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Research Article

Effect of *Cissus quadrangularis* Linn on skeletal growth in the neonates of diabetic rats

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ABSTRACT

Background: Infants of the diabetic mother are known to have reduced bone mineral content and hypocalcemia. Earlier, it has been shown that petroleum ether extract of *Cissus quadrangularis* (PECQ) can enhance the fetal skeletal ossification in normal rats. The present study was designed to evaluate the effect of PECQ on skeletal growth in the neonatal rats of streptozotocin-induced diabetic rats.
Methods: After confirmation of diabetes, the diabetic and non-diabetic female Wistar rats were kept for mating with healthy male rats. After positive vaginal smear test, the pregnant rats were divided into three groups; the normal (non-diabetic) control (NC), diabetic control (DC), and diabetic+CQ (D+CQ) groups. The rats in the D+CQ group were given PECQ (500 mg/kg B.Wt), whereas animals in NC and DC groups were given 0.5% carboxy methyl cellulose, throughout the gestational period. Femur from the 1 week old neonatal rats from each group was collected randomly and subjected to histological analysis.
Results: Thickness of trabecular bone and periosteum was significantly reduced in the neonates of DC rats compared with the neonates of NC group. Pretreatment with PECQ significantly improved the thickness of trabecular bone and periosteum compared with neonatal rats of DC group. No significant differences were observed in the medullary cavity width of femur between the groups.
Conclusion: Data from the present study suggest that the PECQ can effectively attenuate the diabetes-induced reduction in the early skeletal growth. However, further research is warranted to evaluate the exact mechanism of action of phytochemical constituents of PECQ that can cross the placental barrier.

Keywords: Diabetic rats, Skeletal growth, Neonatal rats, *Cissus quadrangularis*, Trabecular bone

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder which will affect the various vital systems of the human body, including the musculoskeletal system.[1] DM in adults has been shown to affect the active bone formation, delay the process of bone fracture healing[1] and reduce the bone mineral content.[2] It has been demonstrated that many nutritional and hormonal factors influence the skeletal growth and bone mineralization in the infancy, puberty and adolescence.[3] Clinical studies have proved that children with Type 1 DM are known have decreased bone mass.[4,5] The decreased bone mass in the children may severely affect the peak bone mass gain in their later life, and this may consequently predispose to osteopenia and other bone related complications.[4] Furthermore, infants of the diabetic mother are known to have deceased mineral content.[6-9] Animal models have shown that diabetes in pregnancy associated with skeletal complications such as delayed fetal skeletal ossification.[10,11] There is a lack of experimental evidence weather the reduced bone mineral content associated with reduced skeletal growth in the diabetic infants. However, a clinical

study by Mughal et al. have questioned such association.[12] In the present study, we evaluated the skeletal growth of 1 week old neonates of diabetic rats by performing the morphometric analysis of the femur. We used the femur as it is the longest bone of the body with a maximum turnover in growing animals.[13]

Cissus quadrangularis (CQ) perennial herb can grow in warm tropic climate, and is distributed in the hotter parts of India, Thailand, Srilanka, Bangladesh, Java, Philippines, West Africa and Ceylon.[14] The pharmacological studies have shown the beneficial effect of CQ against the bone health, blood sugar regulation, weight loss, digestive disorders, menstrual irregularity, cholesterol and triglyceride lowering.[15] Antimicrobial, analgesic, antipyretic and anti-inflammatory activities are the other potential medicinal properties of the CQ.[15] Presence of phytoestrogen steroids and their effect on the bone early regeneration, and quick mineralization has been demonstrated.[16,17] Earlier, Potu et al. have shown the stimulatory effect of petroleum ether fraction of CQ against the fetal skeletal ossification and fetal skeletal growth.[18,19] In the current study, we aimed to evaluate the efficacy of petroleum ether fraction of CQ against the skeletal growth in the offspring of diabetic rats.

