g flax oil, treatment 3: basal diet + 7 g corn oil, and treatment 4: basal diet +7 g flax oil. All rats were housed individually in wire hanging cages with food and water available *ad libitum* and on a 12-h light/dark cycle for 59 days and weighed weekly. The nutrient composition of the flax oil and corn oil were given in **Table 2 and Table 3**.

Sampling and Measurements

At the end of experiment (day 59), rats were anesthetized and venesection was done from left ventricle. Blood sample was taken from each rat and for preventing from coagulation; 30 microliter heparin was added to each blood sample. After venesection, for separation plasma, blood samples were centrifuged and the plasma was collected with sampler and transmitted to clean Ependorf tubes. All samples were kept on ice at the time of collection and promptly frozen at -20 °C.

Both the right and left femurs were taken. After removal of the soft tissue, the bones were weighed and their mass and density were measured. In order to measure the calcium content of bones, first Trichol was used to remove fat from bones and then bones were ashed at 600 °C for 8 hours [**7**]. Afterward, calcium levels were measured by Manganometry method.

Statistical Analysis

Data was analyzed with General Linear Model (GLM) as implemented in the SAS [**8**]. Also Duncan Multi Range Test was performed to compare means where a significant difference was identified ($P < 0.05$).

Table 1: Analysis of basal diet used

Material	Value (%)
Energy	17 k Cal/kg
Moisture	10
Protein	20
Fat	4.5
Fiber	4
Calcium	0.95
Phosphorus	0.65
Sodium	0.5
Lysine	1.15
Methionine	0.33
Threonine	0.72
Tryptophan	0.25

Table 2: Nutrient Analysis of Corn Oil

Nutrients	units	Value per 14 grams of edible portion
Energy	Kcal	885
Protein	g	0
Total lipid(fat)	g	100
Fatty acid, total saturated	g	12.95
Total monounsaturated	g	27.57
Total polyunsaturated	g	54.67
n-6	g	53.22
n-3	g	1.16

Table 3: Nutrient Analysis of Flax Oil

Nutrients	units	Value per 100 grams of edible portion
Energy	Kcal	875
Protein	g	0
Total lipid(fat)	g	100
Fatty acid, total saturated	g	8.8
Total monounsaturated	g	15.5
Total polyunsaturated	g	75.7
n-6	g	14.9
n-3	g	60.7

RESULTS AND DISCUSSION

Body Weight

Results of weekly body weight of rats are shown in **Table 4**. There was no significant difference between treatments (p>0.05). Our results are in agreement with authors [**7, 9, 10, 11**], who reported no significant effects of dietary n-6 and n-3 fatty acidratio on body weight of rats.

Physical Characteristics of Femur Bones

Diameter and length of femur bones are shown in **Table 5**. A significant difference (p<0.05) was observed between treatments. The diameter of femur bones was the highest in treatment 1 (4.81 mm) and the least in treatment 4 (3.65 mm). However, no notable difference (p>0.05) was observed between treatments regarding length of femur bones. Claassen *et al*.[**7**] studied the effect of differentn-6/n-3 fatty acid ratio (3:1, 1:1, 1:3) on length of femur bones and reported that those ratio of n-6/n-3 fatty acid did not have a significant influence on the length of femur bones. Similar observations have been reported by Green *et al*.[**10**]. The weight, mass and density of femur bones are shown in **Table 5**. As observed, the difference between four treatments regarding these characteristics were not significant (p>0.05). The findings are consistent to the study of Green *et al*. [**10**]. Concerning the calcium content of femur bones, the difference between treatments were significant (p<0.05) in a way that treatment 2 had the highest value. Claassen *et al*. [**7**] found that treatments with n-6/n-3 fatty acid ratio of 3:1 significantly increased the calcium content of femur bones compared with other ratio (1:1 and 1:3). In addition, Watkins *et al*. [**12**] reported an increase in the calcium content of femur bones in response to the n-6/n-3 fatty acid ratio of 5:1 compared with 10:1 ratio of n-6/n-3.Linoleic acid (LA) and alpha-linolenic acid (ALA) could increase the absorption and maintenance of calcium in bones [**7**]. Moreover, it has been reported thatn-3 activity includes calcium absorption in bones and differentiation of Osteoblasts [**12**]. However, Green *et al*. [**10**] reported non-significant influence of n-3 fatty acid on calcium content of femur bones. It seems that n-6 or n-3 fatty acid cannot change the calcium content of bones lonely and for increasing the calcium contents of bones, using a mixture of n-6 and n-3 fatty acids is necessary.

Plasma Biomarkers

Table 6 included information regarding the level of blood biomarkers in four treatments. Considering glucose, the dietary n-6 and n-3 fatty acid had no significant effects on the glucose concentration of plasma. Reports showed the positive effects of n-3 on insulin activity and therefore glucose concentration of plasma [**13**]. In our study, treatment 4 which includes only flax oil resulted in less glucose concentration and treatment 3 which includes corn oil produced the highest glucose concentration. Although, the differences were not statistically remarkable, it seems that n-6 and n-3 fatty acids affect glucose concentration in opposite ways. Green *et al*. [**10**] stated that diets supplemented with appropriate ratio of n-6/n-3 could control the level of glucose concentration and prevent diabetes.

