

Effects of Flax Seed Oil on Histological & Biochemical Metamorphosis Induced by Caffeinated Energy Drink in Adult male Albino Rats Bone

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Abstract

Objective: To appraise protective effects of flax seed oil on caffeinated energy drink induced histological (osteoporotic cavities) & biochemical (Alkaline phosphatase) changes in adult male albino rats bone

Method: This was an experimental study conducted at department of Anatomy Shaikh Zayed Postgraduate Medical Institute Lahore for 8 weeks. 32 adult male albino rats weighing (250-300gm) were randomly divided into four groups. Group A (Control) received corn oil 5ml/kg body weight in addition to basal diet daily for 8 weeks. Group B (Experimental) received caffeinated energy drink (15ml/kg body weight) and corn oil (5ml/kg body weight) for 8 weeks. Group C (Experimental) received caffeinated energy drink (15 ml/kg body weight) and 40% flax seed oil (5ml/kg body weight), while group D (Experimental) received caffeinated energy drink (15ml/kg body weight) and 60% flax seed oil (5ml/kg body weight) daily for 8 weeks respectively. All animals were weighed before and after experiment. Blood samples were taken before and after 24 hours of giving last doses for estimation of serum ALP. Furthermore, right femora were used for histological purpose.

Results: Significant difference in mean osteoporotic cavities of femora among experimental groups ($p = 0.000$) was observed. Insignificant difference was found in mean serum ALP ($p = 0.072$).

Conclusion: Flax seed oil helped in alleviating osteoporotic changes induced by caffeinated energy drink in femora of adult male albino rats.

Keywords: Flax seed oil, caffeinated energy drink, osteoporotic cavities, ALP

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Introduction

Caffeinated energy drinks are group of beverages tenanted our markets globally specially in younger population in order to amplify endurance performance.¹

They mainly contain caffeine as a stimulant drug in addition to taurine, glucose, sucrose, glucuronolactone, vitamin B1, B2, B6, B12, artificial flavor and sparkling water.²

Caffeine ($C_8H_{10}N_4O_2$) is the world's most widely used psycho active drug, chemically related to adenine and guanine bases of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).³ It is naturally found in coffee beans, guarana seeds and cocoa beans. Many caffeine based items like chocolate and its products, soft drinks, tea, coffee, ice cream, pain and flu medicines are commonly used in daily life.⁴

Caffeine acting as non-selective phosphodiesterase inhibitor raises intracellular CAMP, activates protein kinases-A, inhibits leukotrien synthesis and reduces

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GABA production in tuberomamillary nucleus thus produces alertness.⁵ Caffeine is metabolized in liver by P450 (CYP1A2) to active methylxanthine, theobromine and theophylline. It crosses blood brain barrier, placenta and can be found in breast milk.⁶

Long term use of caffeine not only leads to addiction and drug dependence but it increases risk of heart disease, type-2 diabetes mellitus, liver disease and bone resorption.⁷ More than 300 mg a day can have deleterious effects on human body and its intoxication can lead to tachycardia, hypokalemia, gastrointestinal disturbances, hallucinations, seizures, arrhythmias and even death.⁸ Caffeine acting as a diuretic causes urinary excretion of calcium phosphorus and magnesium even after several hours of its intake.⁹ It also hinders the absorption of vitamin D, essential for absorption of calcium from intestine. Various human studies revealed that young adults and women who consume adequate calcium and moderate caffeine may have little or no deleterious effects. However, adults and older women who used to take more caffeine than compensated loss of calcium are at higher risk of osteoporosis.^{6,8,9}

The use of functional foods has also been increased universally not only due to their nutritional values but also secure against detrimental health problems.¹⁰ Regarding this aspect flax seed and its various products have occupied a major proportion of ancient medical history along with documented positive effects on bone mass and their biomarkers.^{10,11} Flax seed or linseed (*Linum usitatissimum*), has been cultivated for thousands of years by the ancient civilization of Ethiopia and Egypt for textile fiber and nutrition. Flax seeds come from the flowers of plants and can be pressed into oil or ground into flax seed meal for baking.^{12,13}

