EFFECT OF CAFFEINE AND VITAMIN D3 ON PROLIFERATIVE AND HYPERTROPHY ZONES OF EPIPHYSEAL CARTILAGE OF MICE FEMUR

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ABSTRACT

Objective: To determine the effect of caffeine and vitamin D3 on the height of proliferative and hypertrophy zones of epiphyseal cartilage of developing femur of BALB/c mice.

Study Design: Laboratory based randomized control trial.

Place and Duration of Study: The study was conducted at anatomy department, Army Medical College (AMC), Rawalpindi, in collaboration with National Institute of Health (NIH), Islamabad from Oct 2014 to Oct 2015.

Material and Methods: A total of thirty (30) BALB/c mice (15 male, 15 female), three weeks old and weighing 12-14 g, were taken and divided in to three equal groups of 10 mice each (5 male, 5 female). The control group G1 was given normal diet with water ad libitum. In addition to the same diet, animals in experimental group G2 were given 10 mg of caffeine per 100 g body weight once a day on alternate days through oral gav age for 60 days. All animals of group G3 for 60 days were given caffeine 10mg/100gm body weight on alternate day and vitamin D3 0.1µg per day by oral gav age. At completion of the experiment, effect of caffeine and vitamin D3 on the height of proliferative and hypertrophy zones of epiphyseal cartilage of mice's femur was analyzed.

Results: The mean ± SD of height of proliferative and hypertrophy zones of control group G1 animals was found to be 31.5 ± 4.1µm and 43 ± 2.6µm, respectively. The mean ± SD of height of proliferative and hypertrophy zones of experimental group G2 animals was observed as 59 ± 3.2µm and 72.5 ± 7.2µm, respectively. The mean ± SD of height of proliferative and hypertrophy zones of experimental group G3 animals was measured as 46 ± 4.6µm and 54 ± 4.6µm, respectively.

Conclusion: Caffeine intake altered the height of proliferative and hypertrophy zones of the epiphyseal cartilage of developing femur; however treatment with vitamin D3 ameliorated this effect.

Keywords: Caffeine, Femur, Hypertrophy zone, Proliferative zone, Vitamin D3.

INTRODUCTION

Caffeine, a methylxanthine, forms part of our diet through tea, soft drinks particularly energy drinks and chocolate. It is also an ingredient in some prescription and non-prescription drugs, such as cold, allergy and pain medication. This compound which is extracted from over 60 different plant sources, like coffee beans, tea leaves, cacao seeds and cola nut seeds[1]. Caffeine is socially acceptable and commonly used drug world-wide, unlike most other psychoactive substances[2]. Taking caffeine in moderate quantity makes it a harmless substance that can be used without any negative outcome, but harmful effects can appear if it is ingested in high doses[3]. In recent times, there has been a sharp rise in energy drink consumption leading to caffeine abuse; this is mainly due to aggressive marketing and poor awareness regarding consequences of high caffeine use[4]. Therefore, it is important to understand its impact on the body including influence on cardio-respiratory, endocrine as well as neurological and musculoskeletal systems[5]. Experimental studies on young rats have shown that caffeine not only impairs development and mineral contents of osseous tissues but also inhibits bone mineralization, osteoblast differentiation and formation of extracellular matrix[6]. In Northern American population, coffee consumption considerably increased hip fracture risk by 54.7% and 40.1% in young and elderly women, respectively[7]. The earlier researches conclude that

