Protective effect of *Cissus quadrangularis* Linn. on diabetes induced delayed fetal skeletal ossification

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**ABSTRACT**

**Background:** Delayed fetal skeletal ossification is one of the known complications of maternal diabetes. **Objective:** The present study was designed to evaluate the protective role of petroleum ether extract of *Cissus quadrangularis* (PECQ) on diabetes-induced delayed fetal skeletal ossification. **Materials and Methods:** Female Wistar rats were rendered diabetic with streptozotocin (STZ, 40 mg/kg, intraperitonial) before mating. After confirmation of pregnancy, the pregnant rats were divided into three groups: normal control group, diabetic control group, and diabetic + CQ group. The diabetic + CQ group pregnant rats were treated with PECQ (500 mg/kg body weight) throughout their gestation period. Immediately after delivery, pups were collected from all three groups and processed for alizarin red S–alcian blue staining in order to examine the pattern of skeletal ossification. **Results:** Fewer ossification centers and decreased extent of ossification of forelimb and hindlimb bones were observed in the neonatal pups of diabetic control group as compared to those in the normal control group. PECQ pretreatment significantly restored the ossification centers and improved the extent of ossification of forelimb and hindlimb bones in the neonatal pups of diabetic + CQ group as compared to those in the diabetic control group. **Conclusions:** The results suggested that PECQ treatment is effective against diabetes-induced delayed fetal skeletal ossification. However, further studies on the isolation and characterization of active constituents of PECQ, which can cross the placental barrier and are responsible for the bone anabolic activity are warranted.

**Key words:** Alizarin red-alcian blue, *Cissus quadrangularis*, maternal diabetes, ossification centers, skeletal ossification, streptozotocin

**INTRODUCTION**

Diabetes mellitus (DM) is a group of metabolic disorders with multiple etiologies characterized by impaired metabolism of carbohydrates, proteins, and fat that is caused by complete or relative insufficiency of insulin secretion and/or action of insulin.[1] It has been established that diabetes during pregnancy leads to reproductive abnormalities in the offspring such as altered fetal growth, polyhydramnios, congenital defects, and spontaneous abortion.[2-4] Infants born to diabetic mother are shown to have hypocalcemia and lower bone mineral content at birth.[4,7-9] In addition, hypoplasia of neurocranial, viscerocranial, forelimb, and hindlimb bones have been observed in the fetuses of streptozotocin (STZ)-induced diabetic pregnant rats.[10] The mechanism of ossification defects in the offspring of diabetic rats have been explained by two models:[11] The push model suggests insufficient supply of Ca²⁺ to the fetus due to excessive Ca²⁺ loss in maternal urine[12] or due to decreased capacity of the placenta to transport Ca²⁺.[13] while the pull model explains that the demand for Ca²⁺ is reduced in the fetus due to defects in the process of bone maturation itself, as indicated by reduced cord osteocalcin levels[14] and a decreased number of ossification centers. However, Verhaeghe *et al.* support the latter model.[15]

The plant *Cissus quadrangularis* (CQ) (Veldt Grape or Winged tree-bine), a climber belonging to the family Vitaceae, has been used as an osteogenic/osteoprotective medicine in Ayurveda. In India, CQ is one of the widely used medicinal plants.[16] This plant grows in the hotter regions of India,
Sri Lanka, Malaysia, Java and West Africa. This plant can heal broken bones and thus is commonly known as “bone setter,” or Asthizanthobani in Sanskrit and Hadjod in Hindi. Phytochemical studies of CQ have reported the presence of several compounds such as ascobic acid, carote, calcium, anabolic steroid substances, β-sitosterol, δ-αmyrin, δ-αmyrone, flavonoids, triterpinoids, quercetin, resveratrol, piceatannol, palloid perthenocissin, and phytoesters. Pharmacological studies have demonstrated that unidentified anabolic steroid acts on the estrogen receptors of bone cells and facilitates healing of fractured bones. In vitro studies have suggested that phytosterogens, estrogenic compounds derived from plants, seem to have actions similar to that of estrogen on bone cells. The role of receptor-mediated action of estrogen on osteoblasts and osteoclasts has been studied previously. Furthermore, the stimulatory effect of 17-beta estradiol on the process of bone matrix formation and mineralization in hyperglycemic conditions has been reported.

