In growing pigs, chlortetracycline induces a reversible green bone discolaration and a persistent increase of bone mineral density dependent of dosing regimen

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1. Introduction

Green bone discoloration of pig carcasses is an increasing concern. The use of tetracyclines (TC) had intensified following the emergence of a new genotype of type 2 porcine Circovirus in Canada (Carman et al., 2008), which induces a severe immunosuppressive disease (Segalès et al., 2006) and increases the risk of bacterial infections in 2 to 4 month-old pigs (Gagnon et al., 2008). Tetracycline-induced bone discoloration caused considerable income loss to pig producers, as carcasses were boned to preserve their wholesomeness (Kühne et al., 2000). Indeed, TC residues in bones may be released during digestion (Kühne and Körner, 2001) and afterwards select intestinal antibiotic-resistant bacteria (Kühne and Körner, 2001; Perrin-Guyomard et al., 2001).

The ability of TC to accumulate in bone is well described (Buyske et al., 1960). Tetracyclines, which form colored chelation complexes with metal ions (Albert and Rees, 1956), specifically bind to the calcium ions of bone (Ibsen and Urist, 1961), particularly in new bone (Ibsen, 1985). Moreover, TC decrease bone resorption by inhibition of osteoclasts through direct (inhibition of mature osteoclast production, and apoptosis) and indirect (inhibition of osteoclast enzymes) mechanisms (Bettany et al., 2000; Rifkin et al., 1994).

As TC molecules are released during the process of bone resorption (Milch et al., 1958), we hypothesize that bone discoloration in pigs is reversible, once dietary intake of TC ceases. Moreover, considering that TC inhibit osteoclastic activity, we additionally hypothesize that the use of TC-fortified diets affects bone mineral density (BMD). The objectives of this study were first to evaluate in growing pigs the effects of age at TC treatment onset and dosing duration on bone color and BMD, and second to assess the ability of a quantitative computed tomography (qCT) technique to evaluate the effects of TC on trabecular BMD. It was expected that both bone discoloration and BMD would increase with longer treatment duration and increased age at treatment onset. Chlortetracycline (CTC) was used as the model TC.

2. Materials and methods

2.1. Experimental design

The experimental protocol followed in this study was approved by the bioethical committee of Agriculture and Agri-Food Canada and cared for according to a recommended code of practice (Agriculture and Agri-Food Canada, 1993) and to the guidelines of the Canadian Council on Animal Care (Olfert et al., 1993).

A total of 112 weaned castrated crossbred male piglets with no previous exposure to TC were used. Pigs entered the research facility (Agriculture and Agri-Food Canada, Sherbrooke, Que, Canada) at a mean...
age of 3.8 week (SD = 0.4). Feeds and water were supplied ad libitum during the whole study. Four basic diets were used: nursery-1 (4- to 6-week of age), nursery-2 (6- to 8-week of age), growing (8- to 20-week of age); and finishing (20- to 24-week of age). Control pigs (n = 48) were fed the above mentioned drug-free diets during the whole experiment. Medicated pigs were fed the same diets, but fortified with 800 ppm of CTC hydrochloride (Aureomycin®, 220 G Premix, Alpharma Canada Corporation, Mississauga, ON). Dietary CTC was given starting either at 28- or 84-d of age, and for either a 28 or 56-d duration (coded CTC 28_28, 28_56, 84_28, and 84_56; n = 16 pigs/group). A detailed description of the randomization has been described elsewhere (Guillot et al., 2009), and is depicted in Fig. 1.

2.2. Weighing and euthanasia

At each assessment point, all pigs were weighed. The ones selected to undergo evaluation were sedated with intramuscular azaperone (2–4 mg/kg; Stresnil injection, Merial Canada Inc., Baie d’Urfée, QC), following which they were moved to a quiet room where they received pharmacological euthanasia with an intravenous overdose of sodium pentobarbital (>4 mg/kg; Euthanyl®, Bimeda-MTC Animal Health Inc., distributed by Vétoquinol Canada Inc., Lavaltrie, QC).