METHODS

Plant extract

The air dried stems of the CQ plant was grinded into powder. The powder (1.3 kg) was extracted with 95% ethanol using soxhlet apparatus. Further, the extracted yield (125 g) was suspended in water and partitioned with petroleum ether solvent. The petroleum ether extract of *Cissus quadrangularis* (PECQ) obtained (9.1% w/w) was stored in the plastic containers and maintained at 4°C. The fresh suspension of the PECQ was made daily few hours before use, by diluting it in 0.5% carboxymethyl cellulose (CMC). The dose of the PECQ was fixed based on previous studies.[18,19]

Animals

Wistar rats (8 weeks old), weighing about 170-190 g were housed in the Central Animal House, Kasturba Medical College, Manipal University, Manipal, India. Throughout the experiment, animals were maintained in laboratory conditions in the animal house. All the rats were fed with commercially available diet, and they were allowed free access to the distilled water. Institutional Animal Ethical Committee (IAEC No. IAEC/KMC/68/2010-2011), Kasturba Medical College, Manipal have approved our studies. All the animal experimental procedures were done according to the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India guidelines.

Induction of diabetes

Female Wistar rats weighing 180-220 g were used in the study. After overnight fasting, these rats were rendered diabetes with a single dose of streptozotocin (STZ) (i.p. injection of 40 mg/kg/B.Wt dissolved in 0.1 M citrate buffer, pH 4.5). For 24 hrs rats were allowed to drink 5% glucose added water to overcome the STZ induced hyperglycemia. A week after injection of STZ, the fasting blood glucose levels were measured with accu-chek active glucose strips (Roche Diagnostic India Private Limited, Mumbai). Animals with fasting blood glucose levels >250 mg/dl were considered as diabetic and included in the study.

Experimental design

The non-diabetic and diabetic female rats were mated with normal healthy male rats at 2:1 ratio. After confirmation of pregnancy (vaginal smear test) the diabetic pregnant and non-diabetic pregnant rats were divided into three groups; the normal control (NC) group; diabetic control (DC) group and diabetic+CQ (D+CQ) group. Both NC and DC group rats were given daily 0.5% of CMC (vehicle used to treat with CQ). The D+CQ group rats were treated orally with PECQ at a reference dose of 500 mg/kg B.Wt. The rats in all three groups were treated throughout the gestation. Blood glucose level was measured on 0, 5, 14 and 21 day of gestation. Each pregnant rat was maintained in separate cage 3 days before gestation. On the 8 days after delivery, 12 neonatal rats (two from each rat) in each group were collected randomly. Then, the neonatal rats in all the groups were anesthetized, and right femora were dissected for skeletal analysis.

Tissue processing and staining

The whole right femur was dissected carefully from the hind limb. Soft tissue was removed and fixed in periodate-lysine-paraformaldehyde fixative for 24 hr at 4°C. After fixation, the bones were decalcified in ethylenediaminetetraacetic acid-G solution. The decalcified tissues were then subjected to dehydration through series of alcohol and xylene. Finally, the whole femur was embedded in paraffin wax (58-60°C). Longitudinal sections of the whole femur (5 μm thickness) were taken, and H and E staining was performed.

Morphometric analysis of right femur

The photomicrographs of the H and E stained femur sections were analyzed using Olympus Cellsens Imaging Software (1.6 Version, USA). The thickness of the trabeculae at the metaphyseal region of the lower end and the mid-shaft level was measured. At the mid-shaft level, the thickness of the periosteum was also measured. The width of the medullary cavity of the femur was noted at three levels; at the upper end, mid-shaft level and lower end.

Statistical analysis

Results were expressed as the mean±standard errors of the mean. Graph pad Prism software (5 version; GraphPad Software, Inc. USA) was used for the data analysis. Repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests was used to compare the data for maternal glycaemia. One-way ANOVA, followed by Bonferroni's multiple comparison tests was used to analyze the trabecular bone and periosteal thickness and medullary cavity width. Statistical significance was considered at $p<0.05$.

RESULTS

Effect of PECQ on maternal fasting blood glucose level in pregnant rats

Blood glucose level was estimated at regular intervals during the gestation. Blood glucose level was significantly increased in the DC group throughout the gestation, in comparison to NC group. However, pretreatment with PECQ did not alter the blood glucose level in the D+CQ group when compared to DC group ($p>0.05$; Table 1). This result suggests that the PECQ does not have the hypoglycemic effect.