Alterations in the environmental factors during early development have been shown to permanently program the structure and physiology of the body’s tissues and system. It has been proved that excessive estrogen exposure can result in altered programming of the bone cells, and the same has been shown to have long-lasting effects on fetal skeleton. Plant-derived estrogen mimicking the phytoestrogens are shown to cross the placenta in humans. In past, the beneficial effects of antioxidants and medicinal plants that affect the antioxidant status during pregnancy have been studied on diabetes-induced fetal ossification defects. However, the role of phytoestrogens on fetal skeletal ossification in diabetic rats has not been studied.

Earlier studies have shown that prenatal exposure of petroleum ether extract of CQ (PECQ) stimulates fetal skeletal growth. We observed that the prenatal exposure of PECQ can significantly improve bone mass in the offspring at their adult age (unpublished data). Therefore, the present study was designed to investigate the protective effect of PECQ on diabetes-induced delayed fetal skeletal ossification.

**MATERIALS AND METHODS**

**Plant material and preparation of extract**

Fresh stems of CQ plant were collected in Belgaum, Karnataka, India. The plant was identified and authenticated with the help of a taxonomist. The fleshy stems were washed and air-dried, and then the dried stems were grinded into powder. The powder (1.3 kg) was then thoroughly extracted with 95% ethanol in a soxhlet apparatus, and the yield was concentrated under reduced pressure. Next, the total yield of ethanol extract (125 g) was suspended in water and partitioned with petroleum ether to obtain PECQ with a yield of 11.4 g (9.1% w/w). PECQ was stored in air-and water-proof containers and kept in refrigerators at 4°C. During the experiment, PECQ was diluted in 0.5% carboxy methyl cellulose (CMC) before administration to animals.

**Animals**

With approval of the Institutional Animal Ethical Committee, 8-week-old Wistar rats (weight: 170-190 g) were maintained in the Central Animal House, Kasturba Medical College, Manipal University, Manipal, India, under laboratory conditions of controlled temperature (23 ± 2°C), humidity (50 ± 5%), and a 12-h light–dark cycle. All animals were fed on a commercially available diet and allowed free access to distilled water. All animal studies were approved by the Institutional Animal Ethical Committee (IAEC No. IAEC/KMC/68/2010-2011), Kasturba Medical College, Manipal and conducted according to the prescribed guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

**Experimental design**

Female rats were rendered diabetic by a single intraperitoneal injection of STZ (Sigma Chemical, St. Louis, MO, USA) at a dose of 40 mg/kg body weight dissolved in citrate buffer, 7 days before the mating period described earlier. Normal control rats were injected with citrate buffer only. Animals demonstrating hyperglycemia (>270 mg/dl) within 36 hour of injection were included in the study. After confirmation of the onset of diabetes, the diabetic female rats were allowed to mate with non-diabetic male rats, and non-diabetic female rats were mated with non-diabetic male rats. After confirmation of pregnancy, the pregnant rats were divided into three groups (n = 6). The normal control group, diabetic control group, and diabetic + CQ group. Both the normal and diabetic control groups received equivalent volume of 0.5% CMC orally throughout the gestation period. The diabetic + CQ group rats were treated orally with PECQ at a dose of 500 mg/kg body weight/day in 0.5% CMC throughout the gestation. The dose of PECQ was fixed based on the previous acute toxicity studies, where administration of PECQ extract up to 5000 mg/kg body weight did not show any adverse effects. Blood glucose level and body weight were measured at intervals of 0, 5, 14, and 21 days of pregnancy. Blood glucose levels were measured using accu-chek active glucose strips (Roche Diagnostic India Pvt. Ltd, Mumbai).

**Alizarin red S–alcan blue preparation**

At the end of the gestation, immediately after delivery, pups were collected from all experimental groups. Under anesthesia, the skin, viscera, and adipose tissues were
carefully removed from the neonatal pups. Then the eviscerated pups were processed for alizarin red S–alcian blue (Sigma Chemical, USA) double staining to view the ossified and unossified skeleton.\textsuperscript{42} Ossification centers in the sternum, metacarpus, metatarsus, phalanges, and caudal vertebrae (n = 18 pups in each experimental group) were recorded with the aid of a stereomicroscope.