2.3. Imaging procedures

Before dissection, control and medicated pigs from D28, D84, D140, and D168 were subject to postmortem dual energy X-ray absorptiometry examination of the lumbar vertebrae, the procedures and results of which has been presented in a separate communication (Guillot et al., 2009). Then, the carcasses were subject to quantitative computed tomography (qCT) examination of the lumbar vertebrae for estimation of trabecular bone mineral density (BMD). Shortly, animals were placed on the CT table (CT Hi speed ZX/i, GE Healthcare Canada, Saint-Laurent, QC) in dorsal recumbency, overlying a calibration phantom made of five cylindrical reference rods (Model 3 CT Calibration Phantom, Mindways Software, Inc., San Francisco, CA) parallel to the pig’s longitudinal axis. The qCT scan settings were the following: X-ray tube set at 120 kV and 100 mA, helical mode, pitch of one with a tube rotation time of 1 s, 1 mm slice thickness, 40 cm field of view, 512 × 512 pixel matrix, and bone algorithm. Both L1 and L2 trabecular BMD was estimated using BMD analytical software (QCT PRO™ Bone Investigational Toolkit, version 2.1, Mindways Software Inc., Austin, TX) with the evaluator unaware of the pigs’ treatment status (Guillot et al., 2009).

2.4. Bone discoloration measurements

Finally, following imaging, L1 and L2 vertebrae were manually dissected, stored at −20 °C pending evaluation of bone discoloration, and slightly heated to enable complete manual dissection. Then, green bone discoloration was semi-quantitatively scored as 0 (none), 1 (trace), 2 (light), 3 (medium), or 4 (dark) under blind conditions.

2.5. Lumbar vertebra preparation for physical BMD determination

All L1 and L2 vertebrae were cleaned in 100 °C water bath, degassed in chloroform, and heat dried. Physical BMD was determined first on the vertebral bodies using the Archimedes' principle. All bone specimens were degassed in an ultrasonic distilled water bath. Each specimen was then weighed in air and in distilled water to the nearest 0.1 mg with the electronic scale fitted with a density

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**Fig. 1.** Diagram illustrating time-course of experiment, group sizes, treated groups and assessment points. E = euthanasia; Imaging = dual energy X-ray absorptiometric and quantitative computed tomographic assessment of bone mineral density (BMD) in first (L1) and second (L2) lumbar vertebrae; Dissection = dissection of L1 and L2 and measurement of vertebral body and trabecular BMD.
formed at the 0.05
is its weight in air after degassing, and \( W_{b(a/degas)} \)
where \( W_{b(a/dry)} \) is the dry weight of the bone sample, \( W_{b(w)} \) is its weight in water after degassing, and \( W_{b(w)} \) is its weight in water (Galante et al., 1970).

A 16 mm diameter, rod-shaped trabecular bone sample was cut from the center of each vertebral body, parallel to the long axis. The cortical bone at each base of the rods was removed, and the trabecular bone cores were placed in the agitated chloroform bath, dried at 80 °C, and weighed. Physical BMD measurement of these rods was performed as described above.

2.6. Statistical analysis

The descriptive statistics of body weight (BW) data from control and medicated pigs were examined to assess departure from the normal distribution, after which two-sample \( t \)-tests were performed at the 0.05 \( \alpha \)-level to compare the mean BW between control and treated pigs (at treatment onset) and between treatment duration groups (at the time of switch back to the drug-free feed).

Logistic regression analysis for ordinal data (McCullagh, 1980) was performed to examine the effects of treatment, age at the treatment onset, dosing duration, withdrawal time, BMD, BW, pig age, and feed efficiency on the odds of a bone discoloration equal to 0 (reference value). The statistical significance of the above predictors, their associated polynomials, and all their possible dual interactions was tested with a stepwise-forward algorithm, with a threshold of \( p = 0.15 \) for including these factors in the model, and a threshold of \( p = 0.20 \) for their removal (Hosmer and Lemeshow, 1989). The likelihood ratio test, and the score test for the proportional odds assumption were examined at each step to assess the significance of the newly added predictor (or of the newly reduced model), and the validity of the proportional odds assumption for the response variable (Brant, 1990). Furthermore, the best model was selected based on optimization of the association between predicted probabilities and observed response (Ananth and Kleinbaum, 1997). The odds ratio (OR) and 95% confidence intervals (95% CI) of an imperceptible bone discoloration (i.e. score = 0) were calculated using standard formulae (Hosmer and Lemeshow, 1989).