Regarding PGE2, again the treatment 3 had the highest value. However, the differences among four treatments were not obvious ($p>0.05$). Some researchers [**1, 10, 14**], reported that dietary intake of n-3 cause to decrease in PGE2 concentration of plasma and could prevents occurrence of some health problems such as osteoporosis. High level of n-6 offsets positive effects of n-3 fatty acid because it increases the PGE2 concentration which causes an increase in bone resorption and consequently, results in a sharp decline in bone mass [**1**]. Therefore, as observed here, diets which contain high levels of n-6 fatty acid increased the PGE2 concentration of plasma.

As regard IGF-I, a great influence ($p>0.05$) was observed by implementing treatment 4 which includes flax oil, the source of n-3 fatty acid, indicating the ability ofn-3 to decrease IGF-I concentration. The differences among other treatments were not pronounced ($p>0.05$). While Watkins *et al*. [**6**] reported no significant influence of n-6 and n-3 on concentration of IGF-I. In the study of Green *et al*. [**10**] the effects of n-6 and n-3 on concentration of IGF-I were significant. Li *et al*. [**15**] reported that n-6 and n-3 fatty acids not only influence the PGE2 concentration, but also affect the plasma concentration of IGF-I. They observed that n-3 fatty acid decrease the concentration of IGF-I to the normal level in rats after a period of feeding with sources of n-6 fatty acid.

In the treatment 3, alkaline phosphatase (ALP) was significantly lower than other treatments ($p<0.05$). Liver is responsible for a high percentage of ALP production. In some situations such as growth of bones during fast growth phases, high concentration of ALP is observed in the plasma which is because of osteoblasts activity [**16**]. Watkins *et al*. [**1**] showed that dietary intakes of PUFAs in rats not only stimulate bones development but, also enhance the concentration of ALP in the plasma.

The treatments studied did not show any significant difference regarding calcitonin level ($p>0.05$). Calcitonin activity is toward decreasing plasma content of calcium. However, considering Parathyroid hormone (PTH), the differences among treatments were significant ($P<0.05$) in such a way that

PTH concentration in treatments 3 and 4 were higher than treatments 1 and 2. Our results were inconsistence with finding of Green *et al*. [**10**] who reported non-significant effect of PUFAs on PTH concentration of plasma. PTH activity opposites calcitonin and increases the plasma content of calcium. In treatments 3 and 4, the concentration of calciteriol was significantly higher ($p<0.05$) than treatments 1 and 2 inconsistent with Green *et al*. [**10**] who reported that dietary intake of PUFAs increases the concentration of calcitriol. Calcitriol increase the level of calcium in the plasma by boosting the activity of PTH [**17**].

Non-significant differences were observed between treatments concerning calcium content of plasma. Similar findings were reported by Sharif *et al*. [**18**]. In the treatment 3, the concentration of phosphorus was significantly higher than other treatments.

In conclusion, results obtained here indicated non-significant effects of n-6 and n-3 or mixture of them on body weight, length of femur bones, glucose, PGE2, Calcitonin and calcium concentration of plasma. But their effects on calcium content of femur bones, plasma phosphorus concentration, and the concentration of IGF-I, Alkaline Phosphatase, parathormone and calcitriolin plasma was significant. It should be noted that whereas the effects of n-6 and n-3 fatty acids was not significant on most of appropriate characteristics, in most cases a mixture of them (treatments 1 and 2) had more higher effects. Therefore, it can be recommended that to exploit of the appropriate characteristics of n-6 and n-3 fatty acids, a mixture of them with an appropriate ratio should be used.

Table 4: Weekly body weight (as gram) of rats in four treatments studied

Treatment	Week1	Week2	Week3	Week4	Week5	Week6	Week7	Week8
1	113	136	166	196	235	255	283	311
2	113	137	166	197	234	254	283	311
3	113	135	165	195	232	255	283	312
4	113	135	164	195	232	251	283	311
SEM	0.8	1.5	1.2	1	1.8	2.2	1.7	1.2
P value	0.71	0.34	0.28	0.11	0.17	0.22	0.95	0.46

Table 5: Physical Characteristics of Femur Bones in Four Treatments Studied

Treatment	Diameter mm	Length mm	Weight g	Mass	Density
1	4.81[a]	31.48	0.74	0.098	8.40
2	4.15[ab]	31.45	0.68	0.094	8.00
3	4.40[a]	30.14	0.66	0.090	7.91
4	3.65[b]	28.90	0.65	0.090	8.46
SEM	0.46	2.12	0.07	0.009	0.45
P value	0.01	0.21	0.18	0.93	0.17

[a] Means within a column withdifferent superscript are significantly differ ($P < 0.05$).

Table 6: Biomarkers of blood plasma of rats in different treatments

Treatment	Glucose mg/dl	PGE2 pg/ml	IGF-I ng/ml	ALP IU/L	Phosphorus mg/dl	Calcium mg/dl	Calcitonin pg/ml	PTH pg/ml	Calcitriol pg/ml
1	94.66	38.58	2.64[a]	188.33[a]	5.53[b]	8.57	6.11	29.95[b]	25.64[ab]
2	93.13	37.60	2.74[a]	183[a]	5.16[b]	8.98	6.33	29.80[b]	24.87[b]
3	97.16	41.54	2.54[a]	159.66[b]	6.01[a]	8.59	6.14	34.26[a]	26.52[a]
4	93.03	38.81	1.95[b]	191[a]	5.55[b]	8.40	6.11	33.22[a]	25.94[a]
SEM	2.94	2.24	0.25	9.81	0.22	0.40	0.26	1.01	0.49
P value	0.16	0.24	0.02	0.01	0.01	0.39	0.71	0.001	0.20

[a] Means within a column withdifferent superscript are significantly differ ($P < 0.05$)

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