EFFECTS OF CIPROFLOXACIN ON SECONDARY OSSIFICATION CENTERS IN JUVENILE WISTAR ALBINO RATS

Muhammad Aslam Channa, Muhammad Ashfaq*, Sibghatullah Sanghi,**

Muhammad Azam Qureshi, Muhammad Zahoor Janjua***

Department of Anatomy Mohammad Medical College Mirpurkhas, *Ayub Medical College Abbottabad, ***BMSI, Jinnah Postgraduate Medical Center Karachi and **Department of Pharmacology, Fatima Jinnah Dental College Karachi

Background: Administration of quinolone therapy is controversial during juvenile age as stated by earlier workers. The fluroquinolones are currently not indicated for young children, because of the arthropathy and adverse effect on growing cartilage shown by studies. However the effects of ciprofloxacin on secondary ossification centers has remained undocumented. This study is therefore aimed to determine the risk of Ciprofloxacin administration on neonatal skeletal differentiation by a prospective and comparative animal study model using Wistar albino rats.

Methods: Ciprofloxacin was administered to newly born Wistar albino rat pups at a dose of 20 mg/kg body weight intraperitoneally twice daily from day-1 to day-14 after birth. These animals were killed by deep ether anaesthesia and fixed in 80% alcohol. They were then bulk stained with Alizarin red and Alcian blue. Finally they were cleared in 4% KOH and stored in glycerin. The fore and hind limbs were disarticulated from the axial skeleton and observed under stereomicroscope for evidence of skeletal differentiation in the form of presence of secondary ossification centers in long hones (left humerus and left femur). The time of appearance of these centers were noted and compared statistically with those in control animals. **Results:** The study revealed that the skeletal differentiation in long bones was delayed by 2.4 $\pm$ 0.2 days at both proximal and distal ends in humerus and 2.4 $\pm$ 0.2 days at proximal end and 2.2 $\pm$ 0.2 days at distal end of femur in experimental animals as compared with controls. **Conclusion:** The ciprofloxacin administration during post-natally presents a risk to skeletal differentiation and therefore to its growth upto the age of six weeks is albino rate pups.

Key Words: Ciprofloxacin, Bone differentiation, Ossification centers

INTRODUCTION

The newer quinolones, the fluoroquinolones are represented by Cipro-floxacin, Norfloxacin, Ofloxacin and Tamofloxacin. These agents represent an improvement over their quinolone counterparts in many ways including a wider spectrum of anti-microbial activity, improved pharmacokinetic properties, and clinical efficacy against wider range of diseases.[1] Ciprofloxacin is one of the more active in this calss that possesses an extended spectrum of activity[2], has good bioavailability 80-95% and distributed widely in body fluids and tissues.[3] It has sufficiently long serum half-life to allow twice daily dosing.[4]

Quinolones cause cartilage damage deterioration in young animals and are not recommended for use in children less than 18 years or pregnant or nursing women.[5] After initiation of ciprofloxacin in clinical trials in the early 1980s it has been administered to children despite restrictions. The first report from a child treated with ciprofloxacin was in May 1983.[6] Since that time ciprofloxacin has been continuously used in children and adolescent when conventional therapy failed or was not available.

Studies on the effects of ciprofloxacin on bone growth have been carried out, but any effect on skeletal differentiation has not been reported so far. This study was done to evaluate the effect of ciprofloxacin administration on differentiation of long bones in extremities of postnatal juvenile laboratory Wistar albino rats.

MATERIAL AND METHODS

In this study 30 spontaneously ovulating female & 15 fertile male Wistar albino rats, were 10-12 weeks of age, when taken from the Animal House of BMSI, JPMC, Karachi., The female rats were mated with fertile males of the same strain, according to the method described by Rough[7] allowing one male rat with two female rats in one cage. On next morning the female rats were examined for signs of mating in the form of blood stained vagina or a vaginal plug (a mucoid greenish white material). Presence of any one of these signs was considered as day-1 of pregnancy[8]. The gestation period of the albino rat is usually between 21 and 23 days.[9]

Thirty pregnant albino rats were allowed to deliver their pups. Randomly selected 140 pups were divided into two groups, i.e. A and B, each comprising 70 animals. Sex of these offspring was omitted.

