CISSUS QUADRANGULARIS PLANT EXTRACT ENHANCES THE OSSIFICATION OF FETAL BONES

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Summary

The present study was designed to investigate the effects of plant *Cissus quadrangularis* (Linn) on development of bone during fetal growth period. Pregnant rats were administered with Ethanol extract of *Cissus quadrangularis* (CQ), orally, at the dose of 750 mg/kg body weight from 9th day of gestation till delivery. Skeleton of the newborn pups was studied using Alizarin red S and Alcian blue staining followed by Scion image analysis of long bones in the limbs and interparietal bone. Results showed a significantly increased bone formation in the pups born to CQ treated pregnant rats compared to those born to control rats. The present study demonstrates enhanced bone formation during fetal growth by CQ, which may be related to rich content of calcium, phosphorus and phytoestrogenic property of the plant.

Key words: Ossification, C*issus quadrangularis,* Alizarin red S, Alcian blue, Scion image.

Traditional recipes for treatment of physical and mental ailments exist in all major ancient civilizations of the world. One such recipe popular is the Indian subcontinent involves the use of the extract of *Cissus quadrangularis* plant .*Cissus quadrangularis* is a shrub, with thick and fleshy quadrangular stem. It is an edible plant found in India, Srilanka, Malaya, Java and West Africa (1). The plant and its medicinal properties have been described in literature as early as 1970 (1). Ethnobotanical uses of the plant have been reported (2). Stem and root extract of this plant possess antioxidant and antimicrobial activity (3). Various organic macromolecules ranging from terpenoids to large stilbene derivatives have been isolated from this plant (4). Stem extract contains a high percentage of calcium ions and phosphorus (5). The plant has been documented in Ayurveda, an alternative system of medicine in India, for its medicinal uses in gout, syphilis, venereal diseases, piles, leucorrhoea (6). The stem juice of the plant is used to treat scurvy and irregular menstruation, otorrhoea and epistaxis (7). The roots and stem are most useful for healing of fracture of the bones (7, 8, 9, 10, 11, 12). Phytoestrogenic steroids have been isolated from *Cissus quadrangularis* plant (13, 14, 15), has been shown to influence early regeneration and quick mineralization of bone fracture healing process.

Many studies have focused on assessing the role of estrogens deficiency that plays the major role in the onset of postmenopausal osteoporosis (16, 17, 18,19). Estrogen receptor has been detected in bone cells (Both in osteoblast and osteoclasts (20, 21, 22) suggesting the direct action of estrogens on these bone cells. Several studies have shown that estrogens can modulate bone cell physiology in vitro by direct estrogen receptor mediated mechanism (23, 24, 25). This evidence implicates a direct effect of estrogen on skeleton and alternatively on bone tissue turnover. Natural estrogens such as estradiol were shown to induce permanent changes in the skeleton of adult animals when given as long term treatment (16, 25, 26, 27). Additionally phyto-estrogens which are widely present in our environment seem to have some modulatory effect on bone cells in vitro (28).

The question whether the plant *Cissus quadrangularis*, which is proved to contain phyto-estrogens will increase the bone mass during fetal growth is not addressed. Accordingly the objective of the present study was to evaluate the effect of alcoholic extract of *Cissus quadrangularis* on the intrauterine growth of long and flat bones in rats.

Materials and methods

Animals

Female Wistar rats of 3 months age (200-250 g weight) were housed in the central animal research facility of the Manipal University, Manipal. All animals were allowed to have free access to water and food, and were maintained in 12:12 h dark and light cycle. All experiments were carried out with prior approval from the institutional animal ethical committee. Only the minimum required numbers of rats were used, and they were handled in a humane way. To get pregnant rats of known gestational day, female rats in estrous cycle were mated with young healthy male rats and then, they were subjected for vaginal smear test. Detection of sperms in vaginal smear was considered as day 0 of pregnancy.

Experimental groups

Pregnant rats were divided into control and experimental group (n=6 in each group) Pregnant rats in the experimental group were treated with CQ alcoholic extract, 750 mg/kg/day from gestation day 9 till delivery. The control group received equivolume of saline.

