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INSTRUCTIONS TO AUTHORS

The Thai Journal of Veterinary Medicine publishes articles reporting interdisciplinary investigations concerning veterinary and animal sciences, at all levels of resolution, from basic to clinical, molecular to behavioral, and opinions that are of general interest to the broad community of veterinarians and biological scientists. Clinical or pathological investigations, protocols and reviews will also be considered for publication if they provide significant insight into the structure or function, the pathophysiology of a disease, or its treatment.

In the Journal’s Table of Contents, published articles will be shown under one of the appropriate Section titles listed below.

SECTIONS

*Editorials* A limited amount of space will be available for comments about important scientific points or subjects of topical interest, and will be by invitation only.

*Reviews and Minireviews* will be either by invitation, or submission. The latter will be reviewed by experts in the same manner as other submitted manuscripts.

*Original Articles* should be novel research findings and provide strong evidence for the conclusions. The manuscripts suitable for publication in TJVM should be of extreme importance to scientists in the field as well as interesting to researchers in other disciplines.

*Rapid or Short Communications* These are short communications that describe outstanding new discoveries. This decision will be based on whether the paper reports particularly important findings that are likely to have a high impact in the field of work.

*Clinical or Pathological Reports* These are short reports of original clinical or pathological findings whose importance mean that they will be of interest to veterinarians.

*Diagnostic Forum* is a regular feature of TJVM. This includes Ultrasound Diagnosis, ECG Quiz, Ophthalmology Snapshot and What Is Your Diagnosis, all of which will be by invitation only.

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All manuscripts must be typewritten using TIMES font at 12 point, with double-spacing throughout and with margins at least 2.5 cm wide. Pages should be numbered in succession, the title page being no. 1. Text files should be supplied in Windows Microsoft Word or Word Perfect formats.
Each manuscript should be accompanied by a signed cover letter in which the corresponding Author states: “The work described has not been submitted elsewhere for publication, in whole or in part”. All submissions to TJVM must contain experiments that conform to the ethical standards issued by the National Research Council. If the studies deal with animal experiments, the authors certify in the Materials and Methods section, that the procedures have been approved by the Authors’ Institution’s Ethic Committee, and care was taken to minimize the number of animals used. If the ethical standard governing the reported research is different from those guidelines indicated above, the authors must provide information in the submission cover letter about which guidelines and oversight procedures were followed.

Authors should only use abbreviations sparingly and should always define the abbreviation when first used in the text by placing it in parentheses after the full term. The abbreviations should then be used consistently thereafter and appear at least twice in the text. Drug names should be the official or approved names; trade names or common names may be given where the drug is first mentioned. Trade names should be capitalised and the manufacturer’s name and country given in parenthesis thereafter. The doses of the drugs should be given as unit weight/unit body weight, e.g. mmol/kg or mg/kg. Symbols for physical units should be restricted to the Systems Internationale (S.I.) Units.

Where possible, Authors should also include a list of three or more potential reviewers for their manuscript, with contact information.

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1) Title page
2) Abstract
3) Text
4) Acknowledgments
5) References
6) Tables
7) Figures legends
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Start each subdivision on a new page.

Title Page. The first page of the manuscript should include:
- Title of paper
- Full name of author(s)
- Institutional affiliations and complete mailing address
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Abstract. Submit an abstract of around 250 words that will serve in lieu of a concluding summary. The abstract must be written in complete sentences and succinctly state the objectives, experimental design of the paper, principal observations, and conclusions; it should be intelligible without reference to the rest of the paper.

Key Words. Four to six key words should be included.

Text.
- Original articles: The text should be presented in the following order: INTRODUCTION; MATERIALS AND METHODS; RESULTS; DISCUSSION.
  (i) Introduction. This should provide the scientific rationale for the research that is reported. No results should be presented.
  (ii) Materials and Methods. Procedures used in the research should be described in sufficient detail to permit the replication of the work by others. Previously published procedures should be referenced and briefly summarised. The source of all materials, including animals and human tissue, must be provided.
  (iii) Results. This section presents findings without discussion of their significance. Subsections should be used in order to present results in an organised fashion. The findings may be assisted by high quality illustrations, as necessary, to adequately document the work. Figures should be referred to in the text as Fig.1, Figs. 1, 3-4, etc., and tables as Table 1, Table 1, 3-4, etc.
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exception that results and discussion are combined.

- **Reviews**: Reviews should have an introductory section, followed by several information presentation sections and end with a conclusion section. Section headings should be used to organise the presentation of information.

**Acknowledgements.** It is the corresponding Author’s responsibility to ensure that individuals who are acknowledged for assistance or for providing comments on the manuscript are agreeable to being acknowledged in this way.

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(a) In the text, references should be quoted as the name of the first author and year in chronological order. Multiple authors are indicated by “et al.”, except when there are only two authors, in which case both names are written.

*Examples:*

..... (Garthwaite and Garthwaite, 1995; Morris, 2000).

by Nagy et al. (1999a,b).

Clarkson et al. (2004) stated.......

(b) The reference list should be on a separate page at the end of the manuscript, in alphabetical order and arranged as follows: authors’ names and initials, year, title of the article, abbreviated title of the journal, volume, first and last page numbers.

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*Article in a periodical:*


*Chapter in a book:*


*An entire book:*


*Electronic information:*


(c) A paper which has been accepted for publication but has not yet appeared may be cited in the reference list with the abbreviated name of the journal followed by the words “in press”. Avoid using abstracts, theses or dissertations as references. Unpublished observations and personal communication may not be used as references.

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**Figure 1** Typical lesions of...

**Table 1** Statistical analysis showing....

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Role of the Veterinarian on Animal Welfare

Achariya Sailasuta

The importance of animal welfare has been nowadays increasingly recognized by the governments, academic institutions and the general public. The growing of international public concern towards humane practices involving animals. While, the number of animals used in experimentation is declining in some countries, the adoption of humane slaughter methods is also increasing. The improvement in the welfare of companion animals has often lead these general trends. Today the animal products are largely derived from intensive farming systems, which is considered unacceptable on animal welfare. The veterinary profession is privileged to occupy a position of enormous social influence on animal welfare issue which has traditionally understood welfare primarily the scope of animal health and productivity, and the curriculum also has reflected to this approach.

Animal Welfare on Veterinary Education

Animal welfare has been developed into interdisciplinary science and consequently there is a growing amount of researches. However, the teaching of the science and ethics of animal welfare remains underdeveloped in most of the veterinary schools. An OIE (World Organisation for Animal Health) conference on Animal Welfare in 2004, the importance of animal welfare as a discipline has been discussed to be taught in its own right in the undergraduate veterinary curriculum, although animal welfare should also be considered at the clinical level. There are some suggestions from IVSA (International Veterinary Students Association) 2007 workshop that veterinary students should know about the 5 freedoms during lecturers, clinical practicals, farm and clinic visits and training. The communication to the society about animal welfare should be concerned. To encourage the introduction of animal welfare topics into the veterinary curriculum worldwide, the World Society for the Protection of Animals (WSPA) and the University of Bristol, School of Veterinary Science launched their CD ROM-based “Concepts in Animal Welfare” syllabus in 2003. Since then many veterinary institutes have expressed interest in the syllabus. The syllabus is available and has been organized a series of conferences, workshops and presentations for about 400 veterinary faculties all over the world: Argentina, Brazil, Chile, Colombia, Costa Rica, the Czech Republic and other Eastern European countries, India, Indonesia, Japan, Mexico, the Philippines, Cambodia, Vietnam and Thailand. This syllabus is also being promoted for use as a foundation for teaching animal welfare in related courses such as Animal Science, Agricultural Science and other vocational training. The animal welfare syllabus will play an important role in helping veterinarians develop an improved understanding of animal welfare science and issues, not only during their formal veterinary education, but also their entire veterinary career.

Animal Welfare on Veterinary Profession

The Thai Veterinary Medical Association under the Royal Patronage has achieved the overall goals social contribution or to convey assistance to relief problems
on animal welfare aspects in the veterinary profession since 2001. The Veterinary Volunteer Center (VVC). The VVC has 3 working groups according to their responsibility areas as The Working Group on Veterinary Public Relation, Veterinary Contribution to the Community and Veterinary Information Service. This activity distributed a modern knowledge and ethics for the benefit of the veterinary profession on animal welfare. The expectations are delivered to the Thai society are; to provide prompt veterinary advice for animal life saving, to widely distribute veterinary services to the general public, to develop and improve standards of animal raising or farming in the country, to improve of people’s quality of life relating animal health and food safety issues, to promote and enhance veterinary roles for public acknowledgement, to assist, escort and support the animal welfare issues in the country.

Regards to International veterinary associations, the World Veterinary Association (WVA), Commonwealth Veterinary Association (CVA), World Small Animal Veterinary Association (WSAVA), the Federation of European Companion Animal Veterinary Associations (FECAVA), the Federation of Veterinarians of Europe (FVE) and the World Organization for Animal Health (OIE) have all organized major animal welfare conferences in recent years. This suggests that the level of involvement in animal welfare of these organizations and their veterinary professionals is increasing. That the WVA supports the OIE adopted international standards for animal welfare which recognize the need for humane treatment of sentient animals, while ensuring that these standards are science based. That it is important to achieve acceptance worldwide of animal welfare as an issue of common concern and importance. The Federation of Asian Veterinary Associations (FAVA) has accepted the Universal Declaration of Animal Welfare (UDAW) in the 15th FAVA Congress-OIE Joint Symposium in Bangkok, last October 2008. This establishes the importance of this issue for the animal welfare, which would promote the work of the Federation of Asian Veterinary Associations (FAVA), facilitate regional acceptance and application of international standards.

“The veterinary profession is committed to animal welfare and takes very seriously its obligation to society for the professional management and assurance of animal welfare”

References
The Veterinary Volunteer Center, The Thai Veterinary Medical Association (TVMA) Under the Royal Patronage http://www.thaivma.com
Comparative Effects of Zinc Methionylglycinate and Zinc Sulfate on Hair Coat Characteristics and Zinc Concentration in Plasma, Hair, and Stool of Dogs

Uttra Jamikorn* Thanisara Preedapattarapong

Abstract

There are two forms of Zn supplement used in commercial dog foods, organic and inorganic forms. These forms can influence Zn absorption and utilization of the animals. The goal of the current study was to evaluate the effects of zinc methionylglycinate (ZnMG) compared to zinc sulfate (ZnSO₄) supplementations in commercial dog foods on haircoat characteristics, and Zn concentration in plasma, hair, and stool of the dogs. Eight mature female beagles were randomly divided into two groups of four dogs each. A Cross-over design was used for this study. The treatments composed of 120 ppm Zn supplement of either ZnMG or ZnSO₄. A commercial dry dog food formulated with no Zn supplementation (only from raw materials) was used as the basal diet. Each experimental period lasted 5 wk with the first 2 wk as adaptation period and the last 3 wk as time of Zn supplement. Blood samples were collected for the measurement of serum ALP activity and plasma Zn concentration. Hair was shaved and used to analyze for Zn deposition. Haircoat characteristics were determined under electron microscope. The dogs supplemented with ZnMG had greater hair growth rate, level of Zn deposition in hair, serum ALP activity, amount of Zn absorption ($p<0.05$), and plasma Zn concentration ($p<0.10$). The hair of the dogs received ZnMG supplement appeared to be smoother and lesser fragmented than the dogs received ZnSO₄ supplement. In conclusion, the organic Zn as ZnMG was found to be the form that could enhance the haircoat characteristics and suitable for supplementation into the commercial dry dog foods.

Keywords: dogs, haircoat, plasma, stool, zinc.

*Corresponding author
Introduction

Zinc, a micromineral, presents in the body and the diet at level less than 100 ppm (Hellman and Carlson, 2003). It is widely distributed in many tissues of the body (McDonald, 1995). Zinc is important for many metabolic function, necessary for the maintenance and cofactor of metalloenzymes in all six classes (Case et al., 2000; Kidd et al., 1996). Zinc deficiency occur in the dogs from several causes such as genetic defect that results in diminished intestinal Zn absorption, rapidly growing puppies fed Zn-deficient diet or diets containing substances which prevents the absorption and utilization of Zn, dog food that produced from raw materials which have low amount Zn and varies, and raw materials containing an antinutrition factor such as phytic acids. The first clinical signs of Zn deficiency have been described as alopecia, dull, coarse hair coat, and focal erythemia encircle the eyes, ears, nose, mouth and pressure points (Case et al., 2000; Colombini, 1999). Therefore, Zn is added to most of commercial dog foods to meet the animal requirement. Assessment of Zn status in animal is considered complicate. Concentration of Zn in plasma has been denigrated as a measure of zinc status because it responds to metabolic conditions unrelated to zinc status and because it is insensitive to changes in dietary zinc (King, 1990).
There are two forms of Zn supplement used in the commercial dog foods, organic and inorganic forms. These forms can have an influence on the absorption and utilization of Zn. At present, inorganic Zn such as ZnSO₄ or ZnO are the popular forms commonly used in most commercial dog foods. However, these forms can dissociate to Zn²⁺ in the gastrointestinal tract (GIT) and interact with other substances (e.g., phytic acid) resulting in the formation of strong and insoluble complexes that animal cannot absorb (Wilaison, 2002). Moreover, divalent cations (e.g., Ca²⁺, Cu²⁺ and Fe²⁺) can inhibit Zn absorption possibly due to these cations compete one another for binding ligands in the intestinal lumen or within the cell as well as for receptor sites on the brush border of the enterocytes (Gropper et al., 2005). Only organic mineral in chelated form is stable in a wide pH range encountered within the different segments of the digestive tract and so it does not dissociate before reaching the absorption site (Vandergrift, 1994). It can be absorbed as an intact molecule (Ashmead, 1992). An amino acid which is bound to mineral and acts as a carrier is used to transport that mineral across the intestinal enterocyte and into the circulation.

Nowadays, most animal feed industries are interesting in organic Zn including dog foods industries. However, a few research studies on organic Zn supplementation for dog foods are limited. The hypothesis of the present study is that organic Zn could be utilized more efficiently than inorganic Zn regarding it absorbability. Therefore, the dogs supplemented with ZnMG should have better haircoat characteristics, greater Zn concentration in plasma and hair, greater Zn absorption, but lower Zn excretion in stool than the dogs supplemented with ZnSO₄. The objectives of this experiment was to evaluate the effects of ZnMG supplement compared to ZnSO₄ supplement on hair coat characteristics and Zn concentration in plasma, hair, and stool of dogs.

Materials and Methods

Animals: Eight mature female beagles with body weight of 8.7±0.4 kg were used in the study. The Cross-over design was used as an experimental design. All dogs were housed individually in the metal cages (1.0 x 1.2-m) with a plastic slat floor at temperature between 24.9 and 31.4°C. Each cage was cleaned twice daily. In the pretest period, all dogs were fed a basal diet for 2 wk. The purpose of this period was to reduce the variability of Zn stores of the dogs. At the end of this pretest period, four dogs were randomly allotted to one of two treatments. However, dogs receiving the basal diet would be removed from the test if sign of Zn deficiency were severe. The dogs were fed the basal diets with Zn supplement in the form of solution for 3 wk (test period). Fresh water was available ad libitum throughout the experiment.

Feed and feeding: The commercial extruded dog diet formulated with no Zn supplementation in accordance with the AAFCO (2000) nutrient guide for adult dog was used as the basal diet. The calculated values and chemical analysis of nutrient composition in basal diet are presented in Table 1. This basal diet contained Zn at 58.50 ppm DM as composition of raw materials. Treatments composed of Zn supplementation to meet the minimum requirement for maintenance (120 ppm DM) according to AAFCO (2000) either in the form of ZnMG (20% Zn; 2 mol Met-Gly: 1 mol Zn) or ZnSO₄ (35% Zn; ZnSO₄·H₂O). Each form of Zn was prepared as solution by dissolving ZnMG or ZnSO₄ with deionized water. The amount of food was calculated by using standard equations to determine energy requirements of active adult dogs (Case et al., 2000) and adjusted every 2 wk. Everyday food was weighed and divided into two equal portions and fed to the dogs at 0700 and 1530 h in stainless steel bowls. Zinc solution was served at 15 min after offering each meal.
Data collection:

Data collection of both experiment 1 and 2 were similar. Throughout the experiment, BW of dogs was recorded twice weekly for adjusting the amount of food. Food intake was measured daily.

Table 1 Calculated values and chemical analysis of nutrient composition in the basal diet (DM basis).

<table>
<thead>
<tr>
<th>Item</th>
<th>Calculation</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolizable energy (kcal/g)</td>
<td>3.63</td>
<td>3.67*</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>29.64</td>
<td>25.79</td>
</tr>
<tr>
<td>Ether extract (%)</td>
<td>9.04</td>
<td>7.64</td>
</tr>
<tr>
<td>Crude fiber (%)</td>
<td>1.12</td>
<td>0.81</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.21</td>
<td>5.54</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>1.23</td>
<td>0.95</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>1.05</td>
<td>0.80</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>31.33</td>
<td>58.50</td>
</tr>
</tbody>
</table>

*Calculated by use of equation from NRC (2006): ME, kcal/g = [(3.5 x CP) + (8.5 x EE) + (3.5 x NFE)]/100

Sample collection and determination:

Sample collection and determination were as follow:

Food: Throughout the study, the food sample was collected daily and pooled into plastic bags and stored at -20°C until nutrient content analysis. Food sample was ground through a 1-mm screen mill (cyclotec 1093 sample mill). It was analyzed in duplicate for DM, CP, EE, CF, ash, Ca and P using AOAC (1990) procedures. Concentrations of Zn were determined by atomic absorption spectrophotometer (AAS; Model spectr AA - 300) using Sullivan and Carpenter (1993) method.