Group-A (experimental, n=70) pups were given injection Ciprofloxacin (developed in Bayer Research Laboratories, AG, Germany) at a dose of 20 mg/ kg body weight[10] (0.12 mg in 0.1 ml) intraperitoneally twice daily for 14 days from day-1 after birth.

Group-B (control, n=70) pups were given normal saline in equal volume (0.1 ml)[11] intraperitoneally twice daily for 14 days from day-1 after birth.

Five specimens were then randomly selected for the study from each group for each day mentioned (total 70 pups from group experimental which received Ciprofloxacin and total 70 pups from control group which received Normal saline). Pups were then killed by deep ether anaesthesia and fixed in 80% alcohol after removing their skin and viscera. Pups were then bulk stained in Alizarin red and Alcian blue, cleared in 4% K(OH) revealing ossification centers and were stored in glycerin by the method as described by patton & Kafman.[12]

Left fore and hind limbs were separated from axial skeleton at their joints and viewed by magnification x4 under stereomicroscope. The presence or otherwise of secondary ossification centers in humerus and femur at their proximal and distal ends were observed and recorded. Care was taken to note the measurement of growth plate and its different zones. The said bones were observed throughout the experimental period (day-1 to day-14 postnatally). Even after the detection of primary ossification centers to see appearance of any additional ossification center in shaft or end of these long bones. The mean value of the time of first appearance of secondary center in experimental and control bone is given as mean±SEM. Student 't' test was employed to determine the statistical significance of the results as described in 'introduction of medical statistics'.[13]

RESULTS

The mean time of appearance of secondary ossification centers in major skeletal components of both extremities in experimental and control animals are given in table and shown in figures-1, 2 and 3.

Table-1: Comparison of Time (day) and Appearance of Secondary Ossification Centers in postnatal Experimental animals (group-A) and Control (group-B) at Proximal and Distal ends of Long bones: Left Humerus and Left Femur in Juvenile Wistar Albino rat pups

Bone	Experimental Animals (n=70)	Control (n=70)	Time Delay

Humerus	PE	9.4 ± 0.2*	7.0 ± 00	2.4 ± 0.2
	DE	9.4 ± 0.2*	7.0 ± 00	2.4 ± 0.2
Femur	PE	16.4 ± 0.2*	14.0 ± 00	2.4 ± 0.2
	DE	9.2 ± 0.2*	7.0 ± 00	2.2 ± 0.2

Key:PE = Proximal end; DE = Distal end; n = Total number of animals;

*P<0.001 (highly significant increase)

Figure-1: Photograph of postnatal treated pups whole skeleton with double staining technique, i.e. Alizarin red stained bone and Alcian blue stained cartilage showing comparison of appearance of secondary ossification centers at proximal and distal ends of long bones between experimental [group-A (left)] and control [group-B (right)] animals used in this study.

Figure-2: Photograph of left fore limb (Humerus bone) with double staining technique, i.e. Alizarin red stained bone and Alcian blue stained cartilage showing comparison in presence of secondary ossification centers at proximal and distal ends between experimental [group-A (left)] and control [group-B (right)] animals used in this study.

Key: L = Left; HL = Hind limb

Figure-3: Photograph of left hind limb (Femur bone) with double staining technique, i.e. Alizarin red stained bone and Alcian blue stained cartilage showing comparison in presence of secondary ossification centers at proximal and distal ends between experimental [group-A (left)] and control [group-B (right)] animals used in this study.

Key: L = Left, FL = Fore limb

LEFT HUMERUS

In control animals, the secondary centers of ossification at proximal and distal ends were present on seventh postnatal day in all specimens observed (5 specimens of each bone). No additional ossification center was observed in any of the bones mentioned during the rest of study period.

In experimental animals, the secondary ossification centers at proximal and distal ends were seen to be present on ninth postnatal day in 3 specimens and on 10th postnatal day in 2 specimens (average 9.4 days with delay of 2.4 days).

The mean epiphyseal growth plate thickness in control animals was 131.65±0.63 μm, while ciprofloxacin treated animals was 117.60 ± 1.05 μm. the difference was found to be 14.05±0.37μm.

LEFT FEMUR

In control animals the secondary ossification centers were present at proximal end on 14th day after birth in all specimens observed (5 specimens of each bone) while at distal end were present on 7th postnatal day in all specimens (5 specimens of each bone). Any additional postnatal ossification center was not observed in any of these bones during rest of study.