Flax seed oil is rich in Omega-3, Omega-6 fatty acids (ALA), Polyunsaturated fatty acids (PUFA), eicosapentanoic acid (EPA), docosahexaenoic acid (DHA), lignans, proteins, carbohydrates, Vitamin A, B1, B2, B6, C, E, folic acid and trace minerals like calcium, magnesium, phosphorus, potassium, copper, sodium and folate.¹³ Omega-3 and Omega-6 fatty acids not only reduces inflammatory conditions like rheumatoid arthritis, osteoarthritis & osteoporosis but also provide protection against coronary artery disease, hypertension, hyperlipidemia, diabetes mellitus, chronic kidney disease (CKD), polycystic ovarian syndrome (PCOS) & metabolic syndrome.^{13,14}

Taking into consideration the above mentioned beneficial effects of flax seed oil on human body and due to substantial use of caffeinated energy drinks in our

youth, this study was designed to alleviate the hazardous effects of caffeinated drinks on osteoporosis and raised ALP levels of adult male albino rats.

Material and Methods

Thirty-two adult, healthy male albino rats, age (3-6 months), average weight (250-300 gm) were purchased from university of health science. They were divided into four groups. Group A (control), group B, C and D (experimental groups), each group consisting of 8 rats. All the rats were kept at room temperature of 22±25 °C. They were acclimatized for 7 days and had free access to food and water with ad libitum. A 12 hours light/ dark cycle was maintained.

After acclimatization, rats were divided randomly by lottery method into four groups. The weight of each rat was recorded before experiment and then marked with permanent markers for identification and placed in 4 different cages for 8 weeks.

Group A received corn oil 5ml/kg body weight by gavage for 8 weeks daily in addition to basal diet. Group B received 15ml/kg of caffeinated energy drink and corn oil 5ml/kg body weight daily for 8 weeks. Group C received 15ml/kg of caffeinated energy drink and 40% flax seed oil (100ml oil formed by adding 40ml of flax seed oil and 60ml of corn oil) 5ml/kg body weight daily for 8 weeks. Group D received 15 ml/kg of caffeinated energy drink and 60% flax seed oil (100 ml oil formed by adding 60 ml of flax seed oil and 40ml of corn oil) 5ml/kg body weight daily for 8 weeks.¹⁵

After 8 weeks, all the rats were weighed individually, euthanized for dissection after 24 hrs of last doses. A vertical midline incision was given on each thigh of the rat. The skin was reflected and muscles were removed to view femora. Both right and left femora were dissected out, cleaned and weighed individually. Furthermore; right femora were selected for statistical purpose.

Tissue processing done according to Spencer and Bancroft procedure. Fixed right femora were cut into 3-5 mm small pieces from mid shaft and embedded in paraffin blocks.¹⁶ 5µm thick sections were obtained and stained with haematoxylin and eosin. Slides of mid-shaft of femora were studied under light microscope by using various magnifications and comparison was made between control and experimental groups. Blood samples were taken from each animal via tail for estimation of serum ALP and then allowed to clot. The sera were immediately separated by centrifugation of the clotted blood and stored at -200C till analysis of biochemical

markers.¹⁶

Data was analyzed by using SPSS 20.0. The qualitative variable like osteoporotic cavities was reported by using frequency and percentage of each group. The quantitative variable like serum ALP was presented by using mean and standard deviation (S.D) and comparison among group was made by using One way ANOVA. Tukey's test for post hoc analysis was used where required. Chi-square test used for comparison among groups. Statistically significant p - value was ≤ 0.005

Results

Osteoporotic Cavities: Osteoporotic cavities in femora of all groups were observed. They were (100%) absent in group A (Fig. 1). (100%) present in femora of all rats of group B and the number of osteoporotic foci were more (5-6/field of vision) in this group (Fig. 2).