Blood: At d 14 and d 35, 6 ml of blood was collected from cephalic vein. These collections were performed between 3 and 4 h after offering the meal at 0700 h. Blood samples 5 ml were collected into heparinized polypropylene (PP) tubes and placed on ice then centrifuged at 2000g for 15 mins at 6°C (modified from Brinkhaus et al., 1998). Another 1 ml of blood samples were collected into nonheparinized PP tubes then placed on ice until it was analyzed for serum ALP activity. The separated plasma was stored at -20°C in 5 ml PP tubes until plasma Zn analysis was performed. Plasma was prepared for analysis of Zn concentration by diluting plasma with deionized water (modified from Wedekind et al., 1994) using AAS. Serum ALP activity was determined by automated analyzer (BT2000Plus, Biotecnica Instrument S.p.A.) and test kit Cat. No. AD711AP and AD701AP (Audit diagnostics).

Hair: A patch of similar colored hair was shaved from the dog’s neck using a 20 cm² template on the day before the beginning of the test period (d 14). Thereafter the same patch was shaved on d 35. The hair was handled, collected in plastic bag, weighed and stored at room temperature until Zn analysis was performed. At d 35, chest hair was shaved using a 10 cm² template and collected in plastic bag for analysis of hair condition. Neck hair samples were weighed for determination of hair growth rate (Lowe and Wiseman, 1998) and analyzed for Zn concentrations by AAS using Van den Broek (1988) method. The Zn deposition in hair values were calculated by equation: Zn deposition in hair (μg/21d-20cm²)=[Zn concentration in neck hair sample (μg/g)] x [weight of neck hair sample (μg/21d-20cm²)]. Chest hair samples were screened and photographed under scanning electron microscopy photographic (JSM-5800LV, JEOL) using Kuhlman and Rompala (1998) method.

Stool: All glassware used for Zn analysis was soaked in 10% nitric acid and rinsed (3 times) with deionized water.

From d 25 to d 32, the dogs were dosed orally twice a day, before each feeding, with a gelatin capsule containing 250 mg of chromic oxide, in order to use as an indigestible marker. On the first day of fecal collection (d 29), all stool before 0700 h were removed and discarded from the cages. Fecal output of individual dog was collected and placed into labeled plastic bags from this
point until d 32. Fecal samples of each dog were stored at -20°C and dried at 60°C in a forced-air oven. After drying, the samples were ground through a 1-mm screen mill and collected in labeled plastic bottles at room temperature until further analysis. Fecal samples were determined for Zn and Cr concentrations by AAS using Sullivan and Carpenter (1993) and Williams et al. (1962) procedures, respectively.

Statistical analysis: All data was expressed as mean±SE. Data were analyzed as a Cross-over using the GLM. Each dog represented an experimental unit. The model included period, dog, and treatment, and the error was residual error mean square. The mean differences between treatments were tested by LSD using procedure of SAS (1988). Differences were considered significant when \( p<0.05 \) and were regarded as trends if \( 0.05 \leq p<0.10 \).

Results

No observation of any abnormal behavior and appearance throughout the experiment and also no sign of severe Zn deficiency.

Effects of either ZnMG or ZnSO₄ supplementation on hair coat characteristics and Zn deposition in hair

The hair growth rate and Zn deposition in hair was greater \((p<0.05)\) for the dog supplemented with ZnMG when compared to the dog supplemented with ZnSO₄ (Table 2). Scanning electron microscopy revealed differences between treatments in hair condition with the hair taken from the ZnMG supplemented dogs apparently smoother and less fragmented than ZnSO₄ supplemented dogs (Fig.1).

![Comparison of scanning electron microscopy photographs of a strand of hair from dog supplemented with ZnMG versus ZnSO₄ (Mag. x 1,500).](image)

**Table 2** Hair growth rate and Zn deposition in hair, levels of Zn concentration in plasma and serum ALP activity, amounts of fecal Zn excretion and Zn absorption after receiving either ZnMG or ZnSO₄ supplementation\(^1\).

<table>
<thead>
<tr>
<th>Item</th>
<th>ZnMG</th>
<th>ZnSO₄</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair growth rate (mg/d, 20cm²)</td>
<td>6.2 ±0.34(^a)</td>
<td>3.8 ±0.38(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td>Zn deposition in hair (µg/21d 20cm²)</td>
<td>26.5 ±1.39(^a)</td>
<td>19.2 ±1.79(^b)</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma Zn (µmol/l)</td>
<td>9.0 ±0.09</td>
<td>8.6 ±0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum ALP activity (U/L)</td>
<td>193.9 ±4.77(^a)</td>
<td>164.4 ±7.29(^b)</td>
<td>0.04</td>
</tr>
<tr>
<td>Fecal Zn (mg/d)</td>
<td>11.1 ±0.16(^a)</td>
<td>12.4 ±0.24(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fecal Zn (%)</td>
<td>63.0 ±0.89(^a)</td>
<td>70.2 ±1.36(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td>Zn absorption (mg/d)</td>
<td>6.5 ±0.16(^a)</td>
<td>5.2 ±0.25(^b)</td>
<td>0.02</td>
</tr>
<tr>
<td>Zn absorption (%)</td>
<td>37.0 ±0.88(^a)</td>
<td>29.8 ±1.34(^b)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^1\)Mean±SE

\(^{a,b}\)Mean in the same row with different superscripts differed significantly \((p<0.05)\).
ALP activity when the dogs fed ZnMG was greater ($p<0.05$) than the dogs fed ZnSO$_4$.

Effects of either ZnMG or ZnSO$_4$ supplementation on fecal Zn excretion and Zn Absorption

Table 2 shows the mean value of the fecal Zn excretion and Zn absorption. The dogs supplemented with ZnMG had lower ($p<0.05$) fecal Zn excretion but greater ($p<0.05$) Zn absorption, calculated by subtracted the amount of Zn intake with the amount of fecal Zn excretion, than the dogs supplemented with ZnSO$_4$.

Discussion

Hair coat characteristics and Zn deposition in hair

ZnMG supplementation resulted in greater ($p<0.05$) hair growth rate and more ($p<0.05$) Zn deposition than ZnSO$_4$ supplementation. These result were similar to Lowe et al. (1994b) studied. They reported that the hair growth rate and Zn deposition in hair were greater ($p<0.05$) in dogs fed diet containing zinc amino acid chelate (ZnAAC) than dogs fed ZnO diet. Similarly, França et al. (2005) shown that cats supplemented with Zn proteinate resulted in the greater ($p<0.05$) level of Zn deposition in hair than cats supplemented with ZnO and ZnSO$_4$. Results from these reports demonstrated that animals could utilize Zn in the chelate form better than the inorganic form.

The taken hair of the dogs supplemented with ZnMG showed apparently smoother and less fragmented than the taken hair of the dogs supplemented with ZnSO$_4$. Similar the result was reported by Kuhlman and Rompala (1998) that partial replacement of inorganic Zn, Mn, and Cu with proteinated forms of Zn, Mn, and Cu in diet gave the better hair condition than diet containing inorganic forms of Zn, Mn, and Cu only.

ZnMG supplementation had greater amount of Zn absorption and utilization when compare to ZnSO$_4$ supplementation. ZnMG is absorbed then moved directly into the plasma as an intact molecule. This intact molecule will be metabolized at the target tissue (Albion, 2004). Borges and Silva (n.d.) suggested that the use of minerals chelated to amino acids related to the specific needs of certain tissues and chelated Zn promotes Zn deposition in hair. When the amino acids (chelated to the mineral) are transported to specific tissues, they carry with them the mineral that they are chelated to, ensuring the absorption and deposition of the mineral on the tissue. The target tissue of the current study is hair. Hair has such great requirement of both Zn and sulfur-containing amino acids for proper hair growth rate. ZnMG that used in the present study composed of Zn and sulfur-containing amino acid (methionine). Zn is known to be associated with three key functions in the keratinization process as follow: 1) catalytic roles; 2) structural roles; and 3) regulatory roles (Tomlinson et al., 2004). The availability of sulfur-containing amino acids would affect to the rate of synthesis of high-sulfur hair matrix proteins (Tschammer and Halliwell, 1990). Consequently, if the ZnMG was absorbed readily, proper hair growth and condition would be achieved because optimal both levels of Zn and methionine were provided to the hair follicle.

Zn concentration in plasma and ALP activity

Van den Broek (1988) reported that serum Zn concentrations of normal adult dogs ranged 4.3-16 μmol/l. While the previous report of the normal range values of plasma Zn concentration in dogs were not found. Kirk and Bonagura (1992) reported that serum ALP activity of adult dogs ranged 35-280 U/L. In the present study, plasma Zn concentrations and level of serum ALP activity, the biochemical liver function test of serum, at the end of pretest and the end of test periods were in normal range for both experiments. Serum ALP activity usually was reported in various wide reference ranges. In addition, plasma Zn concentrations and level of serum ALP activity at the end of test period seem to be greater than at the end of pretest period for both experiments. These probably due to all dogs were fed only basal diets in order to reduce the Zn storage in the body.

The plasma Zn concentration tended to be different ($p<0.10$) between ZnMG and ZnSO$_4$ treatments. These results were similar to the reported of Lowe et al. (1994a). At the same time of blood collection (3 to 4 h after meal), they found that dogs fed ZnAAC diet tended to have greater plasma Zn concentration than the dogs fed ZnO diet. Moreover, they found that feeding dogs with ZnAAC diet had greater ($p<0.05$) plasma Zn concentration than ZnO diet. But the significant differences were observed at the different time of peak value between ZnAAC and ZnO. The ZnAAC was observed at 4.5 h while ZnO was observed at 2.25 h. Thus,
the difference between forms of Zn supplementation had affected to plasma Zn determination. According to Valberg et al. (1985), Zn form influenced the Zn transportation from the intestinal lumen to either blood circulation or cell incorporation such as hair. Van den Broek (1993) found that normal beagles had the peak of plasma Zn concentration at 2 h after supplemented with ZnSO₄. In Exp.2, plasma Zn concentration increased \( p<0.05 \) with increasing the amount of Zn supplementation. These results were in agreement with the report by Van den Broek (1993) who found that dogs fed ZnSO₄ increased from 0.50, 0.75, and 1.00 mg Zn/kg BW resulted in increased \( p<0.05 \) plasma Zn concentration.

On the other hand, ALP is a Zn-containing enzyme and Zn is essential to maintain its activity (Gropper et al., 2005). In Exp.1, the dogs supplemented with ZnSO₄ had lower \( p<0.05 \) level of serum ALP activity than the dogs supplemented with ZnMG which could cause by the form of Zn. Since ZnSO₄ possibly provided the exact amount of Zn for ALP less than ZnMG. Although both ZnMG and ZnSO₄ gave the values of serum ALP activity in the normal range.

**Fecal Zn excretion and Zn absorption**

The dogs supplemented with ZnMG had lower \( p<0.05 \) amount of fecal Zn excretion but greater \( p<0.05 \) Zn absorption than the dogs supplemented with ZnSO₄. These results were similar to the study of Lowe et al. (1994b) that Zn excretion was greater \( p<0.05 \) in the dogs fed ZnO diet than the dogs fed ZnAAC diet. In cats, Borges and Oliveira (2003) and França et al. (2005) reported that Zn proteinate supplementation resulted in greater \( p<0.05 \) Zn absorption and retention than ZnSO₄ supplementation.

In fact, soybean meal was used in the most commercial dog foods. In the present study, soybean meal was used as a raw material in basal diet. Furthermore, these basal diets might contain antagonistic substance from soybean meal such as phytic acid. Edwards and Baker (2000) reported that phytic acid from some raw materials had affected on Zn utilization when ZnSO₄ supplemented to soybean meal diet. Because ZnSO₄ can dissociate to Zn²⁺ in GIT and could interact with phytic acid to form strong and insoluble complexes that inhibit Zn absorption by animal (Wilaison, 2002; Gropper et al., 2005). Whereas ZnMG is stable in wide pH range encountered within the different segments of the GIT so it would neither dissociate nor interact with other substances (Vandergrift, 1994). It is transported across the intestinal enterocyte and into the circulation as an intact molecule (Ashmead, 1992).

In conclusion, the organic Zn as ZnMG was found to be the form that could enhance the haircoat characteristics and suitable for supplementation into the commercial dry dog foods.

**Acknowledgement**

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The Efficacy of Tilmicosin against Broiler Chickens Infected with *Mycoplasma gallisepticum* Isolated in Thailand

Somsak Pakpinyo*  Visut Rawiwet  Warod Buranasiri  Supasakol Jaruspibool

**Abstract**

Sixty 1-day-old-female broiler chickens were divided into groups as follows. Thirty birds were randomly selected to be bled from jugular vein for *Mycoplasma gallisepticum* (MG) antibody assay and swabbed at yolk sac for the detection of MG DNA by using PCR. The remaining birds were divided into three groups, 10 birds per group as follows. Group 1 was a sham negative control. Group 2 received MG serving as a positive control, whereas group 3 received MG and simultaneously treated with tilmicosin incorporated into drinking water. Each bird received MG that was injected into the left thoracic airsac, with 0.1 ml of inoculum containing MG organisms $10^6$ CFU. Each group was raised on a wired cage in 3 isolated rooms with similar environmental conditions. Clinical sign and mortality rate were observed during 1-39 days old. Dead birds were necropsied and swabbed from left thoracic airsac for the detection of MG DNA. At 39 days old, all birds were bled, then necropsied to determine the gross airsac and microscopic tracheal lesion scores and simultaneously swabbed at the left thoracic air sac for the detection of MG DNA. Results revealed that the numbers of birds that showed clinical signs, mean gross airsac and microscopic tracheal lesion scores of group 3 were significantly less than those of group 2 ($p<0.05$).

**Keywords**: broiler chickens, *Mycoplasma gallisepticum*, Tilmicosin

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Introduction

*Mycoplasma gallisepticum* (MG) infection is known as a chronic respiratory disease (CRD) in avian species (Kleven, 1998; Ley, 2003). Chickens, turkeys, quails, parrots, pheasants, pigeons, and peacocks are the natural hosts of MG infection (reviewed by Ley, 2003). The mortality rate is low unless a secondary microorganism infection is present. MG infection causes sneezing, conjunctivitis, airsacculitis, and decreased egg production in affected birds. MG organisms of infected birds can be transmitted to the other birds via direct contact; that is, horizontal transmission. In addition, affected breeders can spread MG organisms through their progeny which is called as “vertical transmission” (Ley, 2003).

The economic losses due to decrease in egg production in breeders have been estimated at about 21 eggs/bird, or over US$ 100 millions per year for the US poultry industry (Mohammed et al., 1987). Furthermore, their progeny show decreased feed efficiency, high conversion rate, poor carcass quality, and economic losses due to prevention and treatment costs (Ley, 2003).

For prevention, two types of vaccines; live and inactivated have been widely used in many countries (Ley, 2003) including Thailand. Strains of 6/85, ts-11, and F are commercially available, live, MG vaccines. Because each strain has a different degree of virulence, the use of live vaccines should be carefully considered and followed the manufacturers’ instructions (Kleven, 1998, Ley, 2003). Inactivated MG vaccine is safer but needs 2 doses for optimal protection, plus the cost of individual bird injection and a reduced ability to control long-term infection in multiple age production units (Ley, 2003).

There are several diagnostic methods, clinical signs, histopathology, MG detection and MG serology, all of which are widely used in MG diagnosis (Kleven, 1998). MG detection including MG culture and isolation, and MG polymerase chain reaction (PCR) testing has been
used in most MG laboratories. However, the gold standard for MG diagnosis is MG culture and isolation but this diagnosis requires MG antibody and a fluorescent microscope to determine the immunofluorescence technique (Ley, 2003). MG serology, such as serum plate agglutination (SPA), enzyme linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) tests are widely used in the laboratory; however, each method is limited by sensitivity and specificity (Kleven, 1998). SPA is used as the screening test because of its rapid, high sensitivity, low specificity, as well as being inexpensive. ELISA has been proved to have good sensitivity and more specificity compared to SPA procedure (Kleven, 1998). HI has high specificity but low sensitivity and no commercially available (Kleven, 1998). MG PCR is the rapid, high sensitivity and specificity method (Kempf et al., 1993; Jordan and Pattison, 1996).

Tilmicosin is a semi-synthetic antibiotic classified in group of macrolides and was approved in such countries for veterinary use in respiratory diseases of cattle and swine (Moore, 1996; Moore et al., 1996, Charleston et al., 1998). In vitro study showed that tilmicosin had an activity against Gram negative bacteria associated with respiratory disease including mycoplasmas (Ose, 1987; Jordan and Horrocks, 1996). Shryock et al. (1994) and Charleston et al. (1998) revealed that is effective to treat and control of MG inoculated broilers by tilmicosin administered in the diet and drinking water. For the pharmacokinetic of tilmicosin in chickens, Warren et al. (1997) showed that tilmicosin is distributed into lungs and airsacs in a dose of 2.30±0.72 mg/g of lung following 48 hours of oral administration compared to the minimum inhibitory concentration (MIC) of MG determined by Ose in 1987 (0.048 μg/ml) and Jordan and Horrocks in 1996 (0.0125 mg/ml). In Thailand, Pakpinyo and Sasipreeyajan (2007) reported that the MIC of local MG isolates against tilmicosin ranged 0.099-12.5 mg/ml.

Tilmicosin can be incorporated into feed at 300-500 mg/kg (Shryock et al, 1994) or into water at 50-100 mg/l for either 3 or 5 days (Charleston et al., 1998). Generally, the treatment of poultry diseases in the field usually incorporates into drinking water. Pakpinyo (2005) described that Thai’s MG isolated could cause more severity of the respiratory signs, gross airsac and histopathologic lesion scores, morbidity and mortality compared to the MG reference strain (S6). Due to lack of data of the use of tilmicosin against the Thai’s MG isolated; therefore, this study determined the efficacy of tilmicosin against broiler chickens infected with MG isolated in Thailand. The results of this study may be useful for Thai’s poultry industry. The objective of this study was to determine the efficacy of tilmicosin incorporation in drinking water against broiler chickens infected with MG isolated in Thailand.

