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ORIGINAL ARTICLE

Impact of Caffeine and Vitamin D_3 on the Development of Neonate Mice Femur Growth Plate

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ABSTRACT

Background: High dosage of caffeine can influence histological changes during bone development in the newborns, while vitamin D_3 has also its effect on such changes. Main objectives of the study were to determine the effects of caffeine on the histomorphology of developing femur and the role of vitamin D3 along with caffeine on the femur of BALB/c mice.

Methodology: The animal experimental study was carried out in the National Institute of Health, Islamabad in collaboration with the Anatomy Department, Army Medical College, Rawalpindi, from October 2014 till October 2015. Thirty pregnant mice, weighing 26-28g, were chosen and grouped into 3 equal sets of ten mice each. Control group G_1 was fed normal diet with ad libitum access to water. Experimental group G_2, along with the above diet, was provided caffeine at 10mg/100g body weight as a single dose on every second day using oral gavage for 3 weeks. Experimental group G_3 was administered with caffeine at 10mg/100g body weight on every second day along with vitamin D_3 0.1µg/day for 3 weeks. At completion of the study, neonate mice femurs were analysed to see the changes on the proliferative and hypertrophy zone heights of growth plate.

Results: Proliferative and hypertrophy zones of control group G_1 mice mean height ±SD was measured as 540±10.99µm and 164±6.609µm, respectively, while for experimental group G_2 the same height was observed as 443.5±12.258µm and 138.25±6.129µm, respectively. For experimental group G_3 mice mean height ± SD of these zones was found as 474±3.839µm and 144.25±3.726µm, respectively.

Conclusion: Ingestion of caffeine modified the femur's proliferative and hypertrophy zones height of the growth plate; however, vitamin D_3 dosage mitigated this consequence.

Key words: Caffeine, Femur, Hypertrophy zone, Proliferative zone, Vitamin D_3.

Authors' Contribution:
[1]Conception; Literature research; manuscript design and drafting; [2,] Critical analysis and manuscript review; [3] Data analysis; [4]Manuscript Editing[5].

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Introduction

Caffeine is one of the major components in tea, coffee, carbonated drinks, energy drinks, chocolate derived from cocoa seads[1], and it is the most common psychoactive drug(legal) in the world. The role of caffeine in improving pain has been less known in the past, but now its being increasingly considered.[2] Worldwide utilization of caffeine has been evaluated at 120,000 tons per year.[3]

At present, a great rise in soft drinks ingestion has been seen throughout the world resulting in

caffeine abuse; this is especially due to massive publicity campaign and lack of appropriate understanding regarding outcomes of excessive caffeine use.[4] Experimental studies on young animals have reported that caffeine causes impaired bony ossification and maturation. Moreover, it impedes osteoblast differentiation, formation as well as mineralization of extracellular bony matrix.[5]

Previous research works have investigated that ingesting more than 500-600mg of caffeine in a single day might cause agitation, sleeplessness, anxiety, irritability, nervousness, an upset appetite, tachycardia and even muscle twitching.[6] In females, the half life of caffeine is approximately 5.2 to 5.4 hrs, which becomes longer during pregnancy. The consumption of 300mg of caffeine per day during gestation may potentiate the risk of premature fetal births, reduced crown rump length, micrognathia, cleft palate, limb malformations, hydrocephalus and in severe cases may even cause early death of the developing fetus.[7] The metabolism of caffeine is relatively slower in pregnant females, fetuses, infants and chronic hepatic illnesses.[8]

Vitamin D exists in two forms, specifically ergocalciferol (vitamin D2) – gotten from plants and cholecalciferol (vitamin D3) - in all other dietary sources including skin production of vitamin D.[9] Vitamin D performs a fundamental role in controlling as well as sustaining the mineralization of osseous tissue in all age groups and plays a central role in the regulation of mineral homeostasis and skeletal health.[10]

The previous experimental studies have not sufficiently focused on the histology of the developing long bones in the proliferative and hypertrophy phases. Consequently, the combined effect of caffeine and vitamin D consumption on these developing histological zones has not been investigated. Therefore, objective of this experiment was to find the outcome of caffeine consumption as well as the added effect of vitamin D_3 on the growth plate of neonate femur of BALB/c mice.