In experimental animals the secondary ossification centers at proximal end was seen to be present on 16th postnatal day in 3 specimens and on 17th postnatal day in 2 specimens (average 16.4 days with delay of 2.4 days) while at the distal end the secondary ossification centers were present on 9th postnatal day in 4 specimens and on 10th postnatal day in one specimen (average 9.2 days with delay of 2.2 days).

The mean epiphyseal growth plate thickness in control animals was 139.65±0.39μm while in ciprofloxacin treated animals was 133.05±1.6μm. The difference was found to be 6.6±1.21μm.

DISCUSSION

Appearance of ossification center is the early indication of skeletal differentiation. The effect of ciprofloxacin treatment on skeletal differentiation was therefore studied by determining the time of first appearance of secondary representative ossification center in post-natal humerus and femur in experimental and control animal.

In control animals the secondary centers of ossification in humerus bone at proximal and distal ends appeared at 7th post-natal day and in femora secondary center appeared at proximal end on 14th post-natal day, while at distal end appeared on 7th post-natal day in all specimens observed. Our observation is similar to the findings of Patton and Kaufman[12].

Ciprofloxacin delayed the appearance of secondary ossification centers in humerus at proximal and distal ends by 2.4±0.2 days as compared to control animals. This delay was statistically highly significant ($P<0.001$), whereas, ciprofloxacin delayed the appearance of secondary ossification centers in femora at proximal and distal ends by 2.4±0.2 days and 2.2±0.2 days respectively as compared to control animals. The delay averaged 2.4±0.2 days in fore limb for both ends and 2.4±0.2 days at proximal end and 2.2±0.2 days at distal end in hind limb bones. This concluded that fore limb and hind limb bones were affected by the adverse effect of ciprofloxacin.

In this study the delay in appearance of ossification centers by ciprofloxacin is attributed to tissue accumulation of fluoride which presumabily delayed that calcification process[14] by inhibitory action on hydroxyappetite depostion[15]. The exact histo-chemical mechanism by which ciprofloxacin distrubs the ossification has not been investigated. Our observation is in consistance with the findings of Stahlmann[16] who stated that in animals during early post-natal developmental the epiphyseal growth plate can be damaged. However he neither specified the type of growth plate damage nor clarified the form of the irreversible bone damage. Irreversible bone damage could be either in the form of retarded formation or abnormal formation.

Our study suggests that the growth plate damage is due to chondrocyte proliferation depression as shown by diminuation of proliferative zone of the epiphyseal growth plate. Arora stated that repeated fluoroquinolone administration causes floride accumulation in the bones particularly affecting the linear growth[14].

Linear growth of long bones is through endochondral ossification[17]. This means that linear bone growth may be adversely affected primarily by cartilage growth retardation or secondarily by delay in conversion of the cartilaginous model into the newly formed bone. Since according to Arora floride is deposited into the bone, the bone growth appears to be retarded by the conversion into the newly laid bone. However, our finding of diminuation of the proliferation zone of cartilage in pups favours the idea that even if the bone growth is not entirely affected by the cartilage proliferation retardation, it must be the main determinant factor rather than floride deposition in bone.

In pups fusion of secondary ossification centers of long bones at age 6 weeks[18]. However, in humans the fusion of the secondary ossification centers of long bones occurs at 18 to 25 years. Considering epiphyseal delay of 2.4±0.2 days in pups in relation to 8 weeks age at skeletal maturation. The maturation to delay in maturation duration is 16.8:1. From our study model extrapolated value of delay in maturation in humans would be 478 days. However the ciprofloxacin injection period in pups was 14 days which is 1:3 ratio to the total maturation period.

Therefore if the ciprofloxacin is to be injected in the same ratio to the human skeletal maturation period it would be 7:21 (years). If the ciprofloxacin injection is for 7 years than delay maturation would be 478 days, but in reality ciprofloxacin injection in human children is upto 3 months. That will cause delay of 1:28 days. 478 ciprofloaxacin days delay in human divided by 2.4 ciprofloacin delay in pups would be 199 days ratio. Therefore 2677 days divided by 90 days, 30 times less period of ciprofloxacin injection in human therefore 2.4 days ÷ 30 = 0.08 days, delay would occur in humans if the adverse effect of ciprofloxacin is of similar magnitude. This delay is about 2 hours, since skeletal maturation range in humans varies from 18 to 25 years in which a difference of 2 hour delay is very highly statistically insignificant.