**Materials and Methods**

Sixty 1-day-old female broiler chickens were obtained from commercial breeder farm vaccinated with live MG vaccine at 6 weeks old. Thirty birds were randomized to bleed at jugular vein for MG serology, necropsied then swabbed at yolk sac for MG DNA by PCR procedure. Three cotton swabs were pooled as one sample, totally 10 samples for MG identification. Remaining birds were divided into 3 groups, 10 birds in each group. Each group was raised on a wired cage in 3 isolated rooms with the similar environmental condition and determined as follows.

**Group 1**: sham negative control. Birds were individually injected with 0.1 ml of FMS broth media at the left side of the middle line of the proximal end of the humerus and the pelvic girdle into the left thoracic airsac.

**Group 2**: positive control. Birds were individually injected 0.1 ml of inocula containing MG organisms 1.0x10⁶ CFU into the left thoracic airsac as indicated in group 1.

**Group 3**: treatment. Birds were injected the same as group 2 and simultaneously treated with tilmicosin incorporated into drinking water at 75 mg/l/day for 3 consecutive days.
Feed and water were given ad libitum. All birds were observed for morbidity and mortality rate twice a day. The dead bird was necropsied to observe the airsac lesion, and simultaneously swabbed the left thoracic airsac to culture *E. coli* and to detect MG DNA by PCR procedure. At 39 days old, all remaining birds were bled for serology and identified the number prior to necropsy. The thoracic airsacs were grossly examined for typical of *Mycoplasma* infection, then swabbed for MG DNA by PCR procedure and *E. coli* isolation. Five tracheas from each group were submitted for histopathology. Each trachea was crossly sectioned into 4 pieces (1 proximal, 2 middle, 1 distal part of trachea). Lesion scores of airsacs and tracheas were blindly investigated.

**Airsac lesion score.** The airsac lesion score was grossly evaluated as the following criteria (Kleven et al., 1972): 0: No airsac lesion is observed, 1: Lymphofollicular lesions or slight cloudiness of the airsac membrane are found., 2: Airsac membrane is slightly thick and usually presents small accumulations of cheesy exudates., 3: Airsac membrane is obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one airsac., 4: Lesions are observed as same as 3, but 2 or more airsacs are found.

**Tracheal lesion score.** The tracheal lesion score was microscopically evaluated as the following criteria (Yagihashi and Tajima, 1986): 0: No significant changes are observed., 1: Small aggregate of cells (mainly lymphocytes) is found., 2: Moderate thickening of the wall due to the cell infiltration, and edema commonly accompanied with epithelial degeneration and exudation is present., 3: Extensive thickening of the wall due to the cell infiltration with or without exudation is determined. The maximum tracheal score for each bird was 12.

This experiment was approved by the Authors’ Institution’s Ethic Committee, and care was taken to minimize the number of animals used, issued by the Faculty of Veterinary Science, Chulalongkorn University, Number 44/2549, effective date: 13 September 2549-13 September 2550 (A.D.).

**MG serology.**

**Serum plate agglutination (SPA):** Fresh sera were tested against MG antigen (Nobilis®, Intervet International B.V., Holland), following the manufacturer’s instructions. Briefly, thirty μl of serum were mixed with thirty μl of antigen and then incubated at room temperature for 1-2 min before the result was read. Negative and positive sera were included in each test.

**Enzyme linked immunosorbent assay (ELISA):** Sera were tested with commercial test kits, ProFLOK® (Synbiotics Corporation, USA) following the manufacturer’s directions. Briefly, diluted sera were added onto a MG antigen-coated plate, incubated, washed, and peroxidase labeled, anti-chicken antibody (conjugated antibody) was added. After incubation, the plate was again washed before adding a substrate, and adding the stop solution. The plate was read in an ELISA reader, manufactured by Labsystems Multiskan MS Type 352, Finland. The optical density of the negative, positive controls, and samples were calculated and interpreted according to the manufacturer’s recommendations. For the interpretation, titer levels 0-148, 149-743, and equal or higher than 744 were negative, suspicious, or positive reactors, respectively.

**MG DNA by PCR procedure:** The broth samples were investigated in this study. This method was described by Lauerman (1998). The broth was extracted for the DNA template by centrifugation at 15,000xg, washing with distilled water, following by diluting the pellet with distilled water, boiling for 10 min, storing at -20°C for 10 min, centrifugation, and collecting the supernatant at -20°C until use. The PCR mixture, in a 50 μl volume, contained KCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5’GAGCTAATTGCTGAAAGTTTTGTC3’) and primer R (5’GGTTCCCTTGCGGTATAGCAAC3’) (Qiagen) 10 pmole each, Taq polymerase (Fermentas) 1.25 U and the DNA template, 5 µl (250 ng). The MG strain S6 (ATCC 15302), was used as a positive control. PCR mixtures were amplified in a
DNA thermal cycler (PCR Sprint, Thermo Electron Corporation, Milford, MA) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec over 40 cycles, followed by maintaining 72°C for 5 min. The PCR product was analyzed in 2% agarose gel (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide, visualized by an UV transilluminator and photographed.

Statistical analysis: Morbidity and mortality were analyzed statistically using a nonparametric Kruskal-Wallis test and Mann Whitney U test. The gross airsac and histopathologic tracheal lesion scores were examined by using Fisher’s exact test. Statistical significance was determined as \( p<0.05 \).

Results

The affected birds showed the respiratory signs including coughing, sneaking, watery ocular discharge and nasal discharge. The morbidity of group 1 (sham negative control), 2 (positive control) and 3 (treatment) ranged 0-6 birds. Numbers of sick birds of group 2 were significantly higher than those of groups 3 and 1, respectively. There was one dead bird observed only in group 3 (Table 1).

The numbers of birds aged 1 day old found positive reactors against SPA and ELISA was 0 and 3, respectively. At 39 days old, numbers of positive reactors of groups 1, 2 and 3 detected by SPA, and ELISA was 10, 10 and 9, and 0, 6 and 3, respectively (Table 2).

The mean gross airsac and histopathologic tracheal lesion scores of groups 2 were significantly higher than those of groups 3 and 1, respectively. Group 1 showed the lowest mean airsac and tracheal lesion scores and significant difference was observed compared to group 3 (Table 3).

The MG DNA detected by PCR procedure was not found in birds aged 1 day old. At 39 days old, only groups 2 and 3 detected MG DNA for 7 and 5 birds, respectively (Table 4). For one dead bird of group 3, MG DNA was not detected. *E. coli* could not be cultured from airsac swabs.

Discussion

This study revealed that the broiler chickens inoculated with the local MG isolated and simultaneously treated with tilmicosin (group 3) and the sham negative birds (group 1) had lower morbidity, gross airsac and histopathologic tracheal lesion scores compared to the birds inoculated with the local MG isolated (positive control; group 2). However, one dead bird was found in the treatment group. This dead bird possibly resulted from itself or management, not due to the virulence of MG infection, because none of any gross lesions, no growth of *E. coli* and no MG DNA was observed from this bird.

The antibody detected by the SPA and ELISA of birds aged 1 day old was found 3 out of 30 birds by the ELISA suggesting maternal derived antibody. In addition, no MG DNA was detected at 1 day old prior to separate birds by PCR procedure. The previous reasons indicated that 1 day old birds were free of the MG organisms or no vertical transmission; furthermore, at the 39 days old of birds of group 1 were still absent of MG antibody and organisms. There was a study described that passive transfer of high-titer antibodies could not protect MG infection (McMartin and Adler, 1961). Lam and Lin (1984), Talkington and Kleven (1985) and Whithear et al. (1990) revealed that the antibody titer against MG infection found in the blood did not correlate with the resistance of MG challenge. Therefore, the maternal derived antibody against MG failed to demonstrate any MG protective effect. The MG inoculated groups were found the positive reactors against MG organisms by the SPA and ELISA. From this study, group 2 showed the higher numbers of the positive reactors by the SPA compared with the ELISA. The SPA and ELISA can be used as the diagnosis method of serology, but the sensitivity and specificity of these tests were different. The SPA is much more sensitivity, whereas is lower specificity compared with the ELISA (Kleven, 1998). Furthermore, the SPA detects *E. coli* could not be isolated in all necropsied birds.
Table 1  Morbidity and mortality during 1-39 days old period (n=10)

<table>
<thead>
<tr>
<th>Group</th>
<th>Morbidity</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>2</td>
<td>6b</td>
<td>0a</td>
</tr>
<tr>
<td>3</td>
<td>3c</td>
<td>1a</td>
</tr>
</tbody>
</table>

a,b,c the different superscript in the same column means significant different ($p<0.05$)

Table 2  Numbers of positive samples tested by SPA and ELISA of birds aged 1 and 39 days old

<table>
<thead>
<tr>
<th>Group</th>
<th>SPA 1 day old*</th>
<th>SPA 39 days old</th>
<th>ELISA 1 day old*</th>
<th>ELISA 39 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>2</td>
<td>0/30**</td>
<td>10/10</td>
<td>3/30</td>
<td>6/10</td>
</tr>
<tr>
<td>3</td>
<td>2/9</td>
<td>3/9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Prior to separate birds  **Numbers of positive samples/total samples

Table 3  Mean of gross airsac lesion scores and mean of histopathologic tracheal lesion scores of birds aged 39 days old (Mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Gross airsac lesion scores</th>
<th>Histopathologic tracheal lesion scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.40±0.10 (n=10)</td>
<td>0.79±0.92 (n=10)</td>
</tr>
<tr>
<td>2</td>
<td>2.45±0.14 (n=10)</td>
<td>11.50±0.28 (n=10)</td>
</tr>
<tr>
<td>3</td>
<td>1.83±0.13 (n=9)</td>
<td>7.45±0.70 (n=9)</td>
</tr>
</tbody>
</table>

a,b the different superscript in the same column means significant different ($p<0.05$)

Table 4  Numbers of positive samples detected by PCR of birds aged 1 and 35 days old.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCR 1 day old*</th>
<th>39 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0/10**</td>
<td>7/10</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>5/10</td>
</tr>
</tbody>
</table>

*Prior to separate birds  **Numbers of positive samples/Total samples

IgM (immunoglobulin M), which is the first immunoglobulin produced from the immune response, presented at the first few days until 77 days of post infection (Kleven, 1975; Kleven, 1998). The ELISA detects IgG or IgA, which released after 14 days of post infection. This result also showed that the numbers of positive reactors detected by both SPA and ELISA of group 2 were less than those of group 3. This finding was in accordance with Jordan et al. (1999) described that the infected and medicated poults had fewer numbers of the positive reactors than the infected and unmedicated poults.

The airsac and tracheal lesion scores of group 3 was higher than those of group 1 but was significant lower than group 2 suggesting that tilmicosin possibly reduced...
the lesion scores of the inoculated birds. The result of the present study was similar to Charleston et al. (1998) revealed that the morbidity, the mean lesion score and the proportion of birds with severe lesions decreased after receiving tilmicosin as a treatment of MG infections in chickens. The mean peak plasma concentration of tilmicosin after administration in a single oral dose of 30 mg/kg was 2.09±0.37 μg/ml that is higher than the minimum inhibitory concentrations (MICs) for MG (0.0125-0.1 μg/ml) (Abu-Basha et al., 2007). The advantages of tilmicosin are that tilmicosin can be rapidly absorbed then distribute into tissues including lungs and airsacs as well as phagocyte cells including macrophages, monocytes and heterophils (Warren et al., 1997; Scorneaux et al., 1998; Abu-Basha et al., 2007). Furthermore, the elimination of tilmicosin from the serum and lung was somewhat slow with mean half-lives of 30.18 and 75.74 hours, respectively (Abu-Basha et al., 2007). From rapid absorption, distribution, slow elimination and high plasma concentration, tilmicosin has potential to inhibit or eliminate MG organisms resulting in a small proportion of birds with severe lesions in the group 3.

In conclusion, tilmicosin incorporated in drinking water at a dose of 75 ppm for 3 consecutive days reduced the morbidity rate and the severity of gross airsac and histopathologic tracheal lesion scores in broilers inoculated with MG organisms at 1 day old.

References


Follicular Dynamics Following Estrus Synchronization in Swamp Buffalo Cows (*Bubalus bubalis*)

Akachart Promdireg¹  Giorgio Antonio Presicce²  Fabio De Rensis³  Jinda Singlor¹  Mongkol Techakumphu¹*

Abstract

The objective of this study was to elucidate ovarian follicular dynamics in swamp buffalo cows (*Bubalus bubalis*) following a protocol for estrus synchronization during hot as low breeding season (March to June) and cool as high breeding season (November to February). Nine pluriparous buffalo cows received a progesterone ear implant for 10 days together with the administration of a luteolytic dose of PGF2α at the time of implant removal to start the estrus cycle. Human Chorionic Gonadotrophin was administered whenever ovulation did not occur within the first five days following implant removal. Daily ultrasound monitoring and blood collection for progesterone values were performed starting one day following implant removal. The progesterone monitoring was used to confirm ovulation and to determine luteal function after ovulation. Data analysis was carried out at least for two consecutive cycles in each cycling buffalo in 22 estrus cycles. It was found that 5/22 (22.7%) were characterized by one wave and 17/22 (77.3%) by two waves of follicle development. Within cycles characterized by one wave of follicle development, emergence was recorded on day 2.3±0.5 and 1.8±0.4 during the low and high breeding season, respectively (p>0.05). Day of estrous cycle when the dominant follicle attained the largest size during the low and high breeding season was 13.5±1.2 and 12.6±1.5, respectively (p>0.05). Largest size (mm) of the dominant follicle during the low breeding and high season was 14.5±2.1 and 16.4±2.7, respectively (p>0.05). Within cycles with two follicular waves during the low breeding season, first and second wave emerged on day 1.2±0.3 and on day 11.4±0.8, respectively, and largest diameter of second wave dominant follicle (10.3±1.2 mm) was recorded on day 19.7±1.1. During the high breeding season, first and second wave emerged on day 0.9±0.4 and on day 10.7±0.9, respectively and largest diameter of second wave dominant follicle (12.8±1.2 mm) was recorded on day 18.9±1.7. Pooling data for both 1- and 2-wave cycles and seasons, mean progesterone value at estrus and ovulation was 0.07±0.03 ng/ml, but after ovulation mean progesterone increased to 2.37±0.5 ng/ml at the mid luteal phase and later, decreased at the late luteal phase which was 0.98±0.2 ng/ml. In conclusion, results from this study showed the first time of the typical pattern of follicle wave development in swamp buffalo.

Keywords: estrous cycle, ovarian follicle development, swamp buffalo

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**Introduction**

The continual follicle development within an estrous cycle progressing as sequential waves, encompasses the recruitment of small follicles, a common growth phase and the final selection of the dominant follicle together with the regression of other follicles. Studies on follicular dynamics have extensively been performed in cattle (Sirois et al., 1988; Knopf et al., 1989; Ginther et al., 1989; Sunderland et al., 1994; Lean et al., 1992) whereas only few reports are available in the buffalo species (Presicce, 2006). Mostly, studies have been performed in riverine buffaloes, both in calves and adult animals. Presicce et al. (2003) reported in Mediterranean buffalo calves had a similar pattern of follicle development as previously described by Adams et al. (1994) in cattle calves. In a study by Baruselli et al. (1997) on follicular dynamics in Murrah buffaloes, 1 to 3 waves of follicle development were recorded and the number of waves was found to be linked to the luteal phase and length of the estrous cycle, as previously described in cattle (Ginther et al., 1989). Swamp buffaloes are characterized by estrous cycles of variable length and variable time...
interval between the end of estrus and ovulation (Bodhipaksha, 1987). Such peculiar reproductive aspects can similarly be found in riverine buffaloes as well, being characterized by reproductive efficiency affected by the duration of day length, together with a high rate of silent estrus and seasonal anestrus (Zicarelli, 1997). Swamp buffaloes’ ovarian activity may be characterized by a seasonal low breeding season that last from April to July (summer season) during which estrus cycles are reduced or absent while from November to January (cool season), the reproduction performance is higher. However, no report on follicular dynamics during these two periods has been found. Within this framework, a more in-depth understanding of follicular turnover in swamp buffaloes in the course of favorable (high breeding season) and unfavorable season (low breeding season) could help to improve reproductive function and efficiency, especially in the areas of synchronization of ovulation for artificial insemination and embryo transfer, as well as superovulatory treatments. The aim of this study was therefore to evaluate for the first time the follicular dynamics of swamp buffaloes following an estrus synchronization protocol.

Materials and Methods

Animals: Nine pluriparous swamp buffalo cows, 3 to 10 yrs old (means = 5.4) and weighing between 400 and 600 kg (means = 520), were randomly selected from the heads available at the National Breeding Center, Department of Livestock Development and from the herd of the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. Buffaloes were housed at the Veterinary Student Training Center at Nakorn Pathom province, and fed daily with roughage and water ad lib., together with the supplementation of 4 kg of concentrate containing 14% of protein per animal. This study was conducted on the same animals during March to June 2004 (hot season with average outdoor temperature of 31.7°C, range between 26.0°C and 35.1°C, and Thermal Humidity Index, THI = 84) determined as low breeding season and November 2004 to January 2005 (cool season with average outdoor temperature of 31.3°C, range between 22.7°C and 33.9°C, THI = 81) determined as high breeding season. In order to synchronize estrus, each buffalo was received 6 mg of a progesterone ear implant (Crestar®, Intervet, The Netherlands) for 10 days and a luteolytic dose (25 mg) of PGF2α (Lutalyse®, Pfizer, USA) at the time of implant removal. Ovulation was monitored during the 5 days following implant removal. Two thousands IU of hCG (Chorulon®, Intervet, The Netherlands) were administered at the end of the 5 days from implant removal in the event ovulation had not occurred.