Preparation of extract:

CQ stem were collected from Nalgonda district of Andhra Pradesh, India. A specimen was deposited in the Pharmacognosy Laboratory of the Manipal University, Manipal. The fleshy stems were washed, cut into small pieces, air-dried and crushed into powder. Stem powder was exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The total ethanol extract was concentrated in vacuo till a syrupy consistency is obtained.

Fig 1: *Cissus quadrangularis* plant. Note the fleshy quadrangular stem with nodes at intervals. At nodes we can see a leaf and a tendril

Alizarin red S and Alcian blue staining

Alizarin red S stains ossified bones and Alcian blue stains the unossified cartilages of the skeleton system. All the pups born to control and treated pregnant rats were anaesthetized with anesthetic ether and skin was removed and eviscerated. The pups were then fixed in 95% ethanol for 5 days. Then the pups were placed in acetone to remove fat for 2 days. Thereafter pups were stained with Alizarin red S and Alcian blue solution for 3 days. The stained pups were cleared in 1% aqueous Potassium hydroxide for 48 hours or until skeletons are clearly visible through the surrounding tissue.

Bone length and area measurement

To measure the skeleton length, ossified bone length, and area of the ossified bone, fore limbs, hind limbs and head were photographed along with a measuring scale. Length of the ossified bone, and area of ossified part of the interparietal bone were measured using Scion image programme for Windows (NIH programme).Breifly, Programme was calibrated and the length and area were measured using the grey scale image (BMP) of the original JPEG image. Length of humerus, radius, ulna, femur, tibia and area of interparietal bone were measured (n=6 limbs in each group). The percentage of the total length of bone mineralized in the control and treated groups were measured with Scion image programme.

Results

The oral administration of alcoholic extract of Cissus *quadrangularis* plant caused neither any mortality nor any signs of clinical abnormality in the treated pregnant rats. At necropsy no gross pathological observation could be made in the target organs. There was no mortality observed in the pups born to the treated pregnant rats. Thus it appears that the drug dose given is safe.

Ossification of the humerus:

The scion image data revealed a significant difference in the extent of ossification of humerus bone compared to control pups. Mean length of skeleton ossified in the control pups was 3.23 ± 0.12 mm (mean±SEM,n=6, 63.93 ±1.67% of total length of the skeleton) and mean length of skeleton ossified in Cissus quadrangularis treated pups was 3.85 ± 0.12mm (74.32 ±1.54% of total length of the skeleton, P<0.001, Unpaired t-test (one tailed), Fig-2.

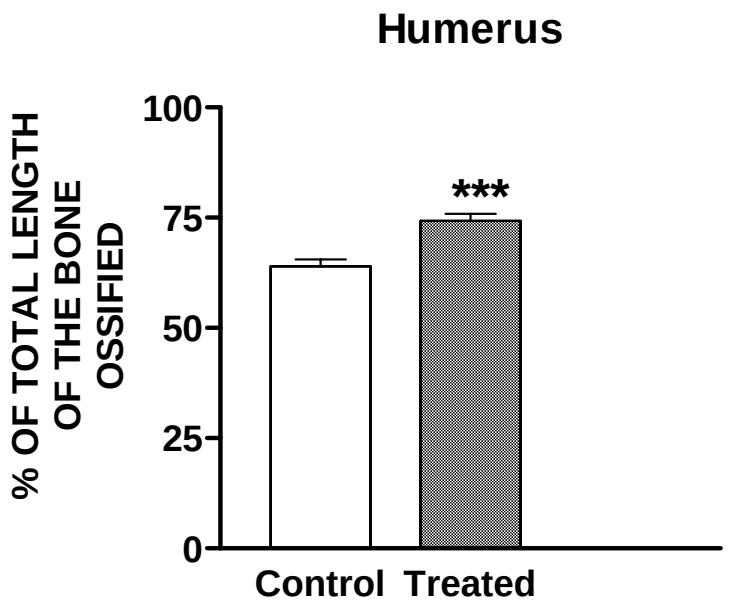

Fig 2: Bargraph showing the percentage of total length of humerus ossified in Control and *Cissus quadrangularis* plant extract treated rats. Note there is a significant increase in the length of ossified part of humerus in treated fetus. *** P<0.001