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Received: 19 Dec 2017; revised received: 16 Apr 2018; accepted: 07 May 2018

consuming more than 500-600 mg of caffeine a day may lead to insomnia, nervousness, restlessness, irritability, an upset stomach, a fast heartbeat and even muscle tremors[8]. However, previous studies have linked even moderate quantities of caffeine to negative health effects. It has been reported that consuming 300 mg of caffeine a day during pregnancy may increase the risk of low birth weight babies, while other research suggests that drinking four cups of coffee a day may increase the chance of early death[9]. Caffeine metabolism is slower among infants, pregnant women and individuals with liver disease. Influence of caffeine is also a function of personal genetic characteristics and lifestyle factors[10]. In early 20th century, the therapeutic use of cod liver oil, having vitamin D as an ingredient, led to a sharp decrease in incidences of rickets. The chemical structure of Vitamin D as fat soluble was determined during 1930s. Vitamin D can be obtained from diet, supplements or produced through endogenous production in the skin. The cutaneous production is very effective and within few minutes of exposure to sun increases vitamin D concentration in the circulation[11]. Vitamin D plays a central role in regulation of mineral homeostasis and skeletal health. It is found in two forms; as ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Ergocalciferol (vitamin D2) is taken from plants, specially mushrooms, while remaining dietary sources including cutaneous production of vitamin D are cholecalciferol (vitamin-D3). Vitamin-D and in particular 1,25 (OH)2D, is of indispensible importance to bone formation[12].

MATERIAL AND METHODS

This study was a laboratory based randomized control trial performed in Anatomy department, Army Medical College Rawalpindi, incooperation with National Institute of Health (NIH), Islamabad. The research was conducted from October 2014 to October 2015 after requisite approval from Army Medical College ethical committee on animal experiments. The convenience non-probability sampling technique was used. Three weeks oldBALB/c mice, weighing 12-14g, 15 male and 15 female, totalthirty healthy (30) in number, were selected for research. Unhealthy or less than three weeks mice were not included in the study. A well-ventilated environment was kept with temperature control between 20-26^{0}C. The mice were randomly divided by lottery method into three groups. Each group contained 5 male and 5 female mice; making a total of 10 animals in a group. Separate cages were used for male and female mice to avoid pregnancy[13]. The mice in control group G1 were provided standard laboratory diet for 60 days. Experimental group G2 mice were administered with caffeine dosage of 10mg/100g body weight, on alternate days, 3 days a week for 60 days through oral gavage. All mice of G3 were given caffeine 10mg/100g body weight on alternate day, three days in a week along with vitamin D3 0.1µg per day by oral gavage for 60 days. At the conclusion of study, the mice were euthanized with ether anesthesia. Subsequently through dissection, the right femur was removed by separating from hip and knee joints. The bone samples were fixed in 10% formalinand the bottles were labelled, accordingly. After fixation, samples were placed into decalcifying solution that is 5% Nitric acid for 18-24 hrs[14]. The bone tissue of right femur was placed in the duly labelled tissue tek cassettes and processed in Leica TP 1020 automatic tissue processor. Paraffin wax having 58^{0}C as melting point was used for infiltration and embedding. for this purpose, paraffin embedding center LEICA EG 1160 was used. The block was solidified on the cold plate. The longitudinal bone tissue sections of 5µm thickness were obtained using rotary microtome. Sections were floated on warm water bath at 45^{0}C; folds were removed and sections were taken on clean slides carefully. The slides were kept in slanting position for 30 minutes to drain excess water and sections were dried in hot air oven for 1 hour at 65^{0}C. Identification details were inscribed by diamond pencil at the edge of glass slides. Sections were stained with hematoxylin and eosin (H&E) stains by using Leica autostainer X. All processing and staining

