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MONITORING AND DISTRIBUTION OF TETRACYCLINES RESIDUE IN BONE AND SOFT TISSUES OF BROILERS

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SUMMARY

The presence of certain antimicrobial agents in meat and other animal products constitute a potential hazard for the consumer and may cause allergic reactions, interference in the intestinal flora, and resistant population of bacteria in the general population, thereby rendering antibiotic treatment ineffective. The present work was conducted to study the correlation between the distribution in bone and soft tissue of tetracycline fluorescence examined under UV-light microscopy and withdrawal period. Semi quantitative histomorphometric analysis of soft tissue and tibia of broilers were performed after tetracycline labeling. For this purpose, 15 broilers approximately 1 kg in weight were subdivided into two groups, one experimental group (10 treated birds) and one control group (5 control untreated birds). Each bird of the experimental group was given intraperitoneally 10 mg of tetracycline and slaughtered after 1, 3, 6, 9 and 12 days of the drug administration. Frozen sections were made from the kidneys and liver. Tibias from both sides were collected; one tibia was fixed in 10 % formal solution, while the other was left unfixed. Both tibias were used for preparation of undecalcified materials. The later consisted of sagittal sections of the metaphyseal plate and cross sections of the diaphysis. All soft tissue and bone sections were examined under the fluorescence microscope. Using image analysis, the thickness of both proliferating and hypertrophying cartilage layers, the front fluorescent line of the metaphysis and the osteoid seams lining the trabeculae in the spongious layer were measured and correlated with the duration of withdrawal.

INTRODUCTION

The presence of certain antimicrobial agents in meat and other animal products constitute a

tential hazard for the consumer and may cause allergic reactions, interference with the intestinal flora, and resistant population of bacteria in the general population, thereby, rendering antibiotic treatment ineffective (Dewdney et al., 1991; Curric et al., 1998).

Food safety and inspection services are responsible for ensuring that inspected meat and meat products are safe, wholesome, free from adulterating residues, and to prevent marketing of animals containing unacceptable residues from antibiotics, drugs, pesticides or other potentially hazardous chemicals (Hubbert et al, 1996).

Tetracyclines (tetracycline, oxytetracycline, and chlortetracycline) are broad-spectrum antibiotics used for their bactericidal action in veterinary and human medicine. These drugs treat many kinds of infections of the skin, bone, gastrointestinal tract, respiratory tract, sinuses, ear, and urinary tract. In chickens, the drugs are used especially for controlling chronic respiratory disease and air sac disease caused by mycoplasma (Calnek et al., 1991).

The European Union maximum residue limits set for oxytetracycline are 100µg/kg in muscle tissue, 300 µg/kg in liver, and 600 µg/kg kidney (Capolongo et al., 2002). In two experiments (Drain, 1962; Gingher, 1980), chickens adminstered 220 mg/kg chlortetracycline in the feed showed residue levels of 0.66 and 0.71 mg/kg in the liver and 0.42 and 0.75 mg/kg in kidney at day 0 after withdrawal of medication. Presalughter withdrawal days for chlortetracycline and oxytetracycline was found to be one and five days respectively.

Detection techniques of tetracyclines in tissue include microbiological inhibition method using Bacillus cereus, Bacillus subtilis, Micrococcus luteus, E.coli (Okerman et al., 2001, 2004; Van Nhiem et al., 2006), and immunochemical methods by using ELISA technique (Lee et al., 2001), receptor technique (in a commercially available test kit "Charm, Sciences, Inc., Malden, MA"; this technique is based on a competitive radioimmunoassay between the target tetracycline and 3H-tetracycline using antibodies bound to microbial receptors which are specific only for tetracyclines (Well, 1996). Microbiological assays, despite being a cost effective method to monitor antibiotic residue they are time consuming while the biochemical methods are more expensive. Other methods were also used including thin layer chromatography, high performance liquid chromatography, and gas chromatography and mass spectrometric procedure (Blanchflower,et al., 1989; Mulders and Van de Lagemaat,1989; Blanchflower et al., 1997; DeRuyck et al., 1999; Pena et al., 1999; Capolongo et al., 2002; Cherlet et al., 2003; Croubels et al., 1994 ; Heller et al., 2006).

Tetracyclines bind to free calcium in newly deposited bone, but not to calcium in the hydroxya-

patite crystals of mature bone. Incorporated tetracycline into bones give fluorescence under UV light microscopy. Tetracycline labeling is one useful method for analyzing the growth and remodeling of bone (Milch et al.1958).