Ossification of Radius and Ulna:

The length of ossified bone in radius and ulna was found to be 2.3 ± 0.11 mm (58.63±2.18% of total skeleton) and 3.34±0.15mm (62.04±1.14% of total skeleton length) respectively in control pups. The length of ossified bone in radius and ulna was found to be significantly increased compared to control pups (Radius: 2.87±0.11mm (71.43% of total length of skeleton, P<0.001; Ulna: 4.05±0.02mm (77.53±0.82% of total length of the skeleton, P<0.001, unpaired t-test, one tailed, Fig-3. Fig-4 shows the photographs of Alizarin red S and Alcian blue stained neonatal pups. Note the increased length of ossified part of humerus, radius and ulna in the *Cissus quadrangularis* plant extract treated groups.

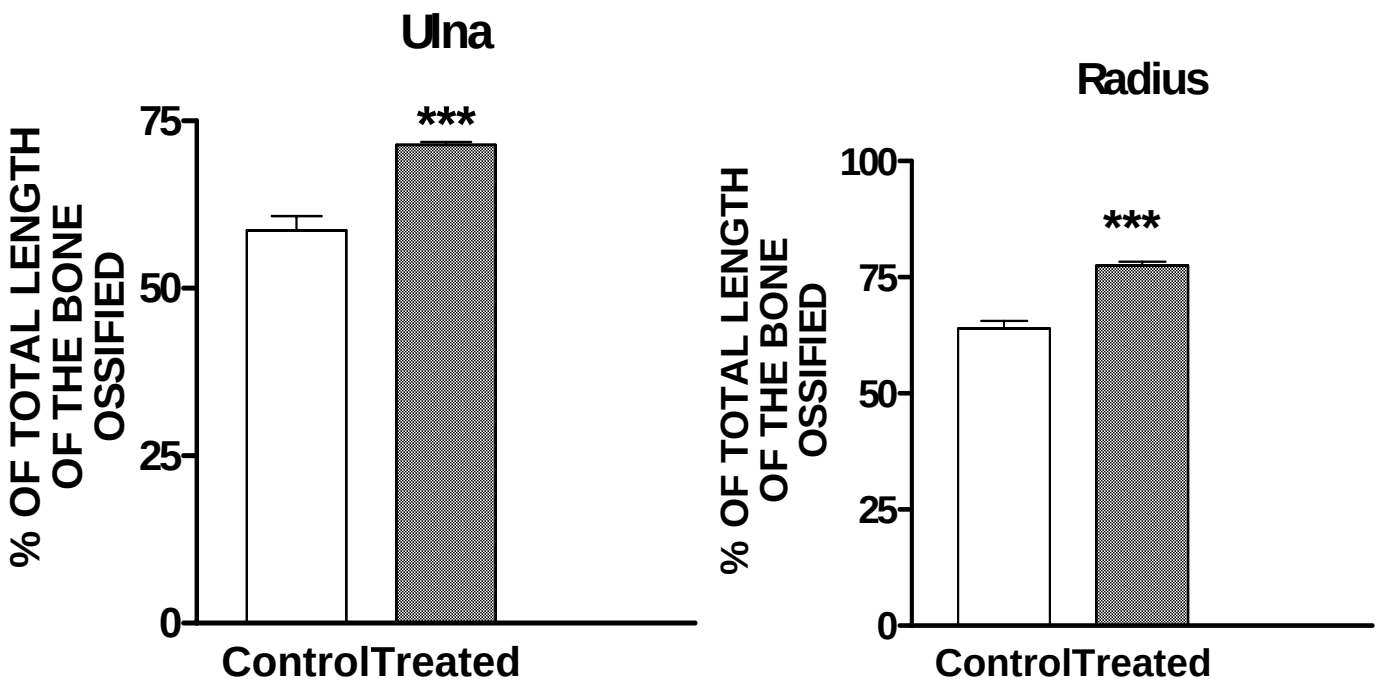

Fig 3: Bargraph showing the percentage of total length of ulna and radius ossified in Control and *Cissus quadrangularis* plant extract treated rats. Note there is a significant increase in the ossified part of ulna and radius*** P<0.001