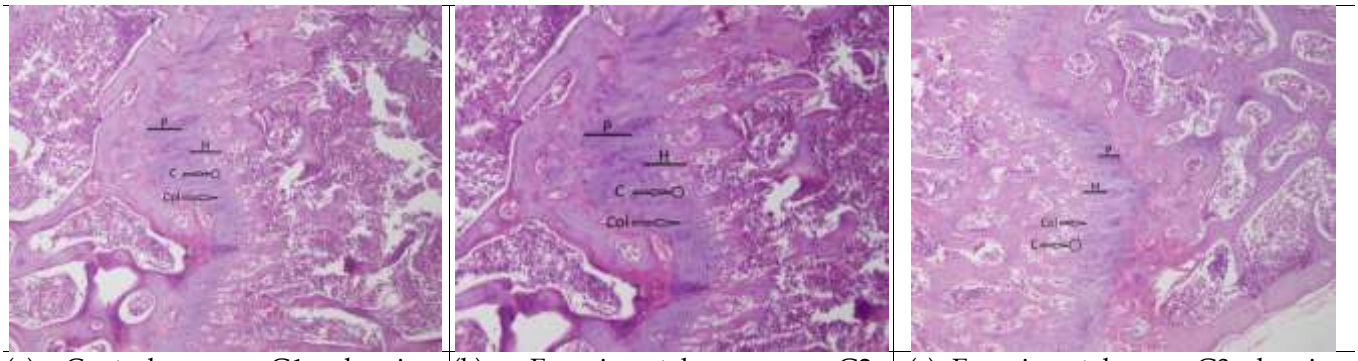
procedures were carried out in histology section of pathology laboratory in Army Medical College. The height of proliferative and hypertrophy zones was recorded in 4X and 10X objective magnifications. The scale of oculometer was aligned parallel to the intact column of chondrocytes of proliferative and hypertrophy zones. Three readings were taken in the central part of each zone and average of the readings was recorded as the height of the zone[15]. Data was analyzed using IBM-SPSS version 20. ANOVA test was performed which was followed by Post-hoc Tukey's test for comparison of quantitative variables between groups. A *p*-value ≤0.05 was taken as significant.

RESULTS

The total number of BALB/c mice was thirty (30). The mean ± SD of height of proliferative and hypertrophy zones of control group G1 animals was found to be 31.5 ± 4.1µm and 43 ± 2.6µm, respectively (table-I) (figure). The mean ± SD of height of proliferative and hypertrophy zones of experimental group G2 animals was measured as 59 ± 3.2 µm and 72.5 ± 7.2µm, respectively. The mean ± SD of height of proliferative and hypertrophy zones of experimental group G3 animals was observed as 46 ± 4.6µm and 54 ± 4.6µm, respectively. The statistical analysis regarding the zones was found to be significant among G1, G2 and G3 groups (table-II), however, it was insignificant within the groups G1, G2 and G3 (p>0.05) (table-III).

DISCUSSION

Caffeine is consumed in variable quantities throughout the world. The potential adverse effects of caffeine on human health have been investigated by researchers in various experimental studies. Caffeine consumption results in variation of two genes due to increase in the rate of caffeine catabolism[16]. The purpose of current research was to investigate the influence of caffeine as well as the combined effect of caffeine and vitamin D3 on the epiphyseal cartilage of developing femur of BALB/c mice. The control group was labeled as G1 and the experimental group G2 was given caffeine while the experimental group G3 was given both caffeine as well as vitamin D3. The results of present study are comparable with the earlier international study regarding the influence of high doses caffeine in male Wristar rats. In current research, the height of proliferative and hypertrophy zones was found significantly greater in caffeine fed rats than that of control group. It was concluded that caffeine seemed to enhance the activity of endochondrocytes and increase thickness of growth plate. Moreover, caffeine had an acute effect on secretion of growth hormone[17]. Hence,



(a): Control group G1, showing normal proliferative (P) and hypertrophy (H) zones, column of chondrocytes (Col) and chondrocyte with lacunae (C).	(b): Experimental group G2, showing increase in height of proliferative (P) and hypertrophy (H) zones.	(c): Experimental group G3, showing decrease in height of proliferative (P) and hypertrophy (H) zones.