**Morphometric analysis of limbs**
Photographs of the double-stained limbs were taken with fixed scales to measure the length of ossified skeleton and that of the total length of the bones using Image J software. Length of the scapula, humerus, ulna, radius, femur, tibia, and fibula were measured (n = 12 limbs in each experimental group), and the percentages of the total length of the mineralized skeletons in the normal control, diabetic control, and diabetic + CQ treated groups were calculated as previously described.\textsuperscript{41}

**Statistical analysis**
Results were expressed as mean ± standard errors of mean. The data was analyzed using Graph pad Prism software (Microsoft, San Diego, CA, USA). Repeated measures ANOVA followed by Bonferroni’s multiple comparison test was employed to compare the data for maternal glycemia and body weight. Ossification centers and the total length of the bone precursor cartilages were analyzed by applying one-way ANOVA followed by the Bonferroni’s multiple comparison test to determine the specific difference between the groups. P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Effect of petroleum ether extract of cissus quadrangularis (PECQ) on glycemia during pregnancy**
Blood glucose level was measured on days 0, 5, 14, and 21 of pregnancy. In normal control rats, normoglycemia was confirmed with mean glucose value <119 mg/dl. In the diabetic control rats, a significant hyperglycemia (P < 0.05) was noted during pregnancy as compared to that in the normal control rats. Furthermore, no alteration in the blood glucose level was observed when diabetic pregnant rats were treated with PECQ [Table 1]. This result indicates that the PECQ administration does not directly interfere with the blood glucose level.

**Effect of PECQ on maternal weight gain during pregnancy**
Throughout pregnancy, an increase in the body weight was observed in the normal control rats. On the other hand, the diabetic control rats showed significantly (P < 0.05) lower body weight on days 0, 5, 14, and 21 of pregnancy as compared to the normal control group. However, PECQ administration to diabetic pregnant mother significantly (P < 0.05) improved the body weight gain on 0, 5, 14, and 21 days of pregnancy [Table 2].

**Effect of PECQ on ossification centers**
Pups born to the diabetic control group rats showed significantly less ossification centers at the time of birth when compared to the pups of the normal control group (sternum, metacarpus, anterior phalanges, posterior phalanges, and caudal vertebrae; P < 0.001 and metatarsus; P < 0.01). Interestingly, pups born to the diabetic mother treated with PECQ showed significantly increased number of ossification centers in the bones at birth (sternum, metacarpus, posterior phalanges, and caudal vertebrae: P <0.001; anterior phalanges: P <0.01; metatarsus: P <0.05) when compared to the pups of the diabetic control group [Table 3 and Figures 1-3]. Therefore, PECQ can influence the fetal ossification process and can prevent diabetes-induced delay in the onset of the ossification during fetal life.

**Effect of PECQ on the ossification of forelimb and hindlimb bones**

**Morphometric analysis**
The Image J data revealed significant differences in the extent of ossification of forelimb and hindlimb bones at birth of pups in the diabetic control group as compared to those in the normal control group. However, pups born to PECQ-treated diabetic mother showed significant improvement in the extent of ossification in the limb bones.

| Table 1: Effect of PECCQ on glycemic levels |
| Groups (n=6) | Blood glucose levels (mg/dl) |
| o day | 5th day | 14th day | 21st day |
|---------|----------|----------|---------|
| C | 74.3±10.88*** | 84.1±9.33*** | 94.5±1.47*** | 94.1±1.18*** |
| D | 279±1.43 | 300±1.21 | 368.8±2.61 | 382.7±4.13 |
| D+CQ | 280.7±1.14 | 292.8±1.26* | 370.7±1.66 | 388±1.77 |