Effect of PECQ on trabecular bone thickness in the metaphyseal and mid-shaft regions of the femur in neonates of diabetic rats

Thickness of trabecular bone was measured at both metaphyseal region and mid-shaft level. The mean thickness of trabecular bone was significantly decreased in the neonates of the DC group in the metaphyseal region ($p<0.001$; Figures 1a and b) and mid-shaft region ($p<0.001$ Figures 2a and b), when compared to neonates of NC group. However, thickness of trabecular bone in these regions was significantly improved in the neonates of diabetic rats treated with PECQ (D+CQ) when compared to neonates of DC group (metaphyseal region: $p<0.01$, Figures 1a and b; mid-shaft region: $p<0.01$, Figures 2a and b). These results suggest that the PECQ significantly influence the skeletal growth in the neonates of the diabetic mother.

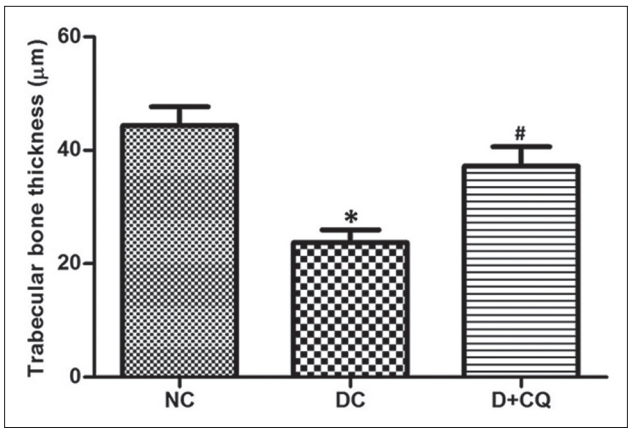

Figure 1a: Effect of petroleum ether extract of *Cissus quadrangularis* on trabecular bone thickness in the metaphyseal region in neonates of diabetic rats.*$p<0.001$, indicate the level of significance in comparison to normal control group. #$p<0.01$ indicate the level of significance in comparison to diabetic control group.

Effect of PECQ on periosteal thickness of femur in neonates of diabetic rats

The mean periosteal thickness at mid-shat region was significantly decreased in the neonates of the DC group when compared to neonates of NC group ($p<0.001$; Figures 2b and 3). Thickness of periosteum was significantly increased in the neonates of diabetic rats treated with PECQ (D+CQ) when compared to neonates of DC rats ($p<0.05$; Figures 2b and 3).

Effect of PECQ on medullary cavity width of femur in neonates of diabetic rats

Medullary cavity width of the femur was measured at the upper end, mid-shaft and lower end. There were no significant differences were observed in the medullary cavity width of the femur between the neonates of NC, DC and D+CQ groups (Table 2). These results suggest that the maternal diabetes does not affect the dimensions of the medullar cavity in their offspring.

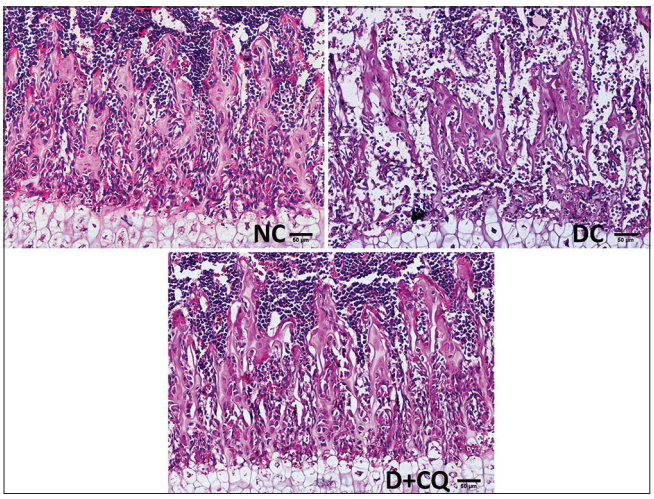

Figure 1b: H and E stained microphotograph showing the trabecular bone at the lower end of the femur. Thickness of the trabecular bone is decreased in the neonates of diabetic control (DC) rats compared to neonates of normal control group. However, neonates born to diabetic mother treated with petroleum ether extract of *Cissus quadrangularis* (CQ) (diabetic+CQ) showed thicker trabecular bone in comparison with neonates of the DC group. Scale bar=50 µm.

Table 1: Effect of PECQ on fasting blood glucose level in pregnant rats.