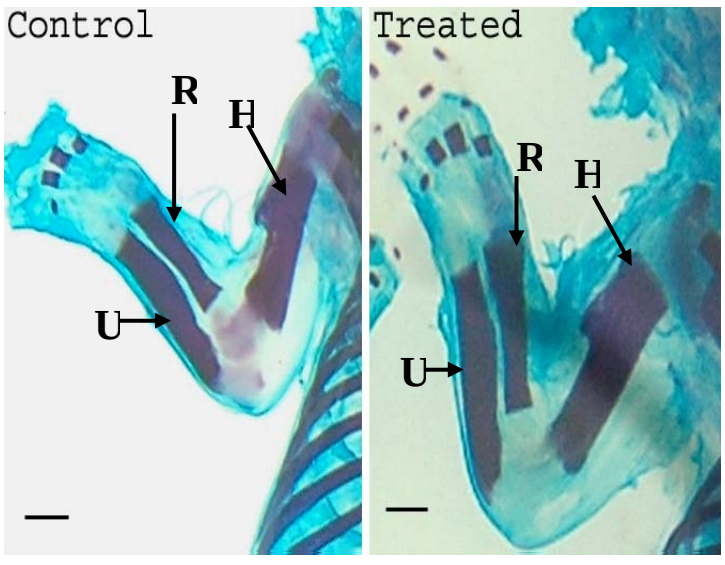

Fig 4: Photograph of Alizarin red S and Alcian blue stained rat forelimb showing the ossified parts of the bones in Control and *Cissus quadrangularis* plant extract treated rats. Note the increased length of Humerus, Radius and Ulna ossified in the treated rats.H-Humerus, R-Radius, U-Ulna, Scale bar =0.5mm in both photographs

Ossification of Femur and Tibia:

Like humerus, radius and ulna, femur and tibia also showed increased extent of ossification. The length of the ossified part of the femur was 2.3±.11mm (52.4±1.5% of total length of skeleton) in control pups and in *Cissus quadrangularis* treated new born pups it was 3.00±0.16mm (64.06±3.15% of total skeleton length, P<0.01). The length of the ossified part of tibia in control was 3.66±0.07mm (67.24±1.01% of total skeletal length and *Cissus quadrangularis* treated new born pups3.75±0.16 mm (74.89±1.37% of total skeleton length, P<0.01). Fig.4. Fig.5 shows the Alizarin red S and Alcian blue stained femur and tibia in control and treated groups. Note there is a significant increase in the length of femur and tibia in treated new born pups.