Follicle development: Follicle development was recorded daily by per rectum and investigated by real time B-mode ultrasound unit equipped with 5 MHz transvaginal probe (Aloka, SSD-210 Tokyo, Japan), starting the day following prostaglandin administration and implant removal. Ultrasound monitoring was always performed by the same person, and follicles with a diameter ≥ 2 mm were recorded and visualized on a sheet map and classified into subordinated and dominant follicles. Heat detection was performed at least twice daily (AM/PM) by use of an epididymectomized bull. Follicular dynamics was monitored for at least 2 consecutive estrous cycles on each cycling buffalo.

Blood collection for hormonal profile: Blood samples (10 ml) from the jugular vein were collected daily from each animal starting the day following implant removal. Samples were stored into sterile plastic tubes without anticoagulant and subsequently centrifuged at 1,500g for 10 min. Serum was stored in Eppendorf tube at -200°C until ready for progesterone analysis. Progesterone monitoring was to confirm ovulation and to determine luteal function after ovulation.

Hormonal assay: Progesterone (P4) values were determined by RIA kit (Hegstag, 1992).

Statistical analysis: When a follicle diameter was
recorded without increase in size for consecutive days, the first day was taken as the day of attained largest diameter described as dominant follicle. The duration of a dominant follicle within the ovary was defined as the interval of time (days) elapsed between its appearance and disappearance as a follicle ≥ 4 mm. Growth rate (mm/d) of each dominant follicle was calculated by the largest size attained divided by the number of days between its appearance as a follicle ≥ 4 mm and its maximum size. The following transformation of the data were made to normalize error distribution: 1) log for maximum size and duration of follicle, and 2) square root for growth rate. Differences between duration of estrous cycle, maximum size of dominant follicles, duration and growth rate of dominant follicles, as well as mean number of total available follicles were tested using t-test. Numerical values are expressed as mean ± SD throughout the text.

**Results**

**Estrous cycles and follicle development**

Nine complete estrous cycles were recorded from 4 buffaloes during the hot season and 13 estrous cycles from 5 buffaloes during the cool season. During summer months, 77.8% (7 buffaloes out of 9) of estrous cycles were characterized by 2-wave development of follicles whereas 22.2% (2 buffaloes out of 9) were recorded as 1-wave cycle. The length of estrous cycle was 22.6±1.9 days in a 2-wave cycle and 25.5±3.5 days in a 1-wave cycle (p<0.05). During winter months, 76.9% (10 buffaloes out of 13) of estrous cycles were characterized by 2-wave development of follicles whereas 23.1% (3 buffaloes out of 13) were recorded as 1-wave. The length of estrous cycle was 22.5±1.9 days in a 2-wave cycle and 20.7±2.8 in a 1-wave cycle (p>0.05). Overall, from the total number of recorded estrous cycles (n = 22), five (22.7%) were characterized by 1-wave of follicle development and seventeen (77.3%) by 2-wave of follicle development (p<0.05).

**One-wave follicular dynamics**

Main parameters referring to follicular dynamics of swamp buffaloes with estrous cycles characterized by one follicular wave during the hot and cool seasons are reported in Table 1. With ovulation time defined as day 0, waves emerged on day 2.3±0.5 and day 1.8±0.4 of the following cycle during the hot and cool season, respectively (p>0.05). Selection of the dominant follicle, characterized by the deviation in growth rate between the first and second largest follicles, occurred on day 7.6±1.8 and day 8.2±1.7 in summer and winter months, respectively (p>0.05). The largest diameter of first subordinated follicle was 8.7±1.7 vs 9.3±2.4 mm in hot and cool season, respectively (p>0.05). The largest size of the dominant follicle was recorded on day 13.5±1.2 and on day 12.6±1.5 during the hot and cool season, respectively (p>0.05). Likewise, the largest diameter recorded was 14.5±2.1 and 16.4±2.7 mm during the hot and cool season, respectively (p>0.05) (Figure 1).

**Two-wave follicular dynamics**

Main parameters of follicular dynamics in estrous cycle of swamp buffaloes characterized by two waves of follicle development during the hot and cool seasons, are reported in Table 2 and 3. During the hot season, the first wave emerged on day 1.2±0.3 of the cycle and was followed by selection of the dominant follicle on day 7.4±1.3. Growth of the dominant follicle continued until day 9.8±0.9, followed by beginning of regression on day 11.3±0.8. The second wave emerged on day 11.4±0.8, with the ovulatory follicle being selected on day 15.8±2.4, and reaching the largest diameter of 10.3±1.2 mm on day 19.7±1.1. During the cool season, the first wave emerged on day 0.9±0.4 of the cycle and was followed by selection of the dominant follicle on day 7.8±0.8. Development continued until day 10.6±1.3 followed by beginning of regression on day 12.4±1.6. The second wave emerged on day 10.7±0.9, with the ovulatory follicle being selected on day 15.4±1.6, and reaching its largest diameter of 12.8±1.2 mm on day 18.9±1.7. The dominant follicle of
Table 1  Follicle end points in swamp buffalos displaying one follicular wave during estrous cycles in the hot and cool season (mean ± SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1-wave (hot season)</th>
<th>1-wave (cool season)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of estrous cycle (%)</td>
<td>2/9 (22.2%)</td>
<td>3/13 (23.1%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Wave onset (day)</td>
<td>2.3 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Wave length (days)</td>
<td>25.5 ± 3.5</td>
<td>20.7 ± 2.8</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Dominant follicle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1-wave (hot season)</th>
<th>1-wave (cool season)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum diameter (mm)</td>
<td>14.5 ± 2.1</td>
<td>16.4 ± 2.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Day of maximum diameter</td>
<td>13.5 ± 1.2</td>
<td>12.6 ± 1.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.6 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Day of deviation</td>
<td>7.6 ± 1.8</td>
<td>8.2 ± 1.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Length of growth phase (days)</td>
<td>11.2 ± 0.8</td>
<td>10.8 ± 0.9</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Largest subordinate follicle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1-wave (hot season)</th>
<th>1-wave (cool season)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum diameter (mm)</td>
<td>8.7 ± 1.7</td>
<td>9.3 ± 2.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Table 2  Follicle end points in swamp buffalos displaying 2 follicular waves during estrous cycles in the hot season (mean ± SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Follicular wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first wave</td>
</tr>
<tr>
<td>No. of estrous cycle (%)</td>
<td>-</td>
</tr>
<tr>
<td>Wave onset (day)</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Wave length (days)</td>
<td>11.8 ± 2.3</td>
</tr>
</tbody>
</table>

Dominant follicle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Follicular wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first wave</td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>10.9 ± 2.1</td>
</tr>
<tr>
<td>Day of maximum diameter</td>
<td>9.8 ± 0.9</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Day of deviation</td>
<td>7.4 ± 1.3</td>
</tr>
<tr>
<td>Length of growth phase (days)</td>
<td>8.4 ± 0.7</td>
</tr>
</tbody>
</table>

Largest subordinate follicle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Follicular wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first wave</td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>7.7 ± 1.6</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>
Table 3  Follicle end points in swamp buffalos displaying 2 follicular waves during estrous cycles in the cool season (mean ± SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Follicular wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first wave</td>
</tr>
<tr>
<td>No. of estrous cycle (%)</td>
<td>-</td>
</tr>
<tr>
<td>Wave onset (day)</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Wave length (days)</td>
<td>12.2 ± 2.1</td>
</tr>
<tr>
<td>Dominant follicle</td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>8.7 ± 1.8</td>
</tr>
<tr>
<td>Day of maximum diameter</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Day of deviation</td>
<td>7.8 ± 0.8</td>
</tr>
<tr>
<td>Length of growth phase (days)</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td>Largest subordinate follicle</td>
<td></td>
</tr>
<tr>
<td>Maximum diameter (mm)</td>
<td>8.1 ± 1.5</td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4  Progesterone values (ng/ml) during estrous cycle with 1-wave and 2-wave follicle development in swamp buffaloes during hot and cool seasons (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>1-wave</th>
<th>2-wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hot</td>
<td>Cool</td>
</tr>
<tr>
<td>Estrus</td>
<td>0.11 ± 0.08</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Mid luteal phase</td>
<td>2.25 ± 0.7</td>
<td>2.06 ± 0.3</td>
</tr>
<tr>
<td>Late luteal phase</td>
<td>1.03 ± 0.4</td>
<td>1.14 ± 0.6</td>
</tr>
</tbody>
</table>

Figure 1.  Follicular development in swamp buffalo
the first wave was recorded more frequently to the right rather than the left ovary (68.7% vs 31.3%; \(p<0.05\)), and the same trend was recorded for the second dominant and ovulating follicle with a frequency of 62.3% vs 37.7% (\(p<0.05\)), respectively. The overall higher incidence of ovulated dominant follicles on the right ovary of all the monitored follicular waves was paralleled by the higher frequency (64.5%) of corpora lutea recorded on the same ovary (Figure 1).

**Progesterone values**

Progesterone values followed a similar trend for both 1- and 2-wave cycles during the hot and cool season. No significant differences were recorded between seasons and at targeted time during the estrous cycle for both kind of wave pattern. Therefore, pooling data for both 1- and 2-wave cycles and seasons, mean progesterone value at estrus and ovulation was 0.07±0.03 ng/ml, but after ovulation, the mean progesterone increased to 2.37±0.5 ng/ml at the mid luteal phase and later, decreased at the late luteal phase was 0.98±0.2 ng/ml. Furthermore, progesterone profiles clearly gave indications that one buffalo was cyclic without apparent signs of estrus. Detailed progesterone values are given in Table 4.

**Discussion**

The need to apply the reproductive technology for improving production and reproductive traits of swamp buffaloes has urged researchers to investigate further on basic aspects of their ovarian physiology. Within this framework, a comparison of follicular turnover in swamp buffaloes between low breeding (hot) and high breeding (cool) seasons has been carried out for the first time in this study. Swamp buffaloes are characterized by a number of physiological features which are mostly similar to those already reported in riverine buffaloes. They suffer from a reproductive function which is heavily affected by the environment, nutrition level and age (Zicarelli, 1997). Ultimately, reproductive efficiency can be related to the fine tuning of ovarian function, the down-stream reproductive organs and hormonal balance. This is why the study of ovarian follicular dynamics has played a role of paramount importance for the understanding, improvement and control of reproductive function in livestock animals (Lucy et al., 1992).

Swamp buffaloes, like their riverine counterpart, are tangentially seasonal breeders (Esposito et al., 1992; Dobson and Kamonpatana, 1986). Therefore their reproductive cycles and follicle development within cycles can be expected to be altered by the unfavorable season leading possibly to a reduced reproductive efficiency. The most disruptive of such deviations from a cyclic pattern of follicle development is the anestrus condition. In riverine buffaloes seasonal anestrus has been differentiated into a deeper and a more temporary or superficial condition (Zicarelli, 1997). In fact, deep anestrus is characterized by a very slow or almost absent follicle turnover which can possibly be resolved only by the exogenous administration of progesterone implant together with gonadotrophin supplementation in order to stimulate ovarian function. On the contrary, a milder degree of anestrus condition, characterized nevertheless by a clear pattern of follicle turnover and presence of large size follicles, can be overcome by the usual treatment with progesterone implant without the need of gonadotrophin supplementation (Presicce et al., 2004). It is clear then, how instrumental can be the understanding of follicular turnover in swamp buffaloes under different seasonal conditions for breeding purposes. The presence of one follicular wave pattern in swamp buffalo in 22% of estrus cycles may suggest the presence of a dominant follicle that persist for more then 10-14 days. Since persistent follicles usually produce oocytes of low quality that are difficult to be fertilized, this could be one of the cause of intrinsic low fertility in swamp buffaloes. Moreover, the presence of one follicular wave may generate a more variable duration of estrus and make prediction of ovulation and identification of the best time for AI more tedious. Therefore, hormonal treatments that reduce the presence of persistent follicles in swamp buffaloes may
be useful approaches to increase infertility.

From previous studies in riverine buffaloes, it appears that 2-wave cycles of follicle development are predominant over 1-wave and 3-wave cycles (Taneja et al., 1996). In this study the same trend was confirmed in both the hot and cool season. In comparison with similar studies performed by Taneja et al. (1996) and by Presicce et al. (2004) in riverine buffaloes displaying a 2-wave cycle, in this study a reduced largest size of 1st and 2nd wave dominant follicles as well as a reduced growth rate of dominant follicles was reported. Although a direct comparison between riverine and swamp type buffaloes has never been carried out, it can be speculated that such differences, albeit not statistically measurable and under the conditions imposed by the studies considered, could be speculatively ascribed to a different genetic makeup of the two subspecies. On the contrary, in a typical 2-wave cycle the expected time of follicle deviation leading to dominance over the second largest and other subordinate follicles found in this study, is in accordance with previous reports by Taneja et al. (1996) and Presicce et al. (2004). Furthermore, in this study growth rate of dominant follicles between 1-wave vs 2-wave cycles has been found similar. Largest attained diameter of dominant follicle in 1-wave cycle in both hot and cool season though, was reported to be larger than the ovulating dominant follicle in a 2-wave cycle. It is known that the fate of the dominant follicle associated to the first wave of follicle development is dependent on CL function and consequently on the level of blood progesterone. Progesterone levels at mid-luteal phase are usually sufficient to reduce LH frequency, determine regression of the first dominant follicle and initiate a new wave of follicle development (Lucy et al., 1992). In this study, recorded larger size of the ovulating dominant follicle in 1-wave cycles when compared to 2-wave cycles, could be ascribed to a longer exposure to LH frequency before its reduction in conjunction to progesterone rise. One-wave cycles of follicle development have been recently reported as normally occurring in suckled buffaloes, and an atypical growth of the dominant follicle has been described as characterized by an initial growth, a regression and a final re-growth followed by ovulation (Awasthi et al., 2006). In accordance with previous studies (Pathak, 1992; Jainudeen and Hafez, 1993), estrous behavior was mostly recorded in the cooler hours of morning and evening, confirming the notion that mating behavior in buffaloes usually ceases during daylight hours (Tailor et al., 1990). In this study a epididymectomized bull was used to detect animals in estrus following synchronization protocol and the corresponding behavior was not found to be significantly affected by the season, although signs of estrus were probably more pronounced during the nights of the cool season. The response of swamp buffaloes to the protocol of synchronization of estrus was not successful in both seasons. As a consequence, all but one buffalo had to be subjected to exogenous hCG administration at the end of the first five days following implant removal. A higher number of ovulations were consequently recorded on a total of nine buffaloes between both seasons. The difficulty in inducing ovulation in swamp buffaloes following a progesterone based synchronization protocol may be speculatively related to an insufficient endogenous LH availability. It is possible that the time given to build up LH reserve in the course of some progesterone based protocols for synchronization of estrus, may not be adequate or else the concentration of circulating progesterone when using Crestar® for a limited amount of time may not reach the critical threshold necessary to unleash LH release following progesterone device removal. Unsuccessful synchronization for estrus and timely ovulation using a progesterone based protocol has also been reported by Presicce et al. (2004) in riverine heifer buffaloes.

The length of the estrous cycle in buffaloes are generally accepted as more variable than those observed in cattle (Bhattacharya, 1974). Cycles ranging between 18 to 26 days are considered normal in buffaloes (Kanai and Shimizu, 1983; Singh et al., 1984)
and length of 1-wave and 2-wave cycles in this study fall therefore within the previously reported range (Presicce et al., 2004; Taneja et al., 1996; Singh et al., 1984). Within 1-wave and 2-wave cycles, no significant differences among the various parameters of follicular turnover under scrutiny have been highlighted in this study between seasons. A possible explanation for such findings can be tracked down on similar climatic conditions that have characterized the two seasons part of this study, in addition to a similar well balanced diet and energy intake given to animals in both seasons. The absence of any real dramatic climatic change between seasons and similar good feeding regimen must have obscured any possible appreciable differences among follicle end points and the cycle as a whole.

In accordance with previous reports by Dobson and Kamonpatana (1986), progesterone values at estrus in both seasons and for both 1-wave and 2-wave cycles were recorded at baseline values and did not rise until day 5 of the following estrous cycle. Overall, in this study the mean progesterone level in cyclic animals at estrus was 0.07±0.03 ng/ml with an increase between day 4 and 7, reaching a peak around day 15 and reduced nearly to baseline levels at approximately day 18 of the estrous cycle. The cyclic pattern of progesterone concentration observed in this study was in line with the changes found in functional CL in the course of the estrous cycle. Between day 16 and 21 of estrous cycle, the expected process of luteolysis resulted in a precipitous fall in progesterone to a basal concentration, as already reported by Chau et al. (1983). Progesterone profile was helpful in highlighting ovarian cyclic condition in one buffalo not showing any signs of estrus, that otherwise would have come unnoticed, in agreement with previous reports of silent estrus in riverine buffaloes (Zicarelli, 1997).

In conclusion, results from this study confirm the typical pattern of follicle wave development in swamp buffalo.