Figure: Fig-Photomicrograph of longitudinal section of 11 week epiphyseal plate (H&E at X500).

in caffeine fed group, larger heights of proliferative and hypertrophy zones resulted into greater longitudinal growth of the bone. Another experimental study demonstrated that caffeine altered osteogenic activity, leading to impaired matrix mineralization, peripubertal longitudinal bone growth and maturation. Caffeine fed rats showed significant decrease of bone mass and osteogenic activity. Early caffeine intake contributed to adverse skeletal effects in later life, permanently transformed the bone composition and appeared to accelerate aging process in bones[18]. Dietary intake of vitamin D in adequate quantity is essential for building and maintaining healthy bones[19]. Vitamin D supplementation decreased bone turnover and increased bone mineral density[20]. In a growing long bone, the cells of growth plate are responsible for the longitudinal growth of bone[21]. Caffeine had an acute effect on increasing the secretion of growth hormone. Hence, the secretion of growth hor-mone (GH) resulted in growth of long bone. Several mechanisms have explained caffeine stimulation of growth hormone secretion. Methylxanthines are phosphodiesterase (PDE) inhibitors, leading to an increase in pituitary cyclic adenosine monophosphate (cAMP) which is involved in GH release[22]. Caffeine also has effect on neurotransmitters. Caffeine has been shown to increase the turnover of norepinephrine and of serotoninin the brain. Both norepinephrine and seroton in stimulate GH secretion in both humans and adult rats[23]. The histomorphological examination regarding the height of proliferative and hypertrophic zones showed statistically significant results. The height of these zones was greater in G2 group (caffeine fed) as compared to G1 and G3 groups. The statistical analysis regarding the zones was significant among G1, G2 and G3 groups (table-II), but it was insignificant within the groups G1, G2 and G3 (p>0.05) (table-III). In experimental group G3, vitamin D3 played a role in abating some effects of caffeine. There was considerable protection of the cytoarchitecture of femur due to vitamin D3. Five to 7% increase in the mean degree of mineralization of bone tissue by vitamin D[24]. Vitamin D deficiency decreased bone mineral density, resulted into increased risk of fractures. Vitamin D stimulated the absorption of calcium from gut and influenced the overall mineralization of the skeleton[25]. Vitamin D has been

Table-I: Mean values of heights of proliferative and hypertrophy zones in control group G1, experimental groups G2 and G3.

Height (μm)	Group G1 Mean ± SD (n=10)	Group G2 Mean ± SD (n=10)	Group G3 Mean ± SD (n=10)	*p*-value
Proliferative zone	31.5 ± 4.116	59 ± 3.162	46 ± 4.595	4.44 × 10-14 *
Hypertrophy zone	43 ± 2.582	72.5 ± 7.169	54 ± 4.595	2.48 × 10-12 *

*p-value ≤ 0.05 is statistically significant

Table-II: Comparison of height of proliferative and hypertrophy zones among groups G1, G2 and G3.

Height (μm)	Group G1 vs. Group G2	Group G1 vs. Group G3	Group G2 vs. Group G3
	p-value	*p*-value	*p*-value
Proliferative zone	2.0×10-12*	6.87×10-7*	7.72×10-7*
Hypertrophy zone	3.7×10-10*	3.4×10-6*	2.0×10-6*

*p-value ≤ 0.05 is statistically significant

Table-III: Comparison of height of proliferative and hypertrophy zones of male and female femur within the groups G1, G2 and G3.

Height (μm)	Male vs. Female G1	Male vs. Female G2	Male vs. Female G3
	p-value	*p*-value	*p*-value
Proliferative zone	0.2731	0.3465	0.1823
Hypertrophy zone	1.0	0.84	0.524

All the p-values are > 0.05; hence insignificant

extensively used for the prevention and treatment of osteoporosis and known as a bone resorbing hormone mainly in in-vitro studies[26].

CONCLUSION

Caffeine instigated increase in height of proliferative and hypertrophy zones of epiphyseal cartilage of BALB/c mice's femur which may be due to stimulation of growth hormone. However, the treatment with vitamin D3 ameliorated this adverse effect of caffeine.

CONFLICT OF INTEREST

There is no conflict of interest to declare in this study by any author.

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