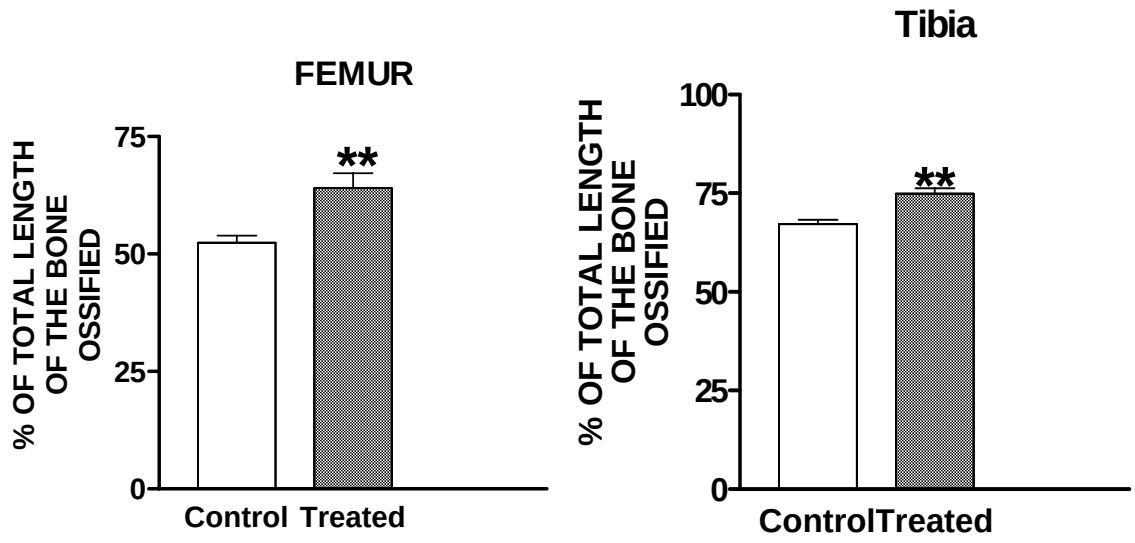

Fig 5: Bargraphs showing the percentage of total length of Tibia and femur ossified in Control and *Cissus quadrangularis* plant extract treated rats. Note there is a significant increase in the ossified part of femur and tibia. ** P<0.01

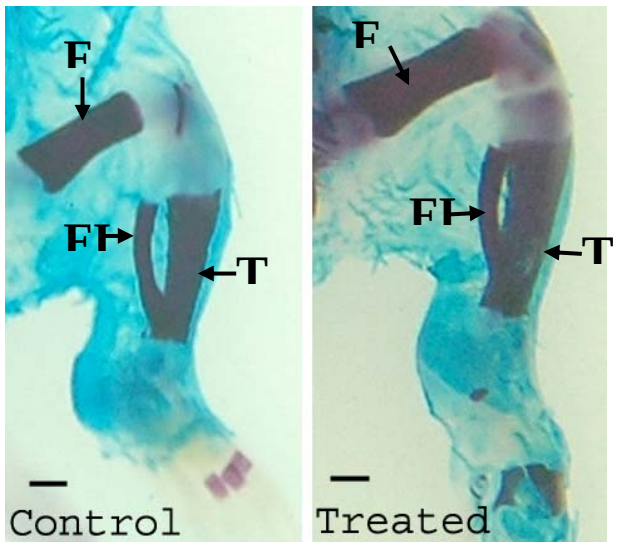

Fig 6: Photograph of Alizarin red S and Alcian blue stained rat hind limb showing the ossified parts of the bones in Control and *Cissus quadrangularis* plant extract treated rats. Note the increased length of femur and tibia in the treated rats.F-Femur,FI-Fibula, T-Tibia, Scale bar =0.5mm in both photographs.

Ossification of interparietal bone:

Area of interparietal bone was measured using Scion Image. There was no significant difference in the area of ossified bone between the control and treated pups, Fig-6. Fig-7 shows Alizarin red S and Alcian blue stained skull part of the new born pups.

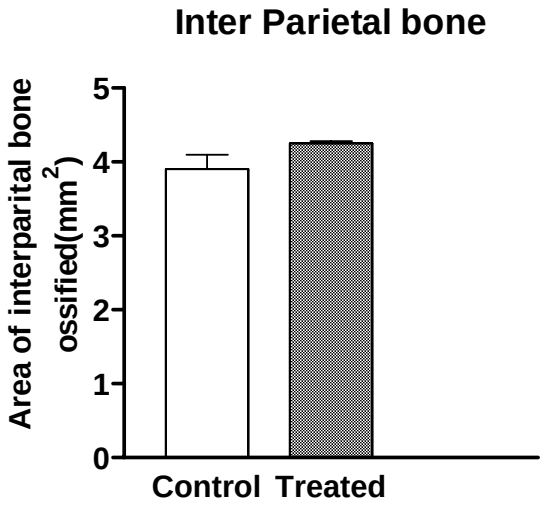

Fig 7: Bargraph showing the area of interparietal bone ossified in Control and *Cissus quadrangularis* plant extract treated rats. Note there is no significant increase in the ossified part of the bone.