Blood glucose levels in the rats of the normal control (C), diabetic control (D), and diabetic+CQ (D+CQ) groups on days 0, 5, 14, and 21 of gestation. Data presented as means±SEM. Statistical analysis with repeated measures ANOVA followed by Bonferroni’s multiple comparison test. Only significant differences versus D group are indicated. ***P<0.001, **P<0.01, *P<0.05

| Table 2: Effect of PECCQ on body weight |
| Groups (n=6) | Body weight (g) |
| o day | 5th day | 14th day | 21st day | Total weight gain (21-0) |
|---------|----------|----------|---------|-----------------|
| C | 179.9± | 197.7± | 218.3± | 300.3± | 120.4± |
| 1.22 | 0.8*** | 2.09*** | 3.24*** | 2.02*** |
| D | 176.8± | 165.5± | 192.1± | 247± | 70.2± |
| 1.53 | 1.4 | 1.66 | 2.39 | 0.86 |
| D+CQ | 181.8± | 172.2± | 208.5± | 275.6± | 93.8± |
| 1.49 | 0.52** | 2.14** | 3.86** | 0.37*** |

Body weight in the rats of the normal control (C), diabetic control (D), and diabetic+CQ (D+CQ) groups on days 0, 5, 14, and 21 of gestation and maternal weight gain (day 21-0). Data presented as means±SEM. Statistical analysis with repeated measures ANOVA followed by Bonferroni’s multiple comparison test. Only significant differences versus D group are indicated. ***P<0.001, **P<0.01, *P<0.05
Effect of PECQ on the ossification of forelimb bones

The mean length of the ossified scapula and humerus in the pups born to the normal control group were 3.55 ± 0.03 mm (70.7 ± 1.08% of the total length of the scapula) and 3.73 ± 0.05 mm (64.19 ± 1.09% of the total length of the humerus), respectively. However, the mean length of the ossified scapula and humerus in the pups born to the diabetic control group were significantly decreased (scapula: 2.41 ± 0.04 mm, 56.01 ± 0.74% of the total length (P<0.001); humerus: 2.32 ± 0.03 mm, 46.18 ± 1.06% of the total length (P<0.001), Figures 4 and 5) indicating the effect of maternal diabetes on fetal skeletal ossification. However, when diabetic pregnant rats were treated with PECQ throughout their gestational period, their pups showed significantly (P < 0.001) higher rate of ossification at the time of birth (scapula: 3.29 ± 0.04 mm, 67.14 ± 1.07% of the total length); humerus: 3.31 ± 0.05 mm, 60.03 ± 1.21% of the total length, Figures 4 and 5) in comparison to the pups of the diabetic control group.

The mean length of the ossified ulna and radius in the pups born to normal control group were 4.23 ± 0.01 mm (73.82 ± 0.95% of the total length of the ulna) and 3.39 ± 0.02 mm (69.53 ± 0.72% of the total length of the radius) respectively. However, the mean length of ossified ulna and radius in the pups of diabetic control mothers were found to be significantly decreased to about 15% as

Table 3: Effect of PECQ on ossification centers

| Bones                | Groups (n=18) | C      | D      | D+CQ   |
|----------------------|--------------|--------|--------|--------|
| Sternum              |              | 5.7±0.1*** | 3.9±0.2 | 5.5±0.3*** |
| Metacarpus           |              | 3.8±0.1*** | 2.6±0.1 | 3.6±0.1*** |
| Anterior phalanges   |              | 3.9±0.1*** | 2.2±0.1 | 3.0±0.2**  |
| Metatarsus           |              | 4.7±0.1*** | 4.1±0.1 | 4.6±0.1**  |
| Posterior phalanges  |              | 4.7±0.1*** | 2.2±0.1 | 3.7±0.1*** |
| Caudal vertebrae     |              | 5.2±0.1*** | 3.5±0.2 | 4.5±0.2*** |

Ossification centers in the pups from the normal control (C), diabetic control (D), and diabetic+CQ (D+CQ) groups at term pregnancy. Data presented as means±SEM of the number of ossification centers identified. Statistical analysis with one-way ANOVA followed by the Bonferroni’s multiple comparison test. Only significant differences versus D group are indicated. ***P<0.001, **P<0.01, *P<0.05

Figure 1: Alizarin red S–alcian blue stained sternum in neonatal pups of the experimental groups at term pregnancy. Ossification center in the sternum (arrow). Fewer ossification centers observed in the D group. PECQ treatment restored the ossification centers to near normal in D + CQ group