Acknowledgments

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Estrogen Receptor Alpha Localization in Thai Swamp Buffalo Oviduct during the Follicular and Luteal Phases

Paisan Tienthai\textsuperscript{1}\* Kriengyot Sajjarengpong\textsuperscript{1} Mongkol Techakumphu\textsuperscript{2}

Abstract

Estrogens are known to be an important modulator for regulation of the reproductive function in female mammals. The specific receptor of estrogens in the buffalo reproductive tract, particularly in the oviduct, has not been explored yet in the expression pattern. This study was, therefore, undertaken to investigate the localization of the estrogen receptor alpha (ER\textsubscript{\alpha}) in the oviduct of Thai swamp buffalo at the follicular and late luteal phases of the estrous cycle. The oviducts were collected from swamp buffalo genital tracts at the local abattoir and separated into the uterotubal junction (UTJ), isthmus, ampulla and infundibulum. Tissue distribution of ER\textsubscript{\alpha} was examined by immunohistochemical technique and the results showed that ER\textsubscript{\alpha} was stained in nuclei of cells and can be detected in all compartments along the entire oviduct at both follicular and late luteal phases. The intensity of immunostaining and proportion of ER\textsubscript{\alpha} positive cells in the epithelium and the subepithelial connective tissue of UJT and the isthmus was significantly higher (\textit{p}<0.05) in the follicular compared to the luteal phase. A higher ER\textsubscript{\alpha} expression during the follicular phase was also found in the ampulla and infundibulum. However, in these parts of the oviduct the increase in ER\textsubscript{\alpha} expression was not statistically significant.

\textbf{Keywords}: estrogen receptor, estrous cycle, oviduct, swamp buffalo

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Introduction

The oviduct plays an important role in the critical processes that take place before the establishment of pregnancy in the uterus such as oocyte maturation, sperm reservoir, sperm capacitation, fertilization of gamete and initial embryonic development (Ellington, 1991; Buhi, 2002). Within the oviduct, the gametes and developing embryos contact with the oviductal epithelial cells and their secretions, therefore, intensive research in co-culture experiments has been done in many species including buffalo (Kumaresan et al., 2005; 2006). In ruminants, the oviductal epithelium has been investigated by scanning electron microscopy (Abe and Oikawa, 1993a; Abe et al., 1993) and the results demonstrate that there are regional and cyclic changes in the morphological features of the oviductal epithelium. It is well established that these changes are correlated with the varying of estrogen and progesterone blood levels throughout the estrous cycle (Brenner et al., 1974; Abe and Oikawa, 1993c). The effects of ovarian steroids are mediated in their actions through specific nuclear receptors (King and Greene, 1984).

Estrogens are considered to be essential for the normal proliferation and differentiation of epithelial cells in the female reproductive tract (Nayak and Ellington, 1977) and appear to regulate the ciliogenesis since ciliated cells are particularly numerous in the bovine oviduct during estrus (Abe and Oikawa, 1993a). Maximum secretory cell differentiation is characterized by having the well-developed secretory granules at follicular phase (Nayak and Ellington, 1977). Therefore, the appearance of an estrogen receptor (ER) for this regulation is also required (Greco et al., 1993; Gorski and Hou, 1995).
It is generally accepted that there are different ER subtypes, i.e. ERα and ERβ (Kuiper et al., 1996). It has been found that ERα is predominant in the oviducts of pigs (Nielsen et al., 2001; Steffl et al., 2004), sheep (Garcia-Palencia et al., 2007) heifers (Bage et al., 2002; Valle et al., 2007) and cows (Ulbrich et al., 2003) in which the ERα has been intensely localized in the epithelium during estrus. To our knowledge no comprehensive investigation has been published on the localization of ER in the buffalo oviduct. The aim of the present study was to investigate the localization of ERα in different parts of the oviducts collected from Thai swamp buffalo during the follicular and luteal phase of estrous cycle.

**Materials and Methods**

**Animals and collection of tissue samples**

The swamp buffalo cows (aged 2-8 years, n=20) were slaughtered at the local abattoir and their genital organs were promptly collected and kept in a cool container at ~4°C for 30 min until being processed in the laboratory. The reproductive organs were examined for normality and the characteristics of the ovarian estrous cycle were classified by changes in the appearance of corpora lutea as described earlier by Ali et al. (2003), i.e. the ovaries at the period of proestrus or estrus were classified as follicular phase (n=8) while the ovaries at late diestrus were classified as luteal phase (n=12). The oviductal samples were cut into four segments, i.e. infundibulum, ampulla, isthmus and uterotubal junction (UTJ), fixed in 10% neutral buffered formalin and embedded in paraffin wax. Thin sections (5μm) were then mounted on Superfrost Plus glass slides (Menzel-Glaser, Freiburg, Germany) and treated according to immunohistochemical procedure.

**Immunohistochemical procedure**

Before immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded alcohol. The immunohistochemical protocol has been described previously by Bage et al. (2002). Briefly, sections were pretreated in a microwave oven at 700 W, in 0.01 M citrate buffer (pH 6.0) for 10 min for antigen retrieval and allowed to cool for 20 min. A standard immunohistochemical technique (avidin-biotin-peroxidase, Vectastain ABC-Elite; Vector Laboratories, Burlingame, CA, USA) was applied to detect the distribution of the estrogen receptor α (ERα). The primary antibody used was a monoclonal mouse antibody to ERα (C-311: sc-787; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, dilution of 1:50). The incubation time for the primary antibody was 1 h at 25°C. Negative controls were obtained by replacing the primary antibody with normal mouse IgG (sc-2025; Santa Cruz Biotechnology Inc.) on an equivalent concentration. Normal bovine uterine horn known to express ERα and PRB was served as positive controls. The site of the bound enzyme was visualized by the application of 3,3’-diaminobenzidine in H2O2 (DAB kit; Vector Laboratories), a chromogen that produces a brown, insoluble precipitate when incubated with the enzyme. Sections were counterstained with hematoxylin and mounted in glycerol gelatin.

**Classification of ERα-positive cells**

Stained sections were evaluated using a light microscope (BX50, Olympus, Tokyo, Japan) equipped with a digital camera (ImagePro6, Tokyo, Japan). Three different tissue compartments were evaluated separately: the surface epithelium, the subepithelial connective tissue and the smooth muscle. Examination of positive immunolabeling for ERα was performed by blinded preparation. The semiquantitative examination of ERα-positive cells was classified to three different levels of intensity in the following staining score criteria: weak, 1; moderate, 2 and strong, 3. Since not all cells were positive in three compartments of uterine tube, the proportion of positive to negative cells was estimated for these tissues. The proportions were estimated into four different levels (marked 1-4): low proportion (<30% of positive cells, 1);
moderate proportion (30-60% of positive cells, 2); high proportion (>60-90% of positive cells, 3) and almost all cells positive (more than 90%, 4).

**Statistical analyses**

Data were analyzed using SAS software (Statistics version 9.1 Cary, NC, USA). Descriptive statistics including the mean and standard deviation (SD) of all parameters were calculated. The score of intensities and proportions was compared between segments and phases using NPAR1WAY procedure of SAS (Wilcoxon rank sum test). Differences with p<0.05 were regarded as statistically significant.

**Results**

The ERα immunoreactive demonstrated positive nuclear labeling in all tissue compartments and also showed differences between segments and estrous phases as depicted in Fig. 1. Variations were observed with respect to the intensity of the positive staining and regarding proportions of positive/negative nuclei in all oviductal tissue compartments. The semiquantitative results of the positive cells by means of ERα intensity and proportion in the epithelium, subepithelial connective tissue or stroma and smooth muscle are described in Table 1.

In the surface epithelium, the immunohistochemical localization of ERα was obvious in all segments of buffalo oviducts during the follicular phase (Figs. 1A, C, E, G). The inset in Figure 1a demonstrates the negative control for ERα immunostaining. The intensity and proportion of ERα immunoreactivity in the UTJ and isthmus show significantly lower (p<0.05) at the luteal phase compared with the follicular phase (Table 1). The distribution of ERα positive labeling in most animals was found to be normally spread out along the surface epithelium of all segments. In the ampulla and infundibulum at the late luteal phase, ERα immunoreaction staining in the bulbous protrusions with nuclei of the epithelium was noticed.

In the subepithelial connective tissue, the ERα localization was stained in the nuclei of undifferentiated connective tissue cells but not all cells (Fig. 1). In addition, the staining intensity and proportion varied depending on the segments, estrous stages and individual animal. However, a stronger intensity and higher proportion were also significantly (p<0.05) observed in the UTJ and isthmus at the follicular phase than both parts at the luteal phase.

The smooth muscle nuclei, but not all nuclei, in the muscular layer of swamp buffalo oviducts reacted to ERα immunolocalization as well as the prominent intensity and proportion tended to be greater in the UTJ and isthmus during the follicular phase but the data were not shown any significant differences (Table 1).
Figure 1 Immunohistochemical staining of ERα in different compartments of the swamp buffalo oviduct at the follicular phase (A, C, E, G) and luteal phase (B, D, F, H) of the estrous cycle. The ERα nuclear positive cells were stained brown in the epithelium (Ep), subepithelial connective tissue (Sp) and muscular layer (Mu) of UTJ (A, B), isthmus (C, D), ampulla (E, F) and infundibulum (G, H) of buffalo oviduct.
Table 1  Immunohistochemical staining of ERα presented as manual scoring (intensity and proportion as shown by mean ± SD) in different tissue compartments of the Thai swamp buffalo oviduct

<table>
<thead>
<tr>
<th>Tissue compartments of oviduct/Phases</th>
<th>UTJ</th>
<th>IST</th>
<th>AMP</th>
<th>INF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>2.6±0.5/2.7±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5±0.4/2.7±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.6/2.6±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.6/2.7±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Luteal</td>
<td>1.5±0.5/1.4±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8±0.6/2.0±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9±0.6/2.0±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9±0.6/2.2±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Subepithelial connective tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>2.4±0.5/2.7±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5±0.5/2.4±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.4/2.4±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.5/2.6±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Luteal</td>
<td>1.5±0.5/1.7±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7±0.7/1.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7±0.7/1.7±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9±0.6/1.9±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>2.6±0.5/3.6±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6±0.5/3.6±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.7/3.0±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.7/2.8±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Luteal</td>
<td>2.0±0.8/3.0±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2±0.9/2.9±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8±0.7/2.4±1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9±0.8/2.2±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The different superscript letters between rows are significantly different (p<0.05)

UTJ: uterotubal junction; IST: isthmus; AMP: ampulla; INF: infundibulum

Discussion

The present study is the first to examine the distribution of ERα localization in Thai swamp buffalo oviduct during the follicular and luteal phase of the estrous cycle. The strongest intensity and highest proportion of ERα immunolabeling was particularly detected in the UTJ and isthmus of Thai swamp buffalo oviduct during the follicular phase whereas ERα immunoreaction in the ampulla and infundibulum did not show any differences between phases.

ERα immunoreaction in this study was detected in the nuclei of the luminal epithelial layer as well as nuclear staining of individual stromal and muscle cells which is in agreement with earlier studies of bovine (Ulbrich et al., 2003; Valle et al., 2007), ovine (García-Palencia et al., 2007) and other mammalian species (Press et al., 1986; Amso et al., 1994; Wang et al., 2000; Nielsen et al., 2001). Considering the presence of ERα immunoreaction in the bovine oviductal epithelium, Ulbrich et al. (2003) and Valle et al. (2007) indicated that ERα protein localization in the isthmus and ampulla was detected during all stages of the estrous cycle but was highest in the early luteal phase. In the buffalo, the appearance of ERα in our observations depicted greater nuclear staining in the epithelial cells particularly in the UTJ and isthmus at the follicular phase and weaker at the late luteal phase except for the ampulla and infundibulum. The present data indicate that the UTJ and isthmic epithelium could be the main target of ERα in the swamp buffalo oviduct and its expression may be up-regulated during follicular phase. Furthermore, the ERα localization in the epithelial cells of the UTJ and isthmus could also be correlated with the ERα mRNA transcripts which were elevated during follicular phase as presented in cows (Ulbrich et al., 2003). During the late luteal phase, a weaker staining intensity of ERα expression, investigated in the buffalo oviductal epithelium, was in agreement with previous reports in rats (Wang et al., 2000) and cows (Ulbrich et al., 2003). This may corresponded by several studies suggesting that progesterone down-
regulated ERα expression in target cells in female reproductive organs (Ing et al., 1996; Wu et al., 1996) including in oviductal cell compartments (Garcia-Palencia et al., 2007). To better understanding of the expression of ERα in Thai swamp buffalo oviduct throughout the estrous cycle, the other stages of cycle, especially the early luteal phase, including the plasma levels of estrogens and progesterone need to be done in the future study.

In the subepithelial layer, most of the connective tissue cells were positive at both phases in the present study but the immunostaining tended to be higher during the follicular phase and strongest in the UTJ and isthmus. Kimmins and MacLaren (2001) indicated that estrogen receptors in connective tissue cells enable them to trigger the steroid responsiveness of the epithelium as shown in the study using knock-out mice (Kurita et al. 2000) and different species (Cooke et al., 1997; Robinson et al., 2001). This mechanism could possibly underlie the present results that observed an intense ERα nuclear staining of connective tissue and epithelial cells in the swamp buffalo oviduct. In addition, the up-regulation of ERα during the follicular phase might account for specific compositional changes in buffalo oviductal fluid occurring at this stage as it occurred in the heifer oviducts (Bergqvist et al., 2005). However, there is no report about the trigger mechanism of ERα in connective tissue on the smooth muscle cells therefore this regulation could be investigated.

Regarding the ERα staining intensity and proportion in the muscular layer of the buffalo oviduct, no significant variation was detected corresponding to that reported in heifers (Valle et al., 2007). The ERα immunoreaction in the present study tended to increase during the follicular phase similar to the studies in women’s oviducts with time of ovulation (Amso et al., 1994) and sow uteruses at proestrus and estrus (Sukjumlong et al., 2003). To date, there is a little known about the effects of estradiol on the muscular cells in the ruminant oviduct during estrous cycle. The study of sheep oviduct reveals that the characteristics of oviductal motility are consistently increased during estradiol treatment (Ayad et al., 1994) and the motility of porcine oviducts shows high activity at estrous (Rodriguez-Martinez and Einarsson, 1982; Rodriguez-Martinez et al., 1982). Therefore, it is speculated that the contractility of buffalo oviduct at follicular phase may involve in the estrogens which is the one important factor among several factors that function through its receptor in smooth muscular cells.

It is known that a marked change in cellular differences occurs in the ampulla and infundibulum of cow oviducts ascribed by scanning electron microscopy, the epithelium of these regions are densely covered with ciliated cells at the follicular phase while the secretory cells are dominated at the luteal phase (Abe and Oikawa, 1993). In the present study, we have found that the secretory cells of the ampulla and infundibulum extend beyond the ciliated cells as bulbous protrusions during the late luteal phase and this phenomenon was not found in the UTJ and isthmus similar to earlier study (Tienthai et al., 2008). Most authors consider this characteristic in the ampulla and infundibulum as a sign of cellular turnover (Wrobel et al., 1993), epithelial regeneration (Walter and Bavdek, 1997) or epithelial renewal (Eriksen et al., 1994). Previous reports suggest that ERα reactive staining is not well detected in ciliated epithelial cells of the cycling rat oviduct but is expressed in connective tissue stromal, muscle and secretory epithelial cells (Okada et al., 2003). Furthermore, the secretory cells in the UTJ and caudal isthmus of bovine oviduct synthesized hyaluronan and glycoproteins which were essential for the formation of sperm reservoir during estrus phase (Suarez et al., 1997; Bergqvist et al., 2005). This investigation might also support the mechanisms of estrogens and ERα in the specific production of secretory epithelial cells in the UTJ and isthmus of Thai swamp buffalo oviduct. In contrast, the appearance of ERα staining in the secretory epithelial cells in the ampulla and infundibulum may be involved in epithelial cell regeneration for the suitable functions in
these segments via intermediate molecules which are produced by ERα subepithelial connective tissue cells as clearly demonstrated in the mouse uterus (Cooke et al., 1997, 1998).

In conclusion, ERα localization was detected in all compartments of the Thai swamp buffalo oviduct at follicular and late luteal phases in which the ERα positive staining was greater in the UTJ and isthmus during the follicular phase, indicating the different regulations of estrogen mediated its own receptor to fulfill the functions of oviduct associated with the segmental variations throughout the estrous cycle.

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References


Blood Haematological-Cholesterol Profile and Antibody Titer Response of Broilers with Added Probiotic Containing both Bacteria and Yeast or an Antibiotic in Drinking Water

Sarinee Kalandakanond-Thongsong1  Boonrit Thongsong2*  Vivat Chavanankul2

Abstract

An experiment was conducted to investigate the haematological and cholesterol profiles and antibody titer responses to both Newcastle disease (ND) and Infectious bronchitis (IB) vaccines in broiler chickens after drinking water supplemented with either a commercial probiotic (an Active Elements®; AE) containing Lactobacillus plantarum and Saccharomyces cerevisiae, an antibiotic Chlortetracycline (CTC), or the probiotic plus the antibiotic. Four hundred one-day-old male Cobb broiler chicks were randomly allocated to 4 treatment groups designated as follow: a non-treated control (T1), a 0.05% CTC (T2), a 1:500 AE (T3), and a combination of 0.05% CTC and 1:500 AE (T4), respectively. The chicks were fed with a commercial broiler diet ad lib. and were reared on rice hull bedding in identical floor pens and in an environmentally controlled experimental room for 6 weeks. The result showed that the IB vaccine antibody titers of the probiotic supplemented broilers group at the 28 days of age were significantly (p<0.05) higher than those of the other groups. At 28 days of age, the chickens originated from the probiotic and antibiotic-probiotic combination groups showed significant (p<0.05) increases in the mean red blood cell count, the mean hemoglobin concentration, mean corpuscular haemoglobin concentration, the number of thrombocyte, monocyte, heterophil but show significant decreases in the number of lymphocyte. Finally at 42 days of age, the total serum cholesterol concentration was significantly higher (p<0.05) in the chickens supplemented with antibiotic and with antibiotic and probiotic as compared to the control chickens. It is concluded that using this probiotic as an alternative for antibiotic in commercial broiler production may be considered.