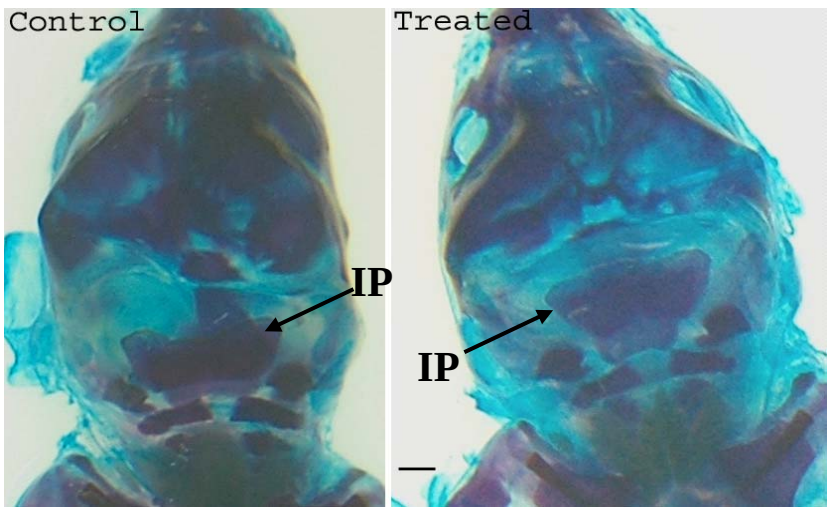

Fig 8: Photograph of Alizarin red S and Alcian blue stained rat skull showing the ossified parts of the inter parietal bone in Control and *Cissus quadrangularis* plant extract treated rats. Note the there is no change in the bone area ossified. IP- interparietal bone, Scale bar =0.5 mm applicable to both.

Discussion

The present study results demonstrate for the first time that maternal treatment with *Cissus quadrangularis* during gestation can dramatically influence the skeletal tissue of developing fetus. In the present study the pups born to the pregnant rats fed with *Cissus quadrangularis* plant extract showed an increased bone formation (extent of intrauterine ossification). In these pups (born to treated pregnant rats) the percentage of the total length of limb skeleton ossified was significantly greater compared to that in pups born to control rats. However such a change was not observed in the interparietal bone, one of the skull bones studied.

It is well known that changes in estrogen levels can dramatically modify bone tissue turnover in both postmenopausal women (27) and adult experimentally induced postmenopausal animals (16, 17). In addition, long term exposure of adult animals to estradiol or diethylstilbestrol induces hyperplasia of bone tissue (17, 28). These data strongly suggest that bone tissue directly respond to estrogens as other estrogen target tissue. Further that transient neonatal exposure of female mice to estrogens have shown to increase the bone mass in the animals during adulthood (25). In addition, sex steroids can influence the development of dimorphism in the female innominate bone (29). Thus these data strongly suggest that alterations of estrogen levels before puberty, in early phase of development can dramatically influence skeletal maturation including final peak bone density. Perturbation in the maternal estrogen level during pregnancy, shown to alter the developing skeleton. (30).

In the present experimental study we have reported an increased bone mass in the pups born to the treated pregnant rats. This may be attributed to the phytoestrogenic compounds isolated from this plant (13) which might have altered the maternal estrogen level. The plant extract not only increased the bone length, but also increased the trabecular density of bone (Unpublished preliminary data). The effect was identical on both male and female pups. Hence data was analyzed together.

Alternatively, the phyto-estrogenic compounds or other unidentified chemical compounds might have altered the steroidal hormonal levels in the pregnant rats, there by influencing the skeleton of the fetus/neonatal pups.

Another possibility is that the phytoestrogens present in the plant extract might have crossed the placental barrier and reached the developing fetus and influenced the skeletal system. This is indeed true in case of study by Magliaccio et al (30), where they have altered the maternal estrogen levels by giving external steroidal hormones and showed alterations in the skeleton of neonatal pups.

Alternatively, the increased bone formation in the *Cissus quadrangularis* plant extract treated pups may be due to rich calcium and phosphorous present in this plant (5). The stem extract of this plant contains a high percentage of calcium ions (4% by weight) and phosphorus, both essential for bone growth (5). In deed using the extract of this plant, high quality calcite crystal has been synthesized as this plant contains high amount of calcium (31). It has been reported earlier that the extract of this plant is very useful in bone fracture healing process (7, 8, 9, 10, 11, 12, 14, 15), which is a process of bone formation in adults. The calcium ions, phosphorous and phytoestrogens present in this plant extract may be made use in the process of ossification and fracture healing.

Thus the plant *Cissus quadrangularis* appears to be very useful in treating the diseases involving deficiency in the bone formation and fracture healing. The active ingredients need to be isolated, characterized and studied further to formulate the therapeutic drugs.

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