Figure 3: Alizarin red S–alcian blue stained foot bones in neonatal pups of the experimental groups at term pregnancy. Ossification center in the metatarsal bone (*), the ossification center in the posterior phalanx (arrow). Fewer ossification centers observed in the D group and PECQ treatment restored the ossification centers to near normal in D + CQ group

Figure 2: Alizarin red S–alcian blue stained hand bones in neonatal pups of the experimental groups at term pregnancy. Ossification center in the metacarpal bone (**), ossification center in the anterior phalanx (arrow). Fewer ossification centers observed in the D group and PECQ treatment restored the ossification centers to near normal in D + CQ group

Figure 4: Percentages of the total length of the ossified portion of the forelimb bones in the neonatal pups of the experimental groups. The pups of the D group showed significant decrease in the length of the ossified portion of forelimb bones in comparison to that of the C group. However, when the diabetic rats were treated with PECQ, the length of the ossified portion of these bones was brought back close to normal. Data presented as means ± SEM. The significant differences versus D group are indicated as ***P < 0.001
compared to the normal control pups (ulna: 2.87 ± 0.08 mm, 55.92 ± 1.04% of the total length (P < 0.001); radius: 2.27 ± 0.04 mm, 54.05 ± 1.14% of the total length (P < 0.001, Figures 4 and 5). Furthermore, the mean length of the ossified ulna and radius in the pups of PECQ-treated mothers was found to be significantly (P < 0.001) increased as compared to that in the pups of diabetic control mothers (ulna: 3.7 ± 0.1 mm, 68.45 ± 1.49% of the total length (P < 0.001); radius: 3.01 ± 0.07 mm, 65.43 ± 1.49% of the total length (P < 0.001, Figures 4 and 5).

**Effect of PECQ on the ossification of hindlimb bones**

The mean length of the ossified femur in the pups of the normal control mothers was 3.03 ± 0.05 mm (n = 12; 54.9 ± 0.78% of the total length of the femur), while the mean length of the ossified femur in the pups of the diabetic control mothers was significantly (P < 0.001) lower as compared to that in the pups of normal control mothers (femur: 2.04 ± 0.05 mm, 44.31 ± 1.11% of the total length, Figures 6 and 7). However, diabetes-induced delayed fetal ossification in the femur was brought back to near normal in the pups of PECQ-treated mothers (2.71 ± 0.04 mm, 50.57 ± 1.16% of the total length, Figures 6 and 7).

The mean length of the ossified tibia and fibula in the pups of normal control mothers were 3.77 ± 0.04 mm (65.49 ± 0.95% of the total length of the tibia) and 3.59 ± 0.05 mm (67.81 ± 0.67% of the total length of the fibula), respectively. However, the ossified portion of these bones significantly (P < 0.001) decreased in the pups of diabetic control mothers (tibia: 2.43 ± 0.04 mm, 51.36 ± 1.07% of the total length; fibula: 2.31 ± 0.03 mm, 51.6 ± 1.03% of the total length, Figures 6 and 7). Similar to the upper limb bones, the mean length of the ossified tibia and fibula were found to be significantly increased in the pups of PECQ-treated diabetic mothers as compared to the pups of diabetic control mothers (tibia: 3.45 ± 0.03 mm, 61.13 ± 1.15% of the total length (P < 0.001); fibula: 3.44 ± 0.04 mm, 63.34 ± 0.87% of the total length (P < 0.001), Figures 6 and 7).

**DISCUSSION**

DM in pregnancy can be divided into clinical diabetes and gestational diabetes.[38] In the present study, we produced animal model for clinical diabetes with STZ administration (40 mg/kg) to rats during their adult life using the venous route, before pregnancy. This model mimics women with uncontrolled clinical diabetes during pregnancy. Diabetic rats had blood glucose levels ≥270 mg/dl and showed reduced body weight during pregnancy. The reduced body weight may be due to the metabolic alterations caused by hyperglycemia/hypoinsulinemia.[39] These findings are similar to those reported in previously performed
Interesting, the gain of maternal body weight during pregnancy was improved with the PECQ treatment when compared to the diabetic group. As PECQ did not interfere in the glycemic condition of the diabetic rats, the positive effect of PECQ on maternal body weight may be attributed to its antioxidant property.\[46\]