In addition, regression models tested the following variables to assess their effects on BMD (physical and qCT-estimated): BW, age, age at treatment onset, treatment duration, withdrawal time and feed efficiency, plus physical BMD (qCT-estimated model 2). All these regression analyses were performed with stepwise-forward procedures, using a threshold for entry and for maintenance in the model of \( p = 0.15 \) and \( p = 0.20 \), respectively, and with backward procedures with a threshold of \( p = 0.10 \) for stay in the model (Kutner et al., 2004). A thorough residual analysis was performed for each model, and predictor variables showing clear non-linear relationships with the response variables were mathematically transformed to improve regression fit. In addition, the effect of dual interactions of the predictors was tested. The final model was selected by optimizing the scatter of residuals over the regression line, coefficient of determination \( (R^2) \) and Mallow’s \( C_p \) criterion (Kutner et al., 2004). All statistical analyses were performed with a statistical software program (SAS system for Windows, 9.2 release, Cary, NC, USA).

3. Results

At D28 and D84, the control pigs and the ones starting a course of dietary CTC had similar BW (\( p > 0.4 \)). The same findings were recorded at the D56 and D112 (long-term medication startpoint), at which the 28 day-medicated pigs and pigs continuing medication up to 56 days had similar BW. Furthermore, all pairs of groups compared had similar ranges of BW data.

An outbreak of intestinal salmonellosis occurred between D140 and D168, causing two deaths and 10 affected pigs. Clinically affected pigs (i.e. with diarrhea, fever, or both) were excluded from the experiment to avoid possible bias associated with the use of parenteral antibiotic therapy. In addition, biosecurity regulations preempted further admission of whole pig cadavers for qCT examination. Therefore, the lumbar region of the D140 and D168 pig cadavers was resected, placed in hermetic bags, and chemically sterilized before submission to the diagnostic imaging service for ex vivo qCT examination, applying the same protocol as already described.

3.1. Bone discoloration

No discoloration was noticed in control pigs. In contrast, the discoloration score was uniform among pigs within a given

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Fig. 2. Probability of obtaining a discoloration score of 0 on the second lumbar vertebra as a function of withdrawal time and CTC treatment duration. (A) Age at the treatment onset of 28 d; (B) Age at the treatment onset of 84 d; color 0, 1 and 2 = none, trace or poor, mild or strong bone green discoloration respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
medication group at treatment completion: 2 for CTC 28_28; 3 for CTC 28_56, and CTC 84_28; and 4 for CTC 84_56. But at D168, differences in discoloration score were noted within treatment groups. The CTC 28_28 pigs had scores of 0 (5 of 6 animals) or 1. The CTC 28_56 had scores of 0 (3 of 6 animals), 1, or 2 (6 of 6 animals). The CTC 84_28 pigs had scores of 0 (1 of 6 animals), 1 (4 of 6 animals), or 2. Finally, equal numbers of the six CTC 84_56 pigs had scores of 3 and 4. The discoloration was limited to bone tissue and was more intense in cortical bone.

To comply with the proportional odds assumption, the original bone discoloration scoring system had to be reduced into three independent categories: 0 = no discoloration; 1 = traces or light bone discoloration; 2 = moderate or dark green discoloration. Fig. 2 delineates the probabilities of obtaining a discoloration score of 0 on second lumbar vertebra, with respect to a score of 1 and to a score of 2. The odds of losing a 0-score (i.e. becoming discolored) increased with increasing dosing duration (OR = 1.151; 95% CI = [1.070–1.239]; p < 0.0001) and the odds of keeping a 0-score increased with the interaction of withdrawal time and age at treatment onset (OR = 1.005; 95% CI = [1.000–1.010]; p = 0.0001). The intensity of bone discoloration was uneven on a given vertebra, which reflects the heterogeneity of bone turnover (Shea and Miller, 2005; Stepenksy et al., 2003). Indeed, a more intense discoloration was noted on the cortical bone, where bone lamellae are more dense as compared to those of the trabecular bone (Shea and Miller, 2005). Besides, discoloration did not occur on the joints or growth cartilage, which are known to be ineffective binding sites for the TC (Milch et al., 1957).