Keywords : broiler, antibody titer, cholesterol, haematology, probiotic, antibiotic

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Introduction

The control of infections and the enhancement of life performance through a non-antibiotic approach are urgently required because increases in microbial resistance to antibiotics and residues in chicken meat products can be harmful to consumers. Probiotics have been used in poultry management not only to enhance production performance (Mohan et al., 1996; Yeo and Kim, 1997; Jin et al., 1998) and to reduce mortality (Vicente et al., 2007) but also to develop and stimulate the immune response (Jin et al., 1997; Rolfe, 2000). The manipulation of gut microbial via the administration of probiotics influences the development of the immune response (Isolauri et al., 2001). In poultry, the probiotics can modulate the systemic antibody response to antigens (Huang et al., 2004; Koenen et al., 2004). This may correlate to the physiological changes in haematological and cholesterol profiles. Probiotic microorganisms (nonpathogenic bacteria and/or yeast) are one of the alternatives for growth promotion in poultry (Abdulrahim et al., 1996; Santin et al., 2001) although their modes of action are not entirely clear and their efficacy is inconsistent.

The *Saccharomyces* species have been widely used in human medicine in combination with therapeutic antibiotic administration to avoid diarrhoea (Surawicz et al., 1989) and in growing pig via oral administration to stimulate sodium dependent glucose absorption into jejunum (Breves et al., 2000). However, the use of them as feed additives to induce physiological changes in terms of haematological-cholesterol levels and the
antibody titer responses to Newcastle disease (ND) vaccine and Infectious bronchitis (IB) vaccine of birds is still under investigation. While other common probiotic strains such as *Lactobacillus* species used in broiler nutrition have a beneficial immunomodulatory effect (Zulkifli et al., 2000a; Koenen et al., 2004), an antibiotic such as Chlortetracycline (CTC) has been suggested for administration in feed or drinking water and in medication programs to promote growth in broilers. However, various results of changes in blood haematology and biochemistry have been found (Mohan et al., 1996; Panda et al., 2006; Murwani and Bayuardhi, 2007). Previously, Thongsong et al. (2008) reported that a combination of bacteria (*Lactobacillus plantarum*) and yeast (*Saccharomyces cerevisiae*) preparation as a probiotic supplement showed beneficially significant effects on accumulated body weight gain during the first two weeks after administration of this probiotic via the drinking water. Additionally, feed conversion ratio (FCR) and antibiotic residues of the probiotic supplemented birds were similar to those of the CTC supplemented birds. Furthermore, the percentage mortality of the probiotic supplemented birds was less than that of the non-treated control chickens. However, there are limited reports of the efficacy of this probiotic on physio-pathological changes in haematological and cholesterol profiles as well as the systemic antibody response after vaccination with some important broiler diseases vaccines.

The objective of the present study was to investigate the effect of the commercial probiotic (Active Elements®; AE, The Long Year Biochem. Coop. Ltd.) as a combination of bacteria and yeast, an antibiotic (CTC) and the probiotic plus antibiotic administration via the drinking water on the haematological and cholesterol profiles and antibody titer responses to ND and IB vaccines in broiler chickens.

**Materials and Methods**

**Animals**

One-day-old, male Cobb chicks were obtained from a commercial hatchery. They were vaccinated with live vaccine against Newcastle disease (ND; VGGA strain) and infectious bronchitis (IB; H120 strain) upon hatching in the hatchery. They were vaccinated against Infectious Bursal Disease (IBD; V877 strain) on day 14 and against ND (B1 strain) and IB (Mass & Conn strain) on day 24 of broiler age. The vaccination programme was conducted according to recommendations from local veterinarians. The experiment was carried out for 42 days and other husbandries were previously published (Thongsong et al., 2008).

The experimental protocol was approved by the Animal Care and Use Committee of Chulalongkorn University.

**Probiotic and antibiotic**

The probiotic (Active Elements®; AE, The Long Year Biochem. Coop. Ltd.) is a commercial liquid preparation. It is composed of both bacteria and yeast: *Lactobacillus plantarum* (1x10^10 cfu) and *Saccharomyces cerevisiae* (1x10^9 cfu). The antibiotic, purchased from a commercial company was chlortetracycline hydrochloride (CTC). The antibiotic and the probiotic were mixed in the drinking water individually at a final concentration of 0.05% CTC or 1:500 AE as suggested by the manufacturer.

**Experimental design**

Four hundred 1-day-old broiler chicks were randomly allocated to 4 treatments, with 4 replicates per treatment and 25 chicks per replicate. The treatments were assigned either with their drinking water containing no additive (T1); 0.05% CTC (T2); 1:500 AE (T3); or a combination of CTC and AE (T4). The drinking water was freshly prepared everyday. For the T4 group, the AE or the CTC was given separately in the morning and in the evening, respectively.

**Collection of blood samples**

Blood samples were collected weekly from the birds via the wing vein of 8-12 birds per treatment before
flock vaccination to assess the systemic antibody titer response to ND and IB. Blood samples were labeled, kept at room temperature for 2 hours and then at 4°C overnight for separated serums. On 28th and 42nd day of the experiment, whole blood was collected to determine the haematological and plasma cholesterol profile. All blood samples were kept on ice. The plasma and serum were kept deep frozen prior to analysis.

Haematological and differential leukocyte examinations

The following haematological parameters such as red blood cell (RBC) and white blood cell (WBC) counts, haematocrit (Hct), haemoglobin (Hb) concentration, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were determined. Differential leukocyte counts to determine the differential percentage of white blood cells; lymphocytes, heterophils, basophils, monocytes and eosinophils, were performed manually for at least 100 leukocytes in blood smears (Ross et al., 1976). The heterophil to lymphocyte (H/L) ratios were used as an index of stress status.

Serological and cholesterol analysis

The serum immune response was evaluated for maternal immunity and acquired immunity in response to vaccination. To determine the specific antibody production in response to ND and IB, a direct haemagglutination inhibition (HI) assay was used. The HI tests were done by standard methods (Alexander 1989) using 8 haemagglutination (HA) units of the La Sota strain of ND virus and 4 HA units of the M 41 strain of IB virus. The plasma cholesterol concentration was determined according to Allain et al. (1974) by enzymatic method.

Statistical analysis

A general linear model procedure (SAS version 9.0; SAS Institute, Cary, NC, USA) was used to estimate the least square means of the different variables. The effect of feed additives was evaluated within the 4x2 or 4x6 experimental design with four treatments (control and feed additives) and weeks 4 and 6 for all haematological and cholesterol profiles or weeks 1-6 for ND and IB titers: $y_{ijk} = \mu + A_i + W_j + (A_xW)_{ij} + e_{ijk}$, where $y_{ijk}$ is the dependent variable; $\mu$ is the overall mean; $A_i$ is the fixed effect of types of feed additive ($i = 1, 2, 3, 4$); $W_j$ is the fixed effect of weeks of blood sampling ($i = 1, 2$ for haematological and cholesterol profiles; $j = 1, 2, ..., 6$ for ND and IB titer determination); $(A_xW)_{ij}$ is the interaction between feed additive and weeks; $e_{ijk}$ is the error term.

To compare the effect of feed additive on a weekly basis, each supplementation was evaluated as the only independent variable: $y_{ij} = \mu + A_i + e_{ij}$, where $y_{ij}$ is the dependent variable; $\mu$ is the overall mean; $A_i$ is the fixed effect of types of supplementation ($i = 1, 2, 3, 4$); $e_{ij}$ is the error term.

The effect of age was evaluated across types of supplementation with weekly blood sampling as the only independent variable: $y_{ij} = \mu + W_j + e_{ij}$, where $y_{ij}$ is the dependent variable; $\mu$ is the overall mean; $W_j$ is the fixed effect of weeks of blood sampling ($j = 1, 2, ..., 6$ for ND and IB titer determination); $e_{ij}$ is the error term.

The data was considered statistically significant at $p<0.05$; tendency was assigned as $0.05 < p< 0.10$.

Results

Haematological indices and differential leukocyte counts

The effects of feed additives on the haematological parameters are shown in Table 1. On day 28 of broiler age (4th week), the data revealed that the total red blood cell count (RBC), Hb and MCHC were significantly higher in broilers receiving AE and CTC+AE. The thrombocyte in AE treated groups was also significantly elevated compared to other groups. MCV was lowered in the CTC, AE and combination of CTC and AE treated groups compared to the control. Although the total white blood cell count (WBC) was unaffected by treatments, the percentages of
heterophil and lymphocyte in the combination group were affected. Consequently, the H:L ratio in this group was significantly elevated. The effects of feed additives on these parameters were lessened as the broilers became older. As shown in Table 1, at the age of 42 days (6th week), the feed additives had no effect on haematological parameters except that the MCV in the CTC treated broilers was smaller than the control group but not different from the AE or AE+CTC groups. The age of broilers had some impact on the level of complete blood count (CBC) in that the CBC was increased in older broilers; consequently, the Hb and Hct were increased as the CBC was elevated with no effect on MCV, MCH and MCHC. Moreover, the percentages of heterophil and lymphocyte were increased and decreased, respectively as the broiler aged; correspondingly the H:L ratio was then elevated. Furthermore, the statistical analysis also demonstrated that there was an interaction between age of broilers and the types of feed additive on the RBC, Hb, MCV, MCHC, thrombocyte, monocyte and basophil.

Total cholesterol in blood plasma

The effects of probiotic and/or antibiotic supplementation on the total cholesterol in broilers on day 28 and 42 of broiler age are shown in Figure 1. The figures show that probiotic and antibiotic supplements induced numerical decreases in total plasma cholesterol on day 28 of broiler age. Subsequently on day 42 of broiler age, there was a markedly elevated level of total cholesterol \( (p<0.05) \) in the antibiotic supplemented groups (CTC and CTC+AE) compared to the control group.

Systemic antibody titer response to Newcastle disease (ND) and Infectious bronchitis (IB)

The effects of feed additives on the weekly antibody titers against Newcastle disease (ND) and infectious bronchitis (IB) are presented in Table 2 and Figures 2-4. The antibody titer response against ND was detected with no significant difference among the experimental groups \( (p>0.05) \). However, the weekly ND titer was changed according to the age of broilers \( (p<0.05) \); we found that the titers dropped dramatically from week 1 to week 4 (Figure 2). After re-vaccination, the titer was increased as was evident in week 5 and then dropped to a lower level on week 6. Interestingly, at week 5 following the re-vaccination, in broilers receiving both CTC+AE, it was likely that their immunity did not respond as well although this failed to appear statistically significant as shown in Figure 2.

For the antibody titer against IB, the statistical analysis demonstrated the effects of feed additives as shown in Figure 3. At week 2, while other groups were able to maintain their antibody titer levels, the broilers receiving both CTC and AE were not; their titers were significantly lower than other groups. While the antibody titers dropped continually from week 1, it is obvious that the broilers receiving AE were able to maintain their titer levels better than others as seen at week 4, their titers were significantly higher than other groups. The age of broilers had a significant effect on the antibody titers against IB as shown in Figure 4. During the first 2 weeks, maternal immunity was maintained and then dropped continually to week 6. The re-vaccination of IB was not likely to affect broiler immunity as the titers continued to drop although were likely to elevate at week 6 (Figure 4). It should be noted that there is an interaction between the age of broiler and the types of feed additive on IB titer \( (p<0.05) \).

Discussion

In this study, we evaluated the effects of feed additives: antibiotic, chlortetracycline; probiotic, the combination of bacteria and yeast \( (L. \) plantarum and \( S. \) cerevisiae, respectively); and the combination of antibiotic and probiotic on haematological profiles, cholesterol level and antibody titers against ND and IB when the feed additives were supplemented for 6 weeks. It is widely accepted that haematological characteristics play an important role in the pathophysiology of chickens such as ascites in broilers (Maxwell et al., 1986; Lunger
### Table 1  The effect of feed additives (antibiotic and probiotic) on haematological profiles of broilers at 4 and 6 weeks.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age (Wk)</th>
<th>control</th>
<th>CTC</th>
<th>AE</th>
<th>CTC+AE</th>
<th>SEM</th>
<th>P value&lt;sup&gt;2&lt;/sup&gt;</th>
<th>A</th>
<th>W</th>
<th>AxW</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (x 10&lt;sup&gt;6&lt;/sup&gt;/μl)</td>
<td>4</td>
<td>1.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.59&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.0115</td>
<td>0.0082</td>
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<td></td>
<td>6</td>
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<td>2.59</td>
<td>2.56</td>
<td>0.05</td>
<td>0.2047</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>4</td>
<td>12.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.86&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>25.75</td>
<td>25.00</td>
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<td>27.88</td>
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<td>0.7605</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>4</td>
<td>135.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>103.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.29</td>
<td>0.0002</td>
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<td>MCH (pg)</td>
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<td>59.59</td>
<td>61.74</td>
<td>61.91</td>
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<td>50.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.50&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>52.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.70</td>
<td>0.0207</td>
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<td>0.8334</td>
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<td></td>
<td>6</td>
<td>56.25</td>
<td>57.00</td>
<td>42.38</td>
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<td>36.38</td>
<td>0.4660</td>
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<tr>
<td>WBC (/μl)</td>
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<td>6694</td>
<td>5356</td>
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<td>5900</td>
<td>591</td>
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<td>265</td>
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<td>Heterophil (%)</td>
<td>4</td>
<td>24.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.45</td>
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<td>Lymphocyte (%)</td>
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<td>68.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.75&lt;sup&gt;e&lt;/sup&gt;</td>
<td>55.50&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.57</td>
<td>0.0196</td>
<td>0.5654</td>
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<td>48.38</td>
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<td>51.50</td>
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<td>0.9050</td>
<td></td>
<td></td>
<td></td>
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<td>Monocyte (%)</td>
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<td>5.88</td>
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<td>6.88</td>
<td>3.88</td>
<td>6.25</td>
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<td>0.69</td>
<td>0.1143</td>
<td></td>
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<tr>
<td>Eosinophil (%)</td>
<td>4</td>
<td>1.13</td>
<td>2.63</td>
<td>1.25</td>
<td>0.38</td>
<td>0.33</td>
<td>0.1005</td>
<td>0.1365</td>
<td>0.0521</td>
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<td>0.63</td>
<td>0.00</td>
<td>0.26</td>
<td>0.5625</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Basophil (%)</td>
<td>4</td>
<td>2.75</td>
<td>3.13</td>
<td>2.63</td>
<td>1.75</td>
<td>0.43</td>
<td>0.7215</td>
<td>0.9964</td>
<td>0.0009</td>
<td>0.3693</td>
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<td>0.88</td>
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<td>0.88</td>
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<td>0.28</td>
<td>0.4612</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H : L ratio</td>
<td>4</td>
<td>0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04</td>
<td>0.0237</td>
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<td>0.2595</td>
</tr>
<tr>
<td></td>
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<td>0.95</td>
<td>1.05</td>
<td>1.01</td>
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<td>0.07</td>
<td>0.8250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Data represents means from each treatment at different ages; CTC: chlortetracycline; AE: Active Element®.

<sup>2</sup>Compares the effect of feed additive on a weekly basis, each supplementation was evaluated as the only independent variable; different superscript denotes statistical significance at \( p < 0.05 \).

<sup>3</sup>Compares the effect of feed additives (A), ages (W; weeks 4 and 6) and the interaction between feed additives and age (AxW).
Table 2  The effects of probiotic and/or antibiotic supplementations on the levels of antibody titer against ND as determined on a weekly basis.

<table>
<thead>
<tr>
<th>Week</th>
<th>Control</th>
<th>CTC</th>
<th>AE</th>
<th>CTC + AE</th>
<th>SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>4.25</td>
<td>4.00</td>
<td>3.75</td>
<td>4.63</td>
<td>0.163</td>
<td>NS</td>
</tr>
<tr>
<td>Week 2</td>
<td>3.13</td>
<td>3.13</td>
<td>3.50</td>
<td>3.50</td>
<td>0.122</td>
<td>NS</td>
</tr>
<tr>
<td>Week 3</td>
<td>2.88</td>
<td>3.13</td>
<td>2.88</td>
<td>3.75</td>
<td>0.186</td>
<td>NS</td>
</tr>
<tr>
<td>Week 4</td>
<td>2.00</td>
<td>1.88</td>
<td>2.63</td>
<td>1.88</td>
<td>0.239</td>
<td>NS</td>
</tr>
<tr>
<td>Week 5</td>
<td>4.00</td>
<td>4.17</td>
<td>4.25</td>
<td>3.17</td>
<td>0.239</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>3.83</td>
<td>2.50</td>
<td>3.50</td>
<td>2.92</td>
<td>0.254</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data represent means from each treatment at different age; CTC: chlortetracycline; AE: Active Element®; NS, $p > 0.05$

Figure 1  The effects of probiotic and/or antibiotic supplementation on the total plasma cholesterol in broilers on day 28 (Wk 4) and 42 (Wk 6) of broiler age.

$^{a,b}$Different superscripts denote significant difference at $p<0.05$.

Figure 2  The effects of age on the levels of antibody titer against ND as determined on a weekly basis. The symbols represent means from each treatment and the symbol with the connected line represents means ± SEM of all treatments.

$^{a,b}$Different superscripts denote significant difference at $p<0.05$.

CTC: Chlortetracycline; AE: Active Elements®
Figure 3  The effects of probiotic and/or antibiotic supplementations on the levels of antibody titer against IB as determined on a weekly basis. The data represent means ± SEM from each treatment.

\(^{a,b}\) Different superscripts denote significant difference at \(p<0.05\).

CTC: Chlortetracycline; AE: Active Elements®

Figure 4  The effects of age on the levels of antibody titer against IB as determined on a weekly basis. The symbols represent means from each treatments and the symbol with the connected line represents means ± SEM of all treatments.

\(^{a,b}\) Different superscripts denote significant difference at \(p<0.05\).