Methodology

The animal experimental study was conducted in the National Institute of Health (NIH), Islamabad in collaboration with the Anatomy Department, Army Medical College, Rawalpindi from October, 2014 till October, 2015 after necessary approval from ethical-committee (No. 02 / CREAM-A / 11 Feb, 2015). Convenience based non-probability sampling technique was used. Healthy pregnant mice, weighing 26-28g, total thirty (30) in number, were taken for the experiment. Non-pregnant and un-healthy female mice were not used in this research. Room environment was controlled between 20-26^{0}C. Using lottery method, pregnant mice were divided into three equal group sets of 10. Control group G_1 was provided with usual laboratory food for 3 weeks. Experimental group G_2 was given pure caffeine of 10mg/100g body weight, on every second day for 3 weeks, using oral gavage needle. Group G_3 was administered caffeine 10mg/100g body weight on every second day, three days a week along with vitamin D_3 0.1µg per day, also through oral gavage needle for 3 weeks. After 21 days of gestation period, the animals were euthanized using ether as anesthesia. When mouse became unconscious, it was placed on a clean sheet of paper on a dissecting board. The abdominal cavity was opened and gravid uterus was exposed to take foetuses out. The total number of foetuses within the uterus of all the animals were counted as 206. Two foetuses were taken randomly from each pregnant animal and right femur of newborns was dissected by separating from hip and knee joints. Fixation of femurs was done in 10% formalin and the bottles were marked appropriately. The samples were decalcified through 5% Nitric acid solution for 18-24 hrs.[11] Later, further processing was done in Leica TP 1020 automatic tissue processor. Infiltration and embedding was performed in paraffin wax using embedding center LEICA EG 1160. Rotary microtome was used to make 5µm thickness longitudinal bone tissue sections on warm water bath at 45^{0}C and slides

were prepared. Staining using Leica auto-stainer X in hematoxylin and eosin was performed in Pathology Laboratory of AMC. Heights of proliferative and hypertrophy zones were measured through ocular micrometer (40X lens). Average of three readings in the central portion of each zone was noted down as the height of the zone.[12] IBM-SPSS version 20 was used for analyses using ANOVA test, while for inter-group comparison of quantitative variables, Post Hoc Tukey`s test was used. A p value ≤ 0.05 was taken as significant.

Results

The mean heights ±SD of proliferative and hypertrophy zones for all the three groups (G_1, G_2 and G_3) as well as statistical findings are given in Table 1. The height of both the zones was decreased in caffeine nourished G_2 group in comparison to the control group G_1 and experimental group G_3 (Fig 1-3). In G_3 experimental group, vitamin D_3 showed a role in diminishing some of the effects of caffeine, through protection of the architecture of osseous tissue.

Table 1: Mean values of zones height of femur (growth plate) of new-borns

Zone	Group G_1 Mean Height ±SD (µm) (n=20)	Group G_2 Mean Height ±SD (µm) (n=20)	Group G_3 Mean Height ±SD (µm) (n=20)	p-value
Proliferative	540.5±10.99	443.5±12.258	474±3.839	< .001*
Hypertrophy	164±6.609	138.25±6.129	144.25±3.726	< .001*

*P value ≤ .05 is statistically significant

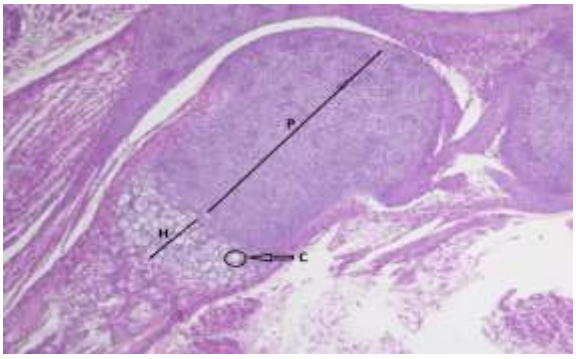

Fig 1. Control group G_1, indicating proliferative zone (P), hypertrophy zone (H), chondrocyte with lacunae (C) and column of chondrocyte (Col) of femur - (40X)