The probability of green bone discoloration was higher with increasing treatment duration, which is consistent with the cumulative deposition of TC in bone (Buyske et al., 1960). This deposition occurs during the processes of bone modeling and remodeling, where the drug molecules are fixed on the newly formed bone by chelating with calcium (Milch et al., 1957, 1958; Milch et al., 2005). At the end of the course of dietary CTC, for identical treatment duration, bone discoloration was darker in older pigs, which may be related to the increase of available chelating sites due to higher bone mass and volume. Besides, similar levels of discoloration were observed among pigs within a given medicated group after terminating the course of dietary CTC, which may result from saturation of bone TC deposition sites. This view is challenged by the loss of drug that parallels bone resorption (Klein and Jackman, 1976; Milch et al., 1958; Stepenksy et al., 2003), a process that uncouples the cation–TC complexes. Hence, it is more likely that this within-group homogeneity in bone discoloration be explained by a dynamic equilibrium between drug deposition and removal. The acidic osteoclastic secretion at the site of bone resorption hinders the ability of released TC molecules to complex anew with the multivalent cations in bone (Jin et al., 2007), which would favor their redistribution to the bloodstream. A study using several dosages of TC may allow confirmation of these hypotheses.

Table 1
Predictors retained in the final linear regression models of the measured or calculated bone mineral density for the second lumbar vertebra, statistical significance, and contribution to the global determination coefficient.a

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Predictor</th>
<th>Estimate</th>
<th>SE</th>
<th>p</th>
<th>Cum. R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>tBMD (g/cm^3)</td>
<td>Intercept</td>
<td>0.3445</td>
<td>0.0114</td>
<td>&lt;0.0001</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Age (d)</td>
<td>0.0008*</td>
<td>1.0 x 10^-4</td>
<td>&lt;0.0001</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Square treatment duration × Age (d^2)</td>
<td>6.9 x 10^-6</td>
<td>2.5 x 10^-8</td>
<td>0.0076</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>0.2101</td>
<td>0.0071</td>
<td>&lt;0.0001</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Square age (d^2)</td>
<td>2.9 x 10^-6</td>
<td>4.1 x 10^-7</td>
<td>&lt;0.0001</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Age at the treatment onset × age (d^2)</td>
<td>1.7 x 10^-6</td>
<td>7.7 x 10^-7</td>
<td>0.0318</td>
<td>0.64</td>
</tr>
<tr>
<td>qBMD (g/cm^3) eq K2HPO4</td>
<td>Intercept</td>
<td>0.4250</td>
<td>0.0122</td>
<td>&lt;0.0001</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BW (kg)</td>
<td>0.0016</td>
<td>0.0004</td>
<td>&lt;0.0001</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Square BW (kg^2)</td>
<td>-5.3 x 10^-6</td>
<td>2.3 x 10^-6</td>
<td>0.0342</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>BW (kg)</td>
<td>0.0016</td>
<td>0.0004</td>
<td>&lt;0.0001</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Square age at the treatment onset (d^2)</td>
<td>2.3 x 10^-6</td>
<td>1.4 x 10^-6</td>
<td>0.0072</td>
<td>0.54</td>
</tr>
<tr>
<td>bBMD, model 1 (g/cm^3)</td>
<td>Intercept</td>
<td>0.1454</td>
<td>0.0192</td>
<td>&lt;0.0001</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>tBMD (g/cm^3)</td>
<td>0.6986</td>
<td>0.0557</td>
<td>&lt;0.0001</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Natural logarithm of BW (kg)</td>
<td>0.0151</td>
<td>0.0040</td>
<td>&lt;0.0001</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Treatment duration × withdrawal time (d^2)</td>
<td>-5.8 x 10^-6</td>
<td>2.1 x 10^-6</td>
<td>0.0065</td>
<td>0.81</td>
</tr>
</tbody>
</table>