CTC: Chlortetracycline; AE: Active Elements®
et al., 2001; Scheele et al., 2003). The normal range values of haematological parameters and differential leukocyte counts reported by Jain (1993) are as follows: RBC = 2.5-3.5 (x10^6/μl), Hb = 7-13 (g/dl), Hct = 22-35 (%), MCV = 90-140 (fl), MCH = 33-47 (pg), MCHC = 21-23 (%), WBC = 12000-30000 (/μl), Heterophil = 15-40 (%), Lymphocyte 45-70 (%), Monocyte 5-10 (%), Eosinophil = 1.6-6.0 (%), Basophil = rare. The values retrieved in this experiment were within the normal range. However, at the 4th week, we found that probiotics had a beneficial effect on haematopoiesis as the parameters related to red blood cells were better than the control. The effect of probiotics on the blood-forming process had been reported previously in piglets (Kander, 2004). This effect was likely to be limited to the growing period of broilers. As we found at the 6th week, none of these parameters relating to haematology was different among treatments indicating that the feed additives no longer had any beneficial effect. Moreover, age was likely to be a factor affecting the blood forming process as the RBC, Hb and Hct were higher at the 6th week compared to those at the 4th week. This data suggest that as the broilers grew up, the ability to synthesize red blood cells also increased.

The differences were not limited to red blood cells, we also found that the percentages of heterophil and lymphocyte were increased and decreased, respectively in broilers receiving both probiotics and antibiotics for 4 weeks. Subsequently, the H:L ratio in this group was elevated indicating that they were stressed. It is readily accepted that the increase in heterophil and H:L ratio are indicators of stress in chickens (Siegel and Gross, 2000; Zulkifli et al., 2000b; Post et al., 2003). The stress in this group may have been caused by the management, as this group received two different types of drinking water, one contained CTC and another contained AE, switching during the day. The switching in drinking water may have led to stress and be reflected by the elevated H:L ratio in this group. However, this effect was no longer seen at the 6th week of broiler age.

Cholesterol levels in avian blood are affected by age, heredity, nutrition and various diseases. In this study, the plasma cholesterol was measured at the 4th and 6th week of broiler age and the values were in the cholesterol range (125-200 mg/dl) in chicken reported by Clarenburg (1992). The plasma cholesterol levels at the 6th week were similar to those previously reported with a value of 120-150 mg/dl (Meluzzi et al., 1992; Panda et al., 2006; Murwani and Bayuardhi, 2007). Previous reports have shown that the serum cholesterol was lower in broilers receiving diets containing probiotics (Mohan et al., 1996; Jin et al., 1998). Additionally, Kalavathy and coworkers (2006) also reported that the cholesterol in the livers and carcasses of broilers was also lower in broilers fed with diets containing Lactobacillus at the 6th week of age. In the current study, we did not find any lowering effect of probiotics on plasma cholesterol at the 4th or the 6th week which is inconsistent with the previous reports. However, it should be noted that the plasma cholesterol at the 4th week seemed to be lower in probiotic groups compare to the control although not significantly. The difference between the results of this study and others could be due to the difference in strain/species of probiotic microorganism used or the route of administration. Further, the age of the broilers also played a part in the level of cholesterol; the older the broilers were the lower was the plasma cholesterol. It may be that cholesterol is required during growing period for the development of muscle and tissue. In order to assess the potential health benefits to consumers of cholesterol levels in broilers, we found that at the 6th week of broiler age, birds supplemented with the antibiotic (chloretetracycline with/without probiotic) showed a significant increase in plasma cholesterol levels. This agrees with the study of Murwani and Bayuardhi (2007) who suggested that antibiotic in feed and the medication programs of broilers can affect the lipid and hepatic metabolism of broilers and be reflected by an increase in serum cholesterol. Based on the studies to date, the mechanism responsible for the cholesterol-heightening effect of antibiotics is interesting.
The effects of probiotics on the immune response of poultry are interesting and complicated. In this study, there was no significant difference in the antibody titer responses to ND among groups. In contrast, Khaksefidi and Ghoorchi (2006) reported that the antibody titers against ND in broilers fed with diets supplemented with probiotics containing Bacillus subtilis was significantly higher at 10 days post-immunization compared to the control birds. The difference in antibody production observed in this experiment could be attributed to the probiotic microorganism and the route of administration. The beneficial effects of probiotics seem to be not only strain-specific but have additional factors or characteristics as well. For another antibody titer against IB, likewise ND, the titer was highest and gradually decreased as the broilers got older. At the second week of age, the broilers receiving a combination of CTC and AE had the lowest antibody titer; it was likely that they were unable to maintain their titers. This may be a stress effect as shown by the H:L ratio in this group later on. The change in differential leukocyte counts could mean that the antibody formation and cell mediated immunity of broilers drinking the probiotic or probiotic plus antibiotic, were affected. At week 4, although the antibodies continually decreased, broilers supplemented with probiotic alone had higher titer levels than other groups. This may be due to two main reasons; firstly, it could be that the probiotic was somehow prolonging the antibody level. Secondly, the probiotic may have enhanced the systemic response to specific antigens which had been reported previously (Haghighi et al., 2005; 2006). From these results, it is obvious that probiotics could be important in populations where the immune response is immature or weak during the life span (Fooks and Gibson, 2002). However, in vivo studies are needed to further elucidate the effects of probiotics.

In conclusion, the administration of probiotics composed of bacteria (L. plantarum) and yeast (S. cerevisiae) in broiler chickens for 6 weeks via the drinking water, starting on the second day of life, had beneficial effects. Interestingly, these effects were more pronounced at a young age during the growing period as can be seen by the higher antibody titer against IB, higher RBC and Hb implying a better blood forming process. Further, we have shown previously that the feed efficiency was also better during the first two weeks (Thongsong et al., 2008). It is then suggested that continually giving probiotics after the developmental period may have no further benefit. Further, it should be noted that at the end of the experiment there was no difference between the supplementation of chlorotetracycline and probiotics in term of performance, antibody titers and haematological profiles. On the other hand, the prolonged use of antibiotics like chlorotetracycline may have had some undesirable effects such as an increase in blood cholesterol. Therefore, using probiotic as an alternative for antibiotic in commercial broiler production should be considered.

Acknowledgements

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References


Effects of the Dietary Inclusion of Fish Meal, Rock Phosphate and Roxarsone on Arsenic Residues in Tissues of Broilers

Kris Angkanaporn¹* Kriengsak Daengprom² Chutarat Kiratisehwe³

Abstract

The effect of dietary ingredients such as fish meal, rock phosphate and roxarsone on the accumulation of arsenic residues in breast meat, liver and heart was examined in Arbor Acre broilers from days 1-43 of age. Broilers receiving diets containing fish meal had a significantly greater amount of arsenic in breast muscle than other groups. There was a positive correlation between muscular arsenic concentration and dietary fish meal levels. Withdrawal of fish meal for a week prior to slaughter reduced the muscular deposition of arsenic. Chicks receiving roxarsone had significantly higher arsenic level in the liver compared to other groups and arsenic levels decreased after withdraw. Neither calcium sources such as rock phosphate nor arsenic-containing growth promoters affected the deposition of arsenic residue in meat. In conclusion, fish meal was the major factor resulting in the arsenic contamination of breast meat in broilers. Roxarsone and rock phosphate do not affect the arsenic level in meat.

Keywords: arsenic, broiler meat, roxarsone, fish meal, rock phosphate

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Introduction

The production of good quality poultry meat is of the utmost importance in poultry farms worldwide. Concern over heavy metal contamination in poultry meat has become an issue of great importance in some countries. Arsenic is one of the elements contaminating the poultry production system in feed ingredients, water or growth promoters. Basically, the concentrations of arsenic in animal tissues depends mainly on the dietary concentrations of arsenic, absorption of arsenic and the homeostatic control mechanism of the body for arsenic (Doyle and Spaulding, 1978). Arsenic level can be used as an indicator of good quality chicken meat and is very important for those countries that export chicken meat. The maximum concentrations of total arsenic (ppm) allowed in frozen chickens in various countries showed that some countries, for instance Austria, France and the Netherlands do not allow the presence of arsenic residue in imported frozen chickens and some of them, such as Czech Republic condemn at a concentration greater than 0.1 ppm. This low level of arsenic residues will affect countries like Thailand, who export approximately 435,000 metric tons with a forecast 5% increase every year. The major markets of Thailand broiler production are the European Union and Japan. In most feed mills, the main feed ingredients used for broiler diets are corn, soybean meal and fish meal. Fish meal is known as a source of arsenic since fish acquires arsenic through the food chain in the sea. The level of arsenic in fish meal is approximately 2-20 ppm (Ammerman et al., 1973). Nonetheless, there are few studies that have reported the contribution and scale of contamination of arsenic in fish meal that will contaminate the meat. Other feed ingredients such as rock phosphate and growth promoters (roxarsone or 3-nitro-4-hydroxyphenylarsonic acid) are also known to contain some arsenic and their effects on meat deposition has not yet been clarified. Roxarsone in one of the phenylarsonic compounds used as feed additives in swine and poultry ration and has the least toxic (El Bahri and Ben Romdane, 1991). Therefore, the
objective of the present study was to examine the effect of fish meal, rock phosphate and roxarsone on the deposition of arsenic in the pectoral muscles, liver and heart of broilers.

Materials and Methods

Animals and diets

Two-hundred and ninety-seven, one day old, male and female Arbor Acre™ broiler chicks were randomly allocated into 11 treatments (3 replicates of 9 chicks in each treatment) based on similar body weights in each replicate. Each replicate of chicks was reared in a single-tier stainless steel cage. The room temperature was between 27-33°C and the relative humidity 60-80%. All chicks were vaccinated with Newcastle disease virus vaccine on day 10 of age. Water and feed were supplied ad lib. The corn-soybean meal based diets were formulated based on NRC (1994) and divided into 3 periods; period 1 from days 1-21, period 2 from days 22-35 and period 3 from 36-43 days. The lighting schedule was changed from Light: Dark (L:D) for 24:0 hr in the first two weeks of age to L:D for 16:8 hr for the rest of the experiment. The chicks were slaughtered at 43 days old. Chicks in treatment 1 were given the basal diets that contained imported dicalcium phosphate (India) as the phosphorus source. This group served as a negative control group. In treatment 2, chicks were given the basal diet plus fish meal and imported dicalcium phosphate. The level of arsenic in this diet was calculated to be approximately 0.5 ppm as shown in Table 2 and the diet was fed for all periods. Chicks in treatment 3 were given diet with the level of arsenic of 0.75 ppm for all three periods. The calculated level of dietary arsenic for chicks in treatment 4 was formulated to be 0.95 ppm and fed for three periods. Chicks in treatments 5 and 6 received diets similar to those given to chicks in treatments 3 and 4, respectively except that the diets were given in the first two periods. Diets free of fish meal (similar to treatment 1) were given in the third period. In treatment 7, chicks were given diet without fish meal and rock phosphate was used as the source of phosphorus while similar diets were given to chicks in treatment 8 except that the phosphorus source was monocalcium phosphate (Biofos™ MCP, USA). Chicks in treatments 9 and 10 were given diet without fish meal and dicalcium phosphate was used as the source of phosphorus. Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) (3-Nitro™, Alpharma, USA) was included in the diet at level 40 ppm (10% premix). The diets were fed for all three periods except in treatment 10 that the diet given in period 3 was diet without fish meal and roxarsone (as same as diet in treatment 1). In treatment 11, diet with fish meal, 40 ppm roxarsone and dicalcium phosphate were given to chicks for all periods. This group served as the positive control group.

Data collection

When chickens were 43 days old, they were tagged with plastic bands at the hock joint. Six chicks in each replicate weighing close to the group average were chosen and sent to the slaughter house. All chickens were fasted for 12 hr prior to slaughter process. The chickens were stunned by a procedure complied with the European Union directives and bled to death. Both sides of pectoral muscle were collected and kept frozen until analysis for arsenic concentration. The whole liver and heart were collected.

Determination of arsenic in feed and muscle

Arsenic in diets and feed ingredients were analyzed using methods in AOAC (1995). Feed samples were dried using dry ashing method. Magnesium nitrate was added onto the samples and dried using hot plate at temperature 375°C. The flask was then put on the oven temperature 450°C for 30 minutes so that the carbonate salt and excess magnesium nitrate will be oxidized. The residue was cool and then added with 2 ml of 8M HCl and 0.1 ml of KI to reduce As⁵⁺ to As³⁺. The solution was analyzed using Hydride Generation Atomic Absorption Spectrophotometer. The standard solution of 0, 0.05, 0.10, 0.15, 0.20 and 0.25μg were analyzed and plotted as the standard curve. The sample was measured
together with reagent blank and was calculated followed the standard curve. The limit of detection (LOD) was 0.07 ng/kg and percent recovery of 90%.

For arsenic detection in breast meat, liver and heart, samples were digested with solution of 65% nitric acid and 30% hydrogen peroxide in microwave digester. Subsequently the digested samples were measured for arsenic using a hydride generation atomic absorption spectrophotometer at wavelength 193.7 nm. The limit of detection of this method was 0.01 ppm.

**Statistical analysis**

Data are presented as mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA) (Steel and Torrie 1982). Multiple comparisons of means were performed using Student Newman Keuls test with a significant level at \( p < 0.05 \). The regression analysis and correlation between the dietary arsenic levels in groups 1-6 and amount of arsenic intake (average level of arsenic in each group \( \times \) average feed intake (g/bird/day) in groups 1-6) and muscular level of arsenic were examined.

### Table 1
Mean arsenic concentrations (ppm) determined in feed ingredients and tap water used in the experiment.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Mean arsenic concentrations (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>0.035</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>0.018</td>
</tr>
<tr>
<td>Full-fat soybean meal</td>
<td>0.037</td>
</tr>
<tr>
<td>Fish meal</td>
<td>12.77</td>
</tr>
<tr>
<td>Dicalcium phosphate (India)</td>
<td>0</td>
</tr>
<tr>
<td>Monocalcium phosphate (Biofos, USA)</td>
<td>4.70</td>
</tr>
<tr>
<td>Rock phosphate</td>
<td>23.6</td>
</tr>
<tr>
<td>Tap water</td>
<td>( 0.64 \times 10^{-3} )</td>
</tr>
</tbody>
</table>

### Table 2
Mean dietary arsenic concentrations (ppm) and overall mean determined in diets used in each period of the experiment. There are three periods of diets fed, period 1 from 1-21 days, period 2 from 22-35 days and period 3 from 36-43 days post-hatching.

<table>
<thead>
<tr>
<th>Group</th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
<th>Weighted mean concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.215</td>
<td>0.153</td>
<td>0.211</td>
<td>0.194</td>
</tr>
<tr>
<td>2</td>
<td>0.459</td>
<td>0.536</td>
<td>0.447</td>
<td>0.483</td>
</tr>
<tr>
<td>3</td>
<td>0.751</td>
<td>0.748</td>
<td>0.766</td>
<td>0.753</td>
</tr>
<tr>
<td>4</td>
<td>0.853</td>
<td>0.927</td>
<td>1.290</td>
<td>0.951</td>
</tr>
<tr>
<td>5</td>
<td>0.778</td>
<td>0.773</td>
<td>0.425</td>
<td>0.718</td>
</tr>
<tr>
<td>6</td>
<td>0.934</td>
<td>1.170</td>
<td>0.273</td>
<td>0.903</td>
</tr>
<tr>
<td>7</td>
<td>0.572</td>
<td>0.581</td>
<td>0.552</td>
<td>0.572</td>
</tr>
<tr>
<td>8</td>
<td>0.420</td>
<td>0.415</td>
<td>0.439</td>
<td>0.422</td>
</tr>
<tr>
<td>9</td>
<td>10.50</td>
<td>10.60</td>
<td>10.40</td>
<td>10.52</td>
</tr>
<tr>
<td>10</td>
<td>10.80</td>
<td>10.40</td>
<td>0.182</td>
<td>8.90</td>
</tr>
<tr>
<td>11</td>
<td>10.70</td>
<td>11.60</td>
<td>10.40</td>
<td>10.47</td>
</tr>
</tbody>
</table>
Table 3  Mean arsenic level (ppm) in breast muscle, liver and heart of broiler at 43 days post-hatching.

<table>
<thead>
<tr>
<th>Group</th>
<th>breast muscle</th>
<th>liver</th>
<th>heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.023±0.018&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.008±0.015&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.015±0.001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.187±0.040&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.131±0.074&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.120±0.025&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>0.327±0.058&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.259±0.165&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.212±0.071&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>0.457±0.190&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.239±0.174&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.275±0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.183±0.060&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.128±0.062&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.133±0.019&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.230±0.080&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.131±0.056&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.151±0.006&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>0.020±0.010&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.015±0.019&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.015±0.013&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>0.023±0.011&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.014±0.022&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.015±0.014&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>0.053±0.020&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.587±0.221&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075±0.022&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>0.023±0.010&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.158±0.064&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.019±0.013&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>0.463±0.090&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.430±0.145&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.305±0.074&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>Mean ± SD, n = 6

<sup>abc,d</sup>Means in the same column with different superscripts differ significantly (p < 0.05)

Figure 1  Regression analysis between dietary weighed concentrations of arsenic (ppm) and muscular concentration of arsenic (ppm)

\[ Y = 0.436X - 0.056 \]

\[ r = 0.63 \]

\[ p < 0.001 \]
Results and Discussion

Table 1 shows the level of arsenic in various feed ingredients used in the experiment. Rock phosphate and fish meal had the respectively high level of arsenic compared to other ingredients. Arsenic concentration in tap water was found to be very low (0.64 ppb). Arsenic levels were different depending on the feed ingredients in each diet (Table 2). Effect of fish meal on arsenic contamination of meat was examined in groups 1-6 of the experiment. In group 1, the diets contained no fish meal and the mean dietary arsenic level of three feeding periods was 0.194 ppm. The arsenic level increased proportionally in groups 2, 3 and 4, respectively (0.483, 0.753 and 0.951 ppm). Increased level of arsenic in fish meal contributed to the enhanced arsenic level in these groups. When fish meal was withdrawn in period 3 of experiment as shown in groups 5 and 6, the mean dietary arsenic level decreased to 0.718 and 0.903 ppm, respectively. The effect of phosphorus source was studied in groups 7 and 8 which these diets comprised arsenic at 0.572 and 0.422 ppm, respectively. In groups 9 and 10, arsenic in the form of growth promoter (Roxarsone) was used. The arsenic concentration was 10.52 ppm in group 9 and the average level decreased to 8.90 ppm since roxarsone was withdrawn in period 3. The diet used in group 11 was similar to those used in the local broiler production. It was found that the average level of arsenic was 10.47 ppm (Table 2).