In the present work, we used this phenomenon, namely, visualization of bound tetracyclines under UV-light to study their distribution in undecalcified bones and soft tissue and to correlate this with the withdrawal time.

MATERIAL AND METHODS

Experimental animals:

A number of 15 broilers, each of 1 kg an average body weight, were used in the present experiment. The broilers were classified into 2 groups. The first group (treated group) contains 10 birds and the second group (control non-treated group) contains 5 birds.

Drug and route of administration:

Tetracycline hydrochloride capsules (Tetracid, Chemical Industries Development, (CID), Gaza, EGYPT.) were used. Birds in the first group were given the drug intraperitoneally each with 10 mg of oxytetracycline in 1 ml distilled water; while, birds in the second group kept untreated. Tow treated birds and one untreated bird were slaughtered together at 1, 3, 6, 9 and 12 days after administration of the drug.

Sampling:

After slaughtering, tissue specimens from the liver and kidneys were taken for preparation of cryostat sections; the rest was fixed in 10 % formalin solution. One tibia was used for preparation of un-decalcified bone sections while the other was fixed in 10 % formalin solution. Bone sections were prepared from sagittal sections at the level of the proximal metaphyseal plate and cross sections from sagittal sections at the level of the proximal metaphyseal plate and cross sections from mid-diaphysis. Bone sections were firstly thinned by grinding with coarse and fine carborundum abrasive-paper to an approximate thickness of 100 μ before being examined by UV-microscope ("American Optics" with descendant light). The distribution of fluorescence was examined and photographed. Digital microphotographs were prepared from all specimens. Images were converted to gray-scale before analysis. Using software "Image J", the intensity of cartilaginous layers of the metaphyseal plate (layer of proliferating cartilaginous and hypertrophic cartilaginous cells) as well as the fluorescent front line at the level of the provisional calcification layer thickness of osteoid seams in bone trabeculae of the primary and secondary spongiosa, and appositional bone growth at the periosteal and endosteal surfaces of the diaphysis in pixels were compared

RESULTS

One day after withdrawal of tetracycline, the fluorochrome was seen as a thin narrow line having a yellowish fluorescence at the inner surface of the Haversian canals in cross section of the tibia and at the line of demarcation of active deposition sites of calcium salts in the appositional surface of the compact bone of the shaft. In bone cortex, some lacunar surface, canalicular walls, and walls of the vascular channels showed yellowish fluorescence (Fig. 1). Similar findings were also seen at the metaphysis occupying the layer of provisional calcification and lamellae of the spongy cancellous bone of the metaphyseal plate (Fig. 2 &fig.3). There was no visually detectable fluorescent tetracycline in the bulk of osteoid seams or bone cortex. In contrast, the later showed relatively intense auto fluorescence especially in ground bone cortex. Bones from birds slaughtered later (2 to 6 days) revealed a shift of the fluorescent band at the sites described above interiorly. There was no increase in width or distribution of fluorescence one day of withdrawal.

The longer the period of time between termination of tetracycline and sampling, the wider were the bands of unlabelled bone interposed between osteoid seams and the labeled band. Areas of nonspecific fluorescence in bone matrix faded 2 days of withdrawal. Formalin solution 10 %, but not freezing, quenched fluorescence by approximately 25 %. Soft tissue, especially the liver and kidneys revealed a faint yellowish fluorescence at the wall of blood vessels one day of withdrawal but not later.