Groups (n=6)	Fasting blood glucose level (mg/dl) during gestation (mean±SEM)			
	0 day	5th day	14th day	21st day
NC	68±2.16	71.66±3.49	79.83±3.58	84.5±5.76
DC	255.83±7.17[b]	275±9.11[b]	290.5±14.55[b]	324.33±15.25[b]
D+CQ	258.33±4.12[b]	270.83±8.84[b]	287.33±10.05[b]	291.16±10.69[b]

[b]p<0.001 significant when compared to NC group. SEM: Standard errors of mean, CQ: *Cissus quadrangularis*, NC: Normal control, DC: Diabetic control, D+CQ: Diabetic+CQ group, PECQ: Petroleum ether extract of *Cissus quadrangularis*

Table 2: Effect of PECQ on femur medullary cavity width in neonates of diabetic rats.

Groups (n=12)	Medullary cavity width of femur (µm) (mean±SEM)		
	Upper end	Mid-shaft level	Lower end
NC	1240±69.42	959.5±54.35	1598±85.06
DC	1155±48.25	913.4±57.71	1541±112.7
D+CQ	1223±81.05	934.6±44.22	1487±91.23

NC: Normal control, DC: Diabetic control, D+CQ: Diabetic+CQ group. No significant changes were observed between the groups. SEM: Standard errors of mean, PECQ: Petroleum ether extract of *Cissus quadrangularis*

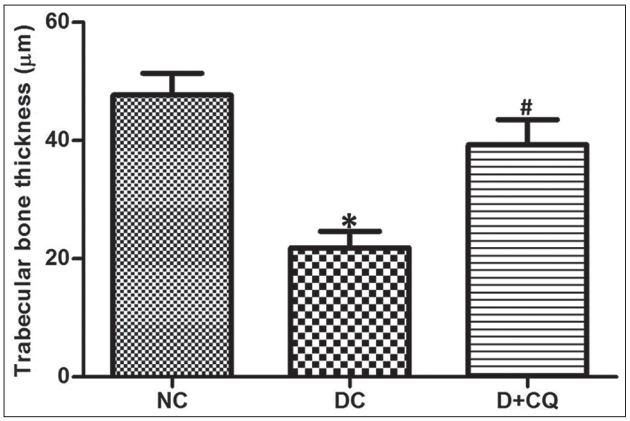

Figure 2a: Effect of petroleum ether extract of *Cissus quadrangularis* on trabecular bone thickness in the mid-shaft region in neonates of diabetic rats, *p<0.001, indicate the level of significance in comparison to normal control group. #p<0.01 indicate the level of significance in comparison to diabetic control group.

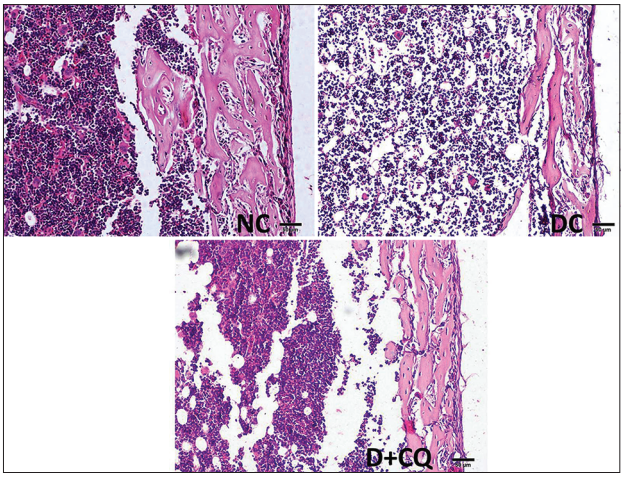

Figure 2b: H and E stained microphotograph showing the trabecular bone and periosteum at the mid-shaft region of the femur. Thinner trabecular bone and periosteum was observed in the neonates of diabetic control (DC) group, in comparison to neonates of normal control rats. However, treating the diabetic mother with petroleum ether extract of *Cissus quadrangularis*, improved the trabecular bone and periosteal thickness (D+CQ) which is nearer to that of NC group. Scale bar=50 µm.