Average concentrations of arsenic in breast muscle, liver and heart are shown in Table 3. Chickens in groups 4 and 11 had the highest amount of arsenic deposit in the breast muscle. Both groups received the diet containing fish meal. When the dietary level of fish meal increased, the level of arsenic in breast muscle also enhanced proportionally as shown in groups 1-4 of the experiment (0.02, 0.19, 0.32 and 0.46 ppm). The withdrawal of fish meal in period 3 markedly reduced (p<0.05) the arsenic contamination as in groups 4 and 6 compared to corresponding groups 3 and 5. Previous studies on arsenic contamination on human food in Thailand were reported based on poultry meat sampling from the markets (Aroonskul et al., 1992; Thaveetiyanont, 1978). Data was varied due to unknown husbandry regimen including diets used. In this study, the chickens were in the same plane of nutrition and environment. Factors affecting the arsenic contamination were studied in the same population of chickens so that they can be compared. There are three possible factors influencing the contamination of arsenic in muscle tissues, arsenic in a form of growth promoter, arsenic in phosphorus sources and arsenic in protein sources such as fish meal. Contamination of arsenic in feeding water is negligible due to its low level as shown in Table 1. From the result, it was clear that fish meal was the most important factor that caused the highest level of arsenic residues in muscle. Feed devoid of fish meal as in group 1 resulted in very small amount of arsenic deposition in muscle. The arsenic accumulation was enhanced when the level of fish meal in diets increased. The regression analysis of the amount of arsenic deposit in muscle and mean weighed values of arsenic in diets used in groups 1-6 were calculated and shown in Figure 1. The increased level of arsenic in these diets was due to arsenic in fish meal since other feed ingredients such as corn, soybean meal and dicalcium phosphate had very low level of arsenic. It was found that the linear equation was $Y = 0.436X - 0.056$ (Y is the level of arsenic in muscle and X is the level of arsenic in diets). The coefficient of correlation was statistically significant ($r=0.63, p<0.001$). From this regression equation, the highest level of fish meal inclusion in broiler diet should be less than 2.18% in order to minimize the level of arsenic in muscle to 0.10 ppm. Although fish meal was still included in the diet as a source of protein riches in sulfur containing amino acids, however, the utilization was reduced due to the effect of heavy metals contamination. The higher the arsenic in fish meal ingested, the more deposition was found in breast muscle. Feed ingredients such as fishes (Bennett, 1981; Pavelka and Sedicek, 1976), prawns (Edmonds and Francesconi, 1987) or shells (Shibata et al., 1992) have the respectively high levels of arsenic as they can accumulate arsenic through environmental contaminants. They can uptake arsenic in the sea in the form of inorganic arsenic for instance arsenate (Edmonds and Francesconi, 1987; Bennett, 1981) to organic arsenic in muscle (Penrose et al., 1977) in the form called arsenobetaine. Jongen et al. (1985) found that human subjects eaten seafood had significantly high level of arsenobetaine excreted in the urine. There was the distribution of $^{74}$As-labelled arsenobetaine.
throughout tissues of human volunteers whom were fed fishes labeled with $^{74}$As arsenobetaine. Moreover, less than 1\% of ingested arsenobetaine was detected in human 24 days after eating (Brown et al., 1990). It is speculated that the form of arsenic in breast muscle was likely to be arsenobetaine as those found in fish. Vahter et al. (1983) studied the metabolism of arsenobetaine in various species of animals. They found that arsenobetaine was highly absorbed in the intestine of mice and there was marked deposition of arsenobetaine in muscle of rabbits. Organic arsenic in the form of arsenobetaine did not have the biotransformation by animal body to other forms as found in inorganic arsenic but it excreted into urine. Organic arsenic in fish was difficult to transform in animal body (Lunde, 1977). In this study, chickens received fish meal for all three periods had substantially high level of arsenic in muscle and the level of arsenic significantly decreased when fish meal was withdrawn from the feed in period 3.

Chickens receiving various sources of phosphorus had very low level of arsenic in breast muscle, liver and heart. Despite high dietary arsenic concentrations, chickens receiving rock phosphate or monocalcium phosphate had very low deposition of arsenic in muscle compared to the control group. It was likely that the level of both ingredients used were low in the diets that made the total level lower than those from fish meal. Inorganic arsenic was found to be highly toxic when received in high amounts (Pershagen, 1981). This form of arsenic will be biotransformed by methylation process. However this process can be maximized when high concentration of inorganic arsenic such as $\text{As}_2\text{O}_3$ was ingested and this harmed the animals (Foa et al., 1984). The metabolites from the biotransformation will be excreted in urine. It is likely that there was little change in the form of inorganic to organic arsenic in chicken compared to marine animals which had high ability (Edmonds and Francesconi, 1987). Inorganic arsenic was well absorbed in chick intestine (Fullmer and Wasserman, 1985) but it was excreted effectively.

Chickens received diets containing roxarsone had low concentrations of arsenic in muscle (group 9). However, it is demonstrated that chicks receiving roxarsone throughout the period (group 9 and group 11) had significantly higher arsenic in the liver than other groups followed by those chicks receiving high dietary fish meal inclusion (groups 3 and 4). In heart muscle, arsenic level was highest in groups 3, 4 and 11. Chicks receiving roxarsone in both groups 9 and 10 had low levels of arsenic in the heart muscle. Those chicks receiving sources of phosphate had low arsenic levels in the liver and heart muscle. Withdrawal of roxarsone in period 3 reduced the level of arsenic in muscle (group 10) ($p$>0.05). Roxarsone is an organoarsenic compound used extensively in broiler industries as a growth promoter. The level of arsenic residues in breast muscle was as low as the control group when the roxarsone was withdrawn from the diet used in period 3. Garbarino et al. (2003) stated that nearly all the roxarsone in feed was excreted unchanged in the manure. Roxarsone can be metabolized by liver and kidney and excreted more easily than arsenic in the form of arsenobetaine that adhered to amino acids in muscle protein. Organic arsenic such as Roxarsone caused low tissue deposition of arsenic although chickens ate it every day. High accumulation of arsenic in chicken receiving roxarsone was in accordance with the result of Chiou et al. (1997) who found that arsenic contents in the liver significantly increased as level of roxarsone increased. Withdrawal of roxarsone in period 3 markedly decreased the arsenic level in the liver compared to those chicks fed roxarsone for the whole period. This finding was similar to the results in pig (Ferslew and Edds, 1979) and growing broilers (Proudfoot et al., 1991) that the arsenic contents in liver were lower after roxarsone was withdrawn from feed.

Conclusion

The present study showed that the arsenic residues in breast muscle of chickens resulted from the arsenic in fish meal. Inclusion of organic arsenic as a growth promoter resulted in little deposition of arsenic in muscle. Similar findings were observed when rock phosphate or monocalcium phosphate was used.

Acknowledgements

Financial support by the Ministry of Commerce, Thailand is acknowledged. We appreciated the valuable comment and assistance of Dr. Anand Sirimongkolkasem from Thai exported broilers association and Ms Pattanee Leksrisompong from Thai feed mill association. The
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References


ECG Quiz
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*Corresponding author

These lead II ECG strips were recorded from a 5 years old, female, spayed Shih Tzu that was referred to the Chulalongkorn University Small Animal Hospital with panting. Physical examination revealed pink mucous membrane, polyuria, polydipsia with normal appetite. Auscultation showed normal lung sound and arrhythmic heart sound. Thoracic radiograph revealed left heart enlargement (VHS=10.2) and mild interstitial lung pattern. All hematological and serum chemistry profiles were within normal limits.

Please answer before turning to the next page.

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Interpretation

Sinus arrhythmia with sinus pause and periodic second degree atrioventricular block

The heart rate was 90 beats per minute. The arrhythmic heart sound is corresponded to the ECG recording which shows some periods of sinoatrial (SA) pause. The pause may not be due to respiratory origin since the patterns of heart rate fluctuation do not decrease or increase progressively like we have seen in respiratory sinus arrhythmia and the periods of sinus pause are random. When heart beat stops, there are no P waves or QRS complexes and the intervals between beats are integers the normal P-P intervals. There are two types of SA pause. If the SA node just stops discharging, the term “sinus arrest” is defined. However, if the sinus impulse is generated but does not get through the conduction path between sinus and right atrium, the term “sinus block” is used. Please notice that there is no escape beat after the period of SA pause.

There are some P waves without the following QRS complexes (circle in tracing I and II). Thus, the periodic second degree AV block is also found. The presence of SA pause with coexisting AV block in this case suggests that the aberrant vagal discharge may be responsible. After atropine administration, the non conducted P wave disappears and the period of sinus pause is shortened. Since the heart rate before giving atropine is within normal limit and no clinical signs occur, therefore, no medication is needed in this case.
Ophthalmology Snapshot

Nalinee Tuntivanich

History

A 1-yr-old male Shih-Tzu was brought in to the ophthalmology clinic, small animal teaching hospital, Chulalongkorn University with a complaint of persistent cloudy cornea. This dog was in the past 2 weeks seen by a veterinarian. Topical antibiotic was prescribed but the cloudy cornea still remained.

Questions

1. What may cause persistent corneal opacity in this dog?
2. What is the treatment of the answer to question 1?

(For better quality, figures can be viewed in the TJVM website.)

Please turn to the next page for answers ......

Ophthalmic examination revealed corneal opacity, which was negative to fluorescein staining test. Slight inflammation of the bulbar conjunctiva was observed, together with mild degree of chemosis on the palpebral conjunctiva.

Figure 1. Ocular adnexa of a Shin-Tzu

Figure 2. Schematic diagram of figure 1.
Answers

Figure 3. Schematic diagram of figure 1 demonstrating cilia (arrow) embedded in the upper palpebral conjunctiva.

Figure 4. Ectopic cilium that impinges directly to the cornea causing constant irritation.

1. Ectopic cilia or atypical distichia.
2. The condition can be resolved by surgery to excise of hair and follicle or cryoepliation to freeze hair follicle prior to hair removal. If unerupted cilia can be seen subconjunctivally, it may be left untreated unless it erupts. Owners should understand that new abnormal hairs can grow from new site after surgery.

Comments

Ectopic cilia are abnormal hairs that may emerge singly or in clumps from meibomian gland through the palpebral conjunctiva. This condition is common in young adult Shih Tzu however other dog breeds can also be seen during the same age. Clinical signs of ocular discomfort such as blepharospasm and lacrimation may be noticed before the emergence of the cilia. If the cilia erupt through the conjunctiva, they usually impinge directly on to the cornea causing intense pain. When this occurs, corneal ulcer is usually present. Because the cilia are very small, they are often misdiagnosed. Careful ophthalmic examination under magnification is therefore necessary.

References

Ultrasound Diagnosis

Phiwipha Kamonrat

History

An eight-year-old, intact male, mixed breed dog was referred to the Chulalongkorn University, Small Animal, Veterinary Teaching Hospital following urine incontinence for a week. This dog also had a clinical sign of anorexia. No constipation or haematuria were reported. The owner noticed the swelling in the left perineal area. On physical examination, the dog was bright and alert. The rectal temperature, pulse rate and respiratory rate were all within normal ranges. Haematological examination revealed a mild anemia (4x10⁶ red blood cells/μl, 12 g/dl haemoglobin, and 36% haematocrit) and a very low platelet count (78x10³ platelets/μl). Biochemical examination showed a mild elevation of serum creatinine concentration (2.6 mg/dl). A large, firm, non-painful, round mass of tissue, approximately 10 cm in diameter, was palpated in the left perineal area. Differential diagnosis included perineal hernia and rectal diverticulum. Pneumocystography was performed, at which time the bladder was observed to be dislocated caudally into the left perineal area. There was no evidence of radiopaque urolithiasis. Ultrasonography was evaluated to further define the perineal hernia.

Ultrasonographic Findings

Real-time, ultrasonographic image was obtained using sagittal (Figures 1A and 2A) and transverse (Figures 1B and 2B) scans of the perineal mass, in ventral recumbency, revealed a large sac with an irregular hyperechoic wall, approximately 3-5 mm thick. A sac contained two well-defined structures. One was a large, predominantly anechoic, fluid-filled mass. The lumen contained some echogenic sediments and the wall was 1.1 mm thick, appeared as two thin hyperechoic lines. This structure was suggestive of herniating urinary bladder. The other adjacent structure was 3.2x4.7x5.5 cm in diameter. This mass had an oval-shaped appearance in sagittal section and echogenic butterfly-shaped in transverse section, corresponding to prostate gland. Two, 4x10 and 5x11 mm, anechoic, intraparenchymal cysts were presented bilaterally with a slightly irregular shape but no discernible wall. A transabdominal ultrasonographic examination was also performed. The urinary bladder and prostate gland were not visualized at normal location of the caudal abdomen and intrapelvic cavity. Other abdominal organs were within normal limits.
Diagnosis

Ultrasonographic diagnosis—A perineal hernia.

Comments

Perineal hernia can be demonstrated radiographically. Plain radiographs may show an air collection in the herniated intestine. Because it is noninvasive, ultrasound examination may be the next logical diagnostic procedure after plain radiography. It is helpful in distinguishing contents of a hernial sac, including loops of intestine, urinary bladder and prostate gland; these are usually readily identifiable by their characteristic sonographic appearance. In most instances, the herniated urinary bladder is easily imaged. It is seen as an anechoic urine-filled structure, surrounded by two thin hyperechoic lines of bladder wall (Finn-Bodner, 1995). The normal prostate gland is characterized as an oval and bilobed gland on the sagittal and transverse scans, respectively. The parenchyma is echogenic, homogeneous and finely textured, with a distinct hyperechoic capsule. The urethra
may be centrally or eccentrically located and seen as an anechoic round structure. Intraparenchymal cysts of varying size and number can be present, as detected in this study; these probably represent dilated acini and ducts secondary to hyperplasia (Mattoon and Nyland, 2002). Intestinal loops may be seen as linear structures containing anechoic fluid, hyperechoic gas with acoustic shadowing or echogenic mucus without acoustic shadowing (Penninck, 2002). The five ultrasonographic intestinal wall layers are readily recognized. Differential diagnosis of perineal hernia includes abscess, cyst, haematoma, lipoma and rectal diverticulum. Surgery performed in this dog confirmed the ultrasonographic diagnosis of a perineal hernia containing the urinary bladder and prostate gland.

References


What is Your Diagnosis
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Signalment
A 4-year-old male Shar Pei.

History
The dog had been continuously lost his weight for 2 months without inappetite. Mild vomiting was sometimes presented an hour after meal. When the dog had liquid food or water, there was no vomiting or regurgitation.

Clinical Examination
The dog was seriously emaciated. None abnormal mass could be found via abdominal palpation. Complete blood count profile revealed mild anemia. Liver and renal profiles were within normal limits.

Radiographic Examination
In addition to plain abdominal radiographs, 60 minutes after 30% w/v barium sulphate suspension administration radiographs of the right lateral and ventrodorsal views were taken to evaluate gastrointestinal tract abnormalities.

Figure 1. A, B. Right lateral and ventrodorsal abdominal radiographs.
Figure 2. A, B. Right lateral and ventrodorsal abdominal radiographs taken at time 60 minutes after 30% w/v barium sulphate suspension administration.

Give your diagnosis and turn to the next page.
Radiographic findings

Plain radiographs (Figures 1A, B) revealed caudal displacement of small intestine, transverse colon and spleen. There were several particles of small radiopaque foreign bodies depositing in the pyloric antrum (Figures 3). The Right lateral and ventrodorsal abdominal radiographs at time 60 minutes after 30% w/v barium sulphate suspension administration (Figures 2A, B) showed retention of most of contrast media in dilated stomach. There was only few amount of barium sulphate passing through small bowel. The right lateral radiograph (Figures 2A, 4) indicated the thickness of the pyloric wall (approximately 1.5 cm.). The ventrodorsal radiograph (Figures 2B, 5) showed a hemispheric filling defect in the pylorus that protrudes into the lumen.

Radiographic diagnosis

Chronic hypertrophic pyloric gastropathy (CHPG), Chronic pyloric obstruction.

Discussion

CHPG can be evaluated by a thickening of pyloric mucosa or muscle or both compartments which is caused by an obstruction of pylorus. This abnormality is usually found in brachycephalic breeds such as boxers and bulldogs. Vomiting after eating solid food is the most common clinical sign that does not occur after eating liquid food or water.

Radiographically, dogs with CHPG have various gastric size from normal to marked enlargement. In order to evaluate this gastric disorder, using barium sulphate swallow will show both delayed gastric emptying (physiological disorder) and abnormal pyloric wall appearance (anatomical disorder). Delayed gastric emptying with retention of most of barium instomach is a significant sign of pyloric obstructive disease. An appearance of a thickening of pyloric wall from contrast radiograph can help to indicate a CHPG. In addition, CHPG may be detected ultrasonographically by measuring a pyloric muscular thickness, which was reported to be greater than 3 mm in mild to moderate and greater than 8 mm in severe chronic pyloric hypertrophy.

References