The morphometric analysis of limb bones and decreased number of onset of ossification centers in the current study revealed influence of maternal diabetes on fetal skeletal ossification in the offspring of diabetic rats. Similar results were reported earlier in studies of the effect of hyperglycemia during pregnancy on fetal skeletal ossification.\[30,31\] In the past, it has been shown that treating animals with PECQ from 9th to 21st day of gestation affects the fetal skeletal ossification and improves the cortical and trabecular bone mass in the offspring.\[40,41\] In the present study, the offspring of diabetic rats treated with PECQ throughout the gestation have shown increased number of ossification centers and improved percentages of the total length of ossified cartilage of limb bones as compared to that in the diabetic group. These results indicate that PECQ has the potential to normalize defective skeletal development in the offspring of diabetic rats. Present morphometric analysis of limb bones, for the first time, revealed that maternal exposure of PECQ to diabetic rats can effectively prevent delayed skeletal ossification in fetal limb bones.

Enhancing effect of PECQ on fetal osteogenesis observed in the present study could be attributed to phytoesthetic, steroid-like compounds present in it that probably act as phytosteroids and effectively cross the placental barrier to affect the fetal skeletal growth.\[27\] Presence of several phytosteroids in the CQ extract has been studied.\[19\] It also been shown that such phytoestrogens can pass through the placental barrier.\[37,38\] The pharmacological effect of PECQ on fetal skeletal osseification can also be explained by the study conducted by Migliaccio et al., where altered maternal estrogen levels during the pregnancy resulted in long-lasting changes in the skeletons of neonatal pups, suggesting the stimulatory effect of prenatal exposure of estrogen on fetal skeletal growth.\[36\]

Reports suggest that ossification defects in the offspring of diabetic rats are due to the deficient bone maturation during the embryonic period.\[15\] An in vitro study showed that PECQ can enhance the mesenchymal stem cell proliferation and facilitate osteoblastogenesis.\[47\] In addition, it has been shown that estrogen stimulates proliferation and differentiation of osteoblasts.\[48,49\] Therefore, the effects of PECQ observed in the present study may be due to the phytoesthetic steroids present in it, which may have acted as an analog of estrogen and enhanced the proliferation and differentiation of the osteoblasts and facilitated the mineralization process in the hyperglycemic conditions. Furthermore, Gopalakrishnan et al. reported that the synthetic estrogen facilitates mineralization in the presence of high glucose in vitro\[34\] and that treatment with estrogen can reverse diabetes-induced osteopenic changes in adult rats.\[33\] Thus, the stimulatory effect of PECQ on defective fetal ossification in maternal diabetes may be due to estrogen-mimicking action of phytosteroids present in PECQ, which may promote fetal bone maturation in diabetic state.

Intra-uterine environment plays an important role in programming the risk of developing chronic diseases such as cardiovascular disease, diabetes, and osteoporosis later in life.\[35\] Poor intrauterine bone growth may program an increased susceptibility and risk for developing osteoporosis later in life.\[36\] Therefore, defective/delayed fetal skeletal development in maternal diabetes may lead to osteoporosis in their offspring later in life. In such cases, PECQ treatment may be useful to normalize the maternal diabetes-induced delay in fetal skeletal ossification and to protect offspring from osteoporosis later in life.

Furthermore, it has been shown that the CQ extract can act as a free radical scavenger,\[51\] and ROS has been proposed for pathogenesis diabetes-induced malformations.\[52\] In addition, decreased rate of malformations were observed in the offspring of diabetic rats fed with inositol, arachidonic acid, and antioxidants.\[53-57\] Therefore, reduced skeletal malformations in the offspring of PECQ-treated diabetic mother may be due to its antioxidant properties as well. However, the antioxidant role of PECQ extract in diabetic rat model needs further evaluation.

CONCLUSION

Our results provide evidence that PECQ treatment can effectively ameliorate the defective skeletal development in the offspring of diabetic rats. This may be due to the antioxidant as well as pro-osteogenic properties of PECQ. Further studies on isolation and characterization of the active chemical constituents of PECQ that can cross the placental barrier and that are responsible for bone anabolic activity are warranted.

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