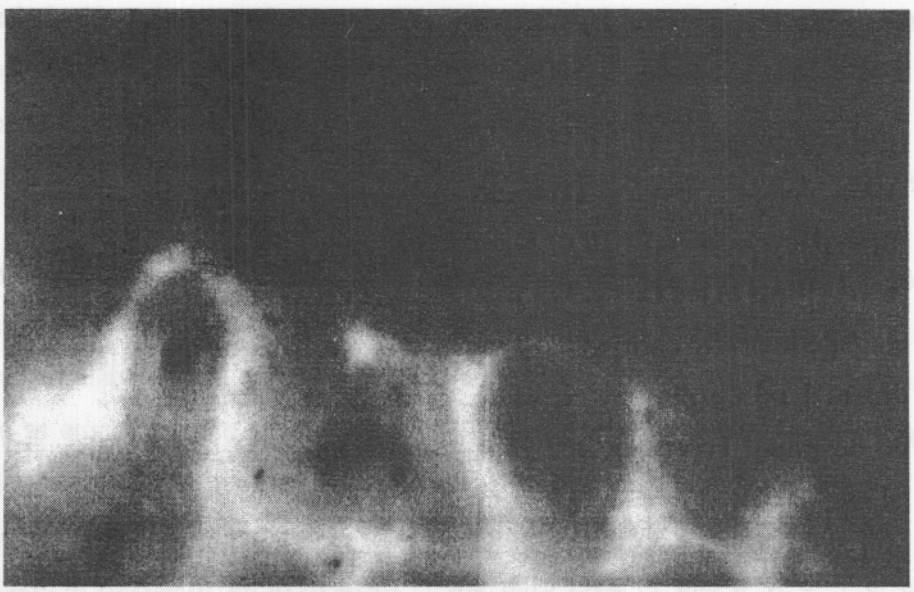

Fig. 1. Bone cortex of the shaft showing fluorescent bands along the lacunar surface, canalicular walls and vascular channels (UV, X 400)

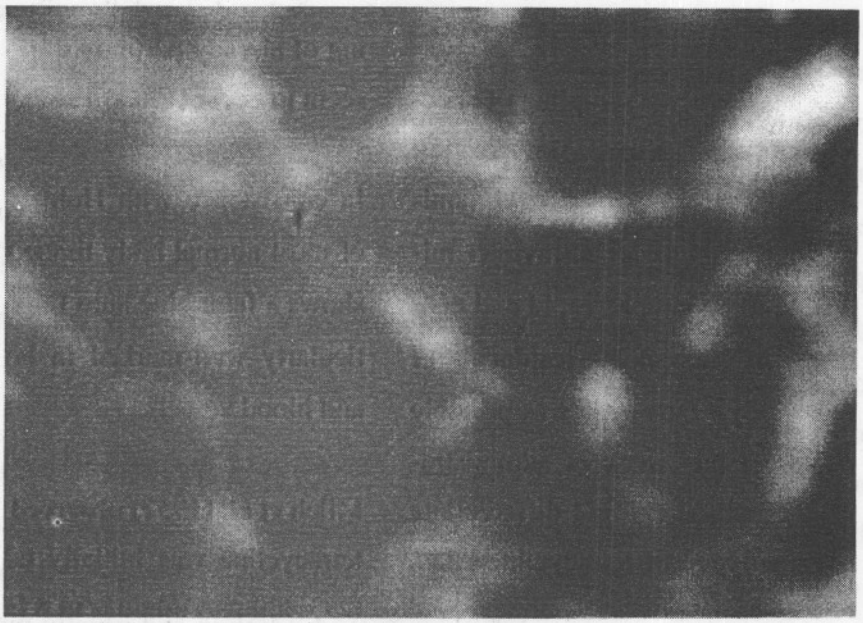

Fig. 2. Metaphysis showing fluorescence of the cancellous bone lamellae (UV, X400)

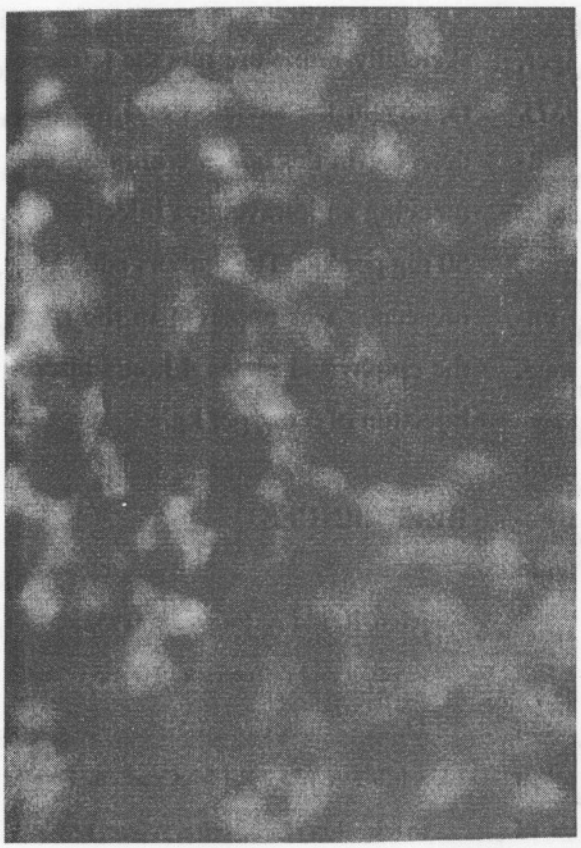

Fig. 3.Metaphysis showing fluorescence of the metaphyseal plate (UV, X 200)

DISCUSSION

Monitoring of residues involves sampling of specified animal population to provide information about the occurrence of residue violation. Examination of bones from birds under ultraviolet microscope was proved to be simple, rapid and economical method to monitor residues of tetracyclines in the body. Detection was possible for a period of at least one week of administration. However, this method can not differentiate between the different types of tetracyclines, i.e., tetracycline, oxytetracycline, chlortetracycline. Storage of bone at 4°C did not appear to influence specific tetracycline fluorescence.