(25%) found in group C and their number were reduced (3-4/field of vision) (Fig.3). They were approximately (100%) absent in group D (Fig.4).



Figure.1 Photomicrograph of longitudinal section of femur (mid shaft) group A, absence of osteoporotic cavities (H&E 10X)



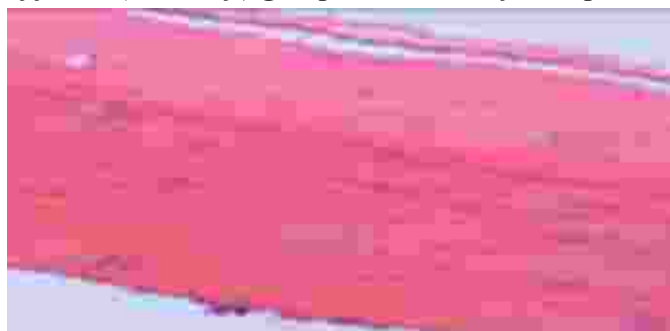
Figure. 2 Photomicrograph of longitudinal section of femur (mid shaft) group B, presence of osteoporotic cavities (yellow arrow) along with disruption of normal architecture of bone tissue (H&E, 10X)

Figure.3 Longitudinal section of femur (mid shaft) group C, reduced no. of osteoporotic cavities (yellow arrow)(H&E, 10X)



(H&E, 10X)

Figure. 4 Photomicrograph of longitudinal section of femur (mid shaft) group D, absence of osteoporotic



cavities & restoration of normal architecture of bone. (H&E, 10X)

The difference among control and experimental groups was significant ($p = 0.000$) (Table 1)

Multiple comparisons of osteoporotic cavities among various groups by one way ANOVA showed signifi-

Table 1: Osteoporotic cavities of femur of rats in control and experimental groups.

Group	OSTEOPOROTIC CAVITIES		Total
	Present	Absent	
A	0 (0.0%)	8 (100.0%)	8 (100%)
B	8 (100.0%)	0 (0.0%)	8 (100%)
C	2 (25.0%)	6 (75.0%)	8 (100%)
D	0 (0.0%)	8 (100.0%)	8 (100%)
Total	10 (31.3%)	22 (68.8%)	32(100%)

$p = 0.000$ * * Significant difference ($p < 0.05$)

cant difference between control group A and experimental group B, also between B, C and D, whereas insignificant difference was found between groups A, C & D (Table 2)

Alkaline Phosphatase (ALP) (u/l):

The mean ALP (u/l) before and after the experiment in

Table 2: Multiple comparisons of osteoporotic cavities of femur of rats in control and experimental groups.

Group	Chi-square	DF	p-value	
A	B	16.000	1	0.000*
	C	2.286	1	0.131 ⁺
	D	-	-	-
B	C	9.600	1	0.002*
	D	16.000	1	0.000*
C	D	2.286	1	0.131 ⁺

* Significant difference (p < 0.05)
⁺ Insignificant difference (p > 0.05)
 - Constant Result

all groups were observed. One-way ANOVA test among groups showed insignificant difference in mean serum ALP (u/l) before and after experiment (p = 0.096 and 0.072) respectively. (Table 3)

While comparing various groups ALP (u/l) before and after experiment. ALP (u/l) in group B was higher

Table 3: Alkaline phosphatase (u/l) in control and experimental groups

	Group	Alkaline Phosphatase (u/l)	
		Mean± SD	p-value
Alkaline Phosphatase u/l before experiment	A	679.38 ± 172.27	0.096 ⁺
	B	539.75 ± 159.16	
	C	482.25 ± 154.54	
	D	529.88 ± 141.99	
Alkaline Phosphatase u/l after experiment	A	694.25 ± 174.39	0.072 ⁺
	B	639.88 ± 174.55	
	C	498.25 ± 160.58	
	D	523.88 ± 143.82	

⁺ Insignificant difference (p > 0.05)

as compared to control group A, however insignificant difference was found in between them. There was also insignificant difference observed in between group C and D as compared to control group A.