a Cum. R² = cumulative coefficient of determination; tBMD = measured trabecular bone mineral density; qBMD = qCT-estimated trabecular bone mineral density; bBMD = measured bone mineral density of the vertebral body.
The TC-induced bone discoloration reversed itself when a sufficient withdrawal time was given, and could entirely vanish in 8 weeks. This finding is consistent with removal through bone resorption (Shea and Miller, 2005; Stepensky et al., 2003), which is a perpetual process (Vaughan, 1981a). Only few studies have focused on the pharmacokinetics and persistence of TC in bones. Following a single dose, a small fraction of TC molecules are retained in the bone of rats (Buyske et al., 1960), dogs (Kelly and Buyske, 1960), and pigs (Black and Gentry, 1984), and persist for several weeks in rats and dogs (Buyske et al., 1960; Kelly and Buyske, 1960). A two-compartment model was proposed in rats to differentiate the elimination speeds of TC, occurring within 1 week from a fast compartment, but occurring over 2 months from a slow compartment representing bone resorption (DeMoss and Wright, 1997). A one-compartment model has also been proposed, where TC had already been excreted from the body but, and under steady state condition, each bone represented an individual compartment, from which TC was considered irreversibly eliminated (Li et al., 1989).

The discoloration disappeared at a slower rate when the age at treatment onset increased, in agreement with the age-related decrease in bone turnover rate (Li et al., 1989). Although a similar discoloration score was observed for each pig within a given medicated group immediately at the end of its treatment course, a large within-group variation in discoloration score was noted at D168. This variable discoloration is likely related to differences in bone remodeling rate among animals, which is affected by several factors such as aging, mechanical constraints including exercise, underlying disease, endocrine factors and local factors (Allen, 2003; Boyde, 2003; Shea and Miller, 2005).

Interestingly, the OR reported in our study are identical to the risk ratio, as the prevalence of a positive discoloration in unexposed cases was null (Zhang and Yu, 1998). For example, as compared with controls, medicated pigs were 1.2 times more at risk of presenting a TC bone discoloration per additional day of age at treatment onset. This risk of bone discoloration was similar for medicated pigs per increment of increased treatment duration.
Trabecular bone, because its turnover rate is higher than that of cortical bone (Vaughan, 1981a), is a valuable site for studying bone metabolism (Akahoshi et al., 2005). The vertebrae, whose trabecular bone benefits from the high vascular supply of its surrounding red marrow, have a high rate of metabolic activity (Li et al., 1999, Shea and Miller, 2005). Therefore, vertebrae were considered the bones of choice to study both accumulation of TC on bone and BMD. Lumbar, as compared with thoracic vertebrae, are commonly used in live studies assessing BMD by qCT due to the minimal impact of respiratory movement (Cann, 1988; Genant, 1985; Steiger et al., 1990). Even if our study was postmortem, we wanted a model that could be used in vivo. Furthermore, samples of trabecular bone of the lumbar vertebrae are also easily obtained. Finally, the trabecular BMD of human L1 and L2 accurately predict those of the entire vertebral body (Cann, 1989; Van Rijn et al., 2003). The successful validation of an increase in trabecular BMD of CTC-medicated pigs by qCT suggests the validity of this technique as a non-invasive tool for assessing the effects of the exogenous factors that potentially affect bone metabolism. A detailed discussion about qCT validation, use and limitations in this study was presented in a separate communication (Guillot et al., 2009).

To conclude, the present study results suggest that a proper dosing regimen design may prevent TC-induced bone discoloration. Practically, the risk of bone discoloration may be minimized by avoiding the continuous use of therapeutic doses of CT for more than 4 weeks, and by limiting its use to pigs at the nursery stage. Bone TC residues are often found in piglet and finishing pig carcasses during deboning or transformation (Kühne et al., 2000), and this is considered a surrogate of antibiotic contamination (Kühne and Körner, 2001). However, further investigations on pharmacokinetics and residue concentration of TC in bone are needed to properly assess the human health risk of consuming bones with green discoloration. We also demonstrated a persistent increase of BMD related to TC medication, which could be assessed with qCT. Therefore, qCT may be a useful non-invasive tool to study TC accumulation in bone.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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