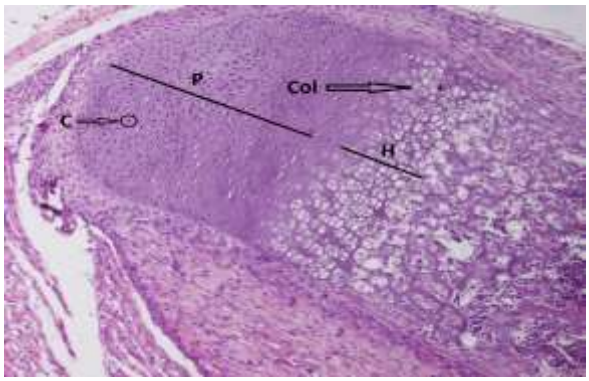

Fig 2. Experimental group G_2, indicating decrease in proliferative (P) and hypertrophy (H) zones' height of femur- (40X)

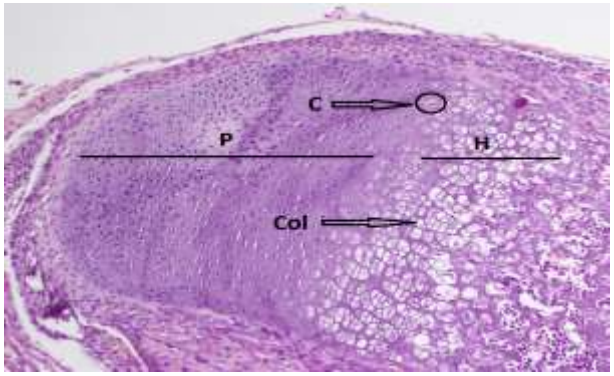

Fig 3. Experimental group G_3, indicating relative increase in proliferative (P) and hypertrophy (H) zones' height of femur - (40X)

Discussion

The present study showed that the height of proliferative and hypertrophy zones of the femur was appreciably reduced in caffeine nourished mice in comparison with the control group. It was determined that the caffein appeared to inhibit the action of endo-chondrocytes and reduce the thickness of epiphyseal cartilage.[13] Thus, in caffeine nourished mice, smaller heights of proliferative and hypertrophy zones caused lesser longitudinal growth of the femur.[14] Similar findings were noted in previous studies in which caffeine altered osteogenic activity, leading to impaired matrix mineralization and maturation[15]

and had detrimental effects on cartilage growth, with severity of adverse effects dependent on dose.[16, 17]

Caffeine passes into off-spring through the placenta, which causes teratogenic transformations and can reduce the formation, growth, and mass of the bones.[18] The mesenchymal stem cells are responsible for ossification of the whole skeleton. The caffeine that goes from the mother to the fetus during pregnancy decreases the osteogenic differentiation of mesenchymal stem cells. The reduction in the osteogenic potential of mesenchymal stem cells is implicated in the pathogenesis of osteopenia resulting from caffeine ingestion.[19]

In another study, significant histological changes were observed in caffeine treated rats. Thickness of epiphyseal plate (particularly proliferating and hypertrophic zones) was reduced in a group which received caffeine. The diaphysis of caffeine treated group manifested thinning out of the outer compact bone with multiple osteoporotic cavities in the bone matrix.[20] The, addition of caffeine in rat chondrocytes cultures diminished cellularity, viability, and matrix synthesis activity.[21] These results match with the findings of current research.

Caffeine fed neonates and young rats demonstrated marked decrease in longitudinal bone growth and maturation.[22] Caffeine ingestion during early years resulted in harmful effects on the bony structure in the subsequent life, hence altering the bone mineral density perpetually, decreasing calcium retention and reducing height of hypertrophic zone.[22] Observations of the present study are also comparable. The cells of epiphyseal cartilage control the longitudinal growth of bone.[23] Therefore, it has been determined that high doses of caffeine can inhibit endochondral ossification in young rats.[24]

Experimental group (G_3) in the current study also showed reduction in detrimental effect of caffeine on bones due to addition of vitamin D in the diet. The role of vitamin D is critical for normal growth and mineralization of the bony skeleton of the developing fetuses.[25] Vitamin D intake improved maternal calcium retention from gastrointestinal tract and enhanced offspring bone mineral density.[26] The current study focused on observing the development of histological zones in the new-born using conventional microscope and thus did not examine the changes in shape, size and number of the cells in the respective zones. It is, therefore, recommended that electron microscope may be used to observe the ultra structural changes in the developing bone zones in future studies.

Conclusion

Caffeine stimulated decrease in height of proliferative and hypertrophy zones of growth plate of new born mice's femur, possibly due to inhibition of endochondral ossification. The dosage of vitamin D_3, however, alleviated this negative influence of caffeine.

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