Discussion

Caffeinated energy drinks have raised special health concerns in the recent past years due to deleterious effects on human body.^{3,8} Public interest towards functional food has been developed due to potential health benefits of their ingredients. According to European commission, functional foods not only provide basic nutrition but also improve physical and mental health and decrease the risk of diseases.^{12,13}

Flax seed and its various ingredients have potential health benefits due to their important ingredients like PUFA, MUFA, ALA in form of Omega-3, omega-6 fatty acids, Vit A, B, C, E, trace minerals like calcium, phosphorus, magnesium, potassium and copper.^{12,13,14}

In this study the presence or absence of osteoporotic cavities in all four groups were observed. It was significant (p = 0.000) (table 1). Multiple comparison of presence of osteoporotic cavities among various groups showed significant difference between control group A and experimental group B (p=0.000), between group B and D (p = 0.000) respectively, while insignificant difference was found in between A, C and D (table 2). According to Omaima et al, oral doses of caffeine lead to urinary excretion of calcium, phosphorus and magnesium even after several hours of consumption. Secondly, it inter-feres with absorption of vitamin D necessary for absorption of calcium from intestine. Uncompensated losses of calcium would be a risk factor for the development of osteoporosis.¹⁷

Another possible reason of the presence of osteoporotic cavities in group B was due to caffeine consumption which besides causing urinary excretion of calcium, phosphorus and magnesium also stimulated glucocorticoid production which in turn reduced osteoblastic activity and calcium absorption in gastrointestinal tract.^{9,18}

Treatment of group C and D with 40% and 60% of flax seed oil respectively helped in reduction of osteoporotic cavities due to antioxidant activity of flax seed oil which help to restore osteoporosis.^{11,19} It was in agreement with the work of Watkins et al, who reported that dietary omega-3 and omega-6 PUFA not only lower PGE-2 production but also increase EPA and DHA through modulation of cytokines, thus decreased bone resorption and osteoporosis. An over production of PGE-2 would impair bone by reducing the activity of osteoblas.¹⁹ Ilesanmi et al, had also clarified the pathogenesis of osteoporosis in postmenopausal women due to over-pro-duction of cytokines lead to imbalance between bone formation and resorption.²⁰ Costa et al, observed that flax seed ALA content increased HDL cholesterol and decreased the concentration of cholesterol which increa-sed the differentiation of osteoblasts and decreased osteoclast bone resorption.²¹

In the present study the mean serum ALP before and after the experiment in all groups was also observed. There was insignificant difference in mean serum ALP before experiment among all group (p = 0.096), however

after the experiment it was also insignificant ($p = 0.072$) (table 3). Multiple comparison of serum ALP among groups also showed insignificant difference in between control and experimental groups. The increase in ALP in experimental group B was due to caffeinated drink.

It was in accordance with the work of Taiwo et al, who also observed raised ALP in male and female rabbits at day 14 and 21 after administration of caffeinated drink. He also elaborated the reason of raised ALP in experimental groups as compared to control was due to hepatic canalicular obstruction associated with inflammation and hepatocellular injury.²²

The gradual reduction of ALP in group C and D was due to protective effect of flax seed oil in these groups. The results of present research work were in relevance with the work of Boulbarounet al, who not only observed improvement in micro architecture of bone but also in reduction of serum tartrate resistant acid phosphatase (TRAP) and Alkaline phosphatase (ALP) in adult female rats suffering from osteoporosis when treated with 10% flax seed and sesame seed oil.²³ Tariq et al also concluded in study that serum ALP could be used as an index of decrease in bone mineral density.²⁴

Conclusion

Flax seed oil helped in mitigating caffeinated energy drink induced osteoporosis and raised serum ALP. It should be added in diet to minimize bone and mineral loss both in younger and elderly group.

Conflict of interest: None

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Authors Contribution

MS: Conceptualization of Project

AB: Data Collection

AR: Literature Search

AZ: Statistical Analysis

AB: Drafting, Revision

AW: Writing of Manuscript