Fixation of tetracycline antibiotics in living tissue has been known for years (Helonder and Bottiger, 1953; Milch et al., 1957, 1958, Rall et al., 1957). Most of the tetracyclines bind with newly deposited calcium salts in bone is permanent and remains in situ until the labeled bone is resorbed (Frost, 1962). Microscopical examination of native undecalcified bone, either compact or spongy bone, may be a tool with which antibiotic treatment residue can be successfully identified and monitored. The correspondence in expansion and time between the site of initial mineralization and osteoid seams.

Amprino and Engstrom (1952) noted that about 70 % of all the mineral ever deposited in lamellar bone matrix is laid down in the initial 4 days of mineralization in the zone of demarcation. Labeling of already partially mineralized bone does not seem to occur (Frost, 1962).

In consistence with Holmes (1963), examination of most normal body tissue under ultraviolet light shows a faint blue auto fluorescence which is particularly well-marked in bone, cartilage, tendon and blood vessels.

Milch et al. (1957) observed that in the healthy rat tetracycline was initially taken up by all tissues but within 30 minutes to 6 hours of an intraperitoneal injection and usually within 12 hours, it was lost by soft tissue and the induced fluorescence was then confined to the bones and teeth where it was persisted for at least 10 weeks. In young skeletally immature rats the tetracycline appeared rapidly in the skeleton and the fluorescence was detected in the bones within 30 seconds of commencing an intravenous injection at a dose rate of 50 mg per kg. The authors added that striking fluorescence was apparent in the metaphyses under the epiphyseal plates 4 hours after intraperitoneal injection of 5 mg per kg.

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رصد وتوزيع متبقيات مركبات التيتراسيكلين فى العظام والأنسجه الداخليه لدجاج التسمين

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يمثل وجود بعض المضادات الحيويه فى اللحوم والمنتجات الحيوانيه خطرا كامنا على صحة المستهلكين وربما يحدث أعراض حساسيه أو يتداخل مع البكتريا المعويه، وتكاثر البكتريا المقاومه للمضادات الحيويه، قد يجعل العلاج بالمضادات الحيويه غير ذى جدوى. وقد أجريت هذه التجربه لدراسة العلاقه بين توزيع متبقيات مركبات التيتراسيكلين فى العظام والأنسجه الرخوه، عن طريق فحص وميض مركبات التيتراسيكلين باستخدام الميكروسكوب الفلورسنتى ، والمدة اللازمه لأنسحاب هذه المركبات من الجسم . وقد أجريت دراسه تحليليه قياسيه نسيجيه شبه كميه لقياس وميض مركبات التيتراسيكلين فى الأنسجه الداخليه وعظمة الساق.

ولهذه الدراسه استخدمت ١٥ دجاجه تزن كل منه حوالى كيلوجراما واحدا ، قسمت الى مجموعتين المجموعه الأولى (المعالجه) بها ١٠ دجاجات تم حقنها ب ١٠ ملج من مركب التيتراسيكلين عن طريق البريتون، وتركت المجموعه التانيه (الضابطه) بها ٥ دجاجات دون أى علاج. وقد تم ذبح دجاجتان من المجموعه الأولى ودجاجه واحده من المجموعه الضابطه بعد ١، ٣، ٦ ، ٩ و١٢ يوما من حقن الدواء. وقد أخذت مقاطع نسيجيه على الميكروتوم الثلجى من الكلى والكبد.

أما عظمتى الساق اليمنى واليسرى ثبتت احداها فى محلول الفورمالين ١٠% وتركت الأخرى دون تثبيت لتحضير مقاطع من عظمتى الساق دون نزع الكالسيوم ،وقد أخذ مقطعا طوليا عند رأس العظمه وأخر عرضيا عند جسم العظمه. كل المقاطع من الأنسجه الداخليه والعظام تم فحصها تحت الميكروسكوب الفلورسنتى. وباستخدام الكومبيوتر تم تحليل صور الميكروسكوب الفلورسنتى التى تم التقاطها لرأس وجسم الساق وأخذت القياسات لتحديد العلاقه نسبة الوميض الفلورسنتى للدواء ومدى انسحابه من الجسم.