Therefore we consider the ciprofloxacin is very safe as far as its action on delayed epiphyseal closure is concerned. The time of appearance of secondary epiphysis is inversely related to the epiphyseal closure. Therefore the extrapolated values of delay in animals need to be confused in humans.

CONCLUSION

Since ciprofloxacin significantly inhibited the ossification process 2.4±0.2 days (delayed $P<0.001$), as compared with control. The ciprofloxacin administration post-natally presents a risk to skeletal differentiation and therefore to its growth upto the age six weeks in abino rat pups.

REFERENCES

1. Patterson-Reid D, Illnois-Park A. Quinolone toxicity– Methods of assessment. Am J Med 1991;91(Suppl 6A):35S-37S.

2. Patterson-Lance R, Lissack LM, Canter K, Asching CE, Counie C et al. Therapy of lower extremity infections with ciprofloxacin in patients with diabetes mellitus, peripheral vascular disease or both. Am J Med 1989; 86: 801-8.

3. Katzung BG. Quinolones. In:Basic and Clinical pharmacology. 8th ed. New York:MC Graw Hill 2001:797-801.

4. Gillman GA, Hardman JG; Limbird LE. The Quinolones. In: Goodman and Gillman's. The parmacological Basis of Therapeutics. 10th ed. New York: MC Graw Hill; 2001, Vol. II: 179–1183.

5. Pradhan KM, Arora NK, Jena A, Susheela AK, Bhan MK. Safety of Ciprofloxacin therapy in children, magnetic resonance image, body fluid levels of fluoride and linear growth. Acta paediatr 1995;84:555-60.

6. Hample B, Hulmann R, Schmidt H. Ciprofloxacin in paediatrics. World-wide clinical experience based on compassionate use. Safety report. Pediatr Infect Dis J 1997;16(1):127-9.

7. Rough R. Reproductive system. In: The mouse. 2nd ed. Minneapolis: Burgess Pub Co, 1968:269-99.

8. Chang HH, Schwartz Z, Kaufman MH. Limb and other postcranial skeletal defects induced by amniotic sac puncture in mouse. J Anat 1996;189:37-49.

9. Greene EC. Anatomy of rat. Philadelphia: American Philosophical Society, 1968; Vol. XXVIII, pp 5-30.

10. Martindale W. The complete drug reference 33rd ed, Swectman, S.C. (ed) London, Chicago: Pharmaceutical press, 2002: 182-5.

11. Lori EK, Sulik KK. Experimental foetal alcohol syndrome proposed pathogenic basis for a variety of associated facial and brain anomalies. Am J Gen Med 1992; 44: 168-76.

12. Patton J, Kaufman H. Timing of ossification of limb bones and growth rates of various long bones of the fore and hind limbs of the prenatal and early postnatal laboratory mouse. J Anat 1995;186:175-85.

13. Bland M. Introduction of medical statistics. 1st ed. Oxford: Oxford University Press, 1987: 165-187.

14. Arora NK. Are fluoroquinolones safe in children? Indian J Pediatr 1994;61(6):601-3.

15. Junqueira LC, Carneiro J, Keeley RO. Endochondral ossification. In: Basic histology. 7th ed. Lond: Appleton & Lange. 1992: 151-156.

16. Stahlmann R. Children as a special population at risk – quinolones as an example for xenobiotics exhibiting skeletal toxicity. Arch Toxicol 2003; 77(1): 7-11.

17. Williams PL, Bannister LH, Berry MM, Collins P, Dyson M, Dussek JF et al. Histogenisis of bone. In: Gray's Anatomy. 38th ed. New York: Churchill Livingstone, 1995, 471-80.

18. Slonaker JR. The normal activity of the albinorats from birth to natural death, its rate of growth and the duration of life. J Animal Behavior 1912;2:20-42.

Address for Correspondence:

Dr. Muhammad Aslam Channa, H.No.837/137-II-A, Golimar, Latifabad # 2, Nawabshah.

Phone: 0241-62631