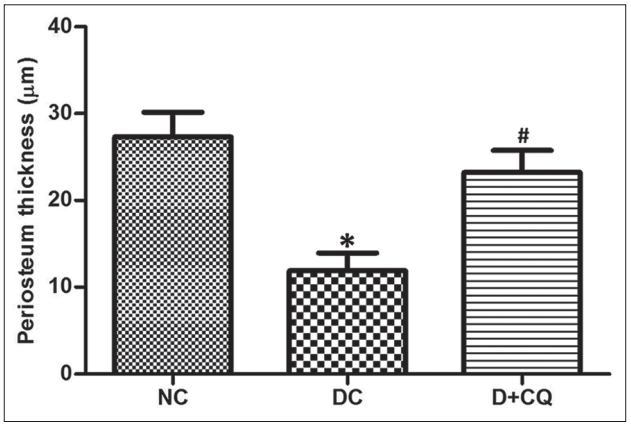

Figure 3: Effect of petroleum ether extract of *Cissus quadrangularis* on periosteal thickness of femur in neonates of diabetic rats, *p<0.001, indicate the level of significance in comparison to normal control group. #p<0.01 indicate the level of significance in comparison to diabetic control group.

DISCUSSION

At birth, infants of the diabetic mother are known to have decreased bone mineral content and hypocalcaemia.[6-9] Even the infants of maternal gestational DM are known decreased bone strength.[20] The decreased bone mineral content could be due to the increased bone resorption.[21] It has been demonstrated that in the infants of the diabetic mother, the osteoclast induced osteopenia may be due to the decreased fetal cyclic movements.[22] We hypothesize that the decreased bone mineral content in the newborn may affect the normal skeletal growth. In the present study, the thickness of the

trabecular bone and periosteum was significantly decreased in the neonates of the DC rats compared with neonates of the NC group. These results confirmed the direct association between the reduced mineral content and reduced skeletal growth.

Studies in humans and experimental animal models have shown that estrogens significantly affect the bone turnover in postmenopausal conditions.[23-25] An *in vitro* study demonstrated the modulation of bone activity by the estrogenic compounds such as estradiol (E2).[26] It has been postulated that this activity could be due to the direct action of E2 on the biologically active estrogen receptor on the bone cells.[27,28] This receptor mediated action of E2 has been confirmed by finding of such receptors on the bone cells.[29,30] Earlier, studies have also shown that exposure to estrogen in the initial period of the development can significantly affect the bone growth and subsequently increase the bone mass gain and final peak density in the adulthood.[31] Even the maternal exposure of the estrogen has been shown to influence the fetal bone tissue development.[32] Further, studies suggest that phytoestrogens can cross the placental barrier,[33,34] and can behave like an estrogen-like compounds and acts directly on the estrogen receptors of fetal bone cells. The positive effect of PECQ observed in the present study could be due to the phytoestrogens present in it,[16,17] which might have crossed the placental barrier and influenced the fetal bone tissue. The phytoestrogens of PECQ might have also shown the effect by altering the maternal estrogen levels or other steroid hormonal levels.[18]

It has been suggested that the hypocalcaemia in the offspring of diabetic rats are due to a defective bone maturation in the fetus itself as a result, fetus minimizes its Ca^{2+} requirements.[35] Earlier Potu et al. have shown that PECQ can increase the mesenchymal stem cell proliferation and subsequently their differentiation into osteoblasts.[36] In an *in vitro* study, the Gopalakrishnan et al. have demonstrated the positive effect of synthetic estrogen on bone mineralization process in hyperglycemic conditions.[37] In an *in vivo* study synthetic estrogen significantly ameliorated the experimental diabetes-induced osteopenic changes in adult rats.[38] Therefore, the beneficial effect of PECQ on neonatal skeletal growth in maternal diabetes may be attributed to the estrogen like action of phytosteroids present in it, which might facilitated the bone growth in hyperglycemic conditions.

CONCLUSION

Treating the diabetic mother with PECQ during gestation can effectively attenuate the reduced skeletal growth in their neonates. As PECQ does not show the hypoglycemic effect, the positive effect of PECQ on the skeletal growth of diabetic neonatal rats may be due to its pro-osteogenic properties. However, further evaluation is needed to understand the mechanism of its phytochemical constituents that can cross the placental barrier and act on the fetal bone cells in hyperglycemic conditions.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Animal Ethics Committee

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