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HISTOLOGICAL AND HISTOCHEMICAL PICTURE OF ALKALINE PHOSPHATASE IN *STAPHYLOCOCCUS AUREUS* MASTITIS

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ABSTRACT

Cryostat sections of 7 µm thickness, obtained from the udder of diseased (*S. aureus* was isolated both from the milk and udder parenchyma) and clinically normal control animals (no isolation of *S. aureus*) were incubated for histoenzymatic demonstration of alkaline phosphatase (ALP). The results demonstrated that the epithelial cells of the diseased udder presented significantly ($P < 0.001^{***}$) higher levels of ALP than the endothelial cells of the healthy udder (7.69 ± 0.49 *versus* 5.11 ± 0.11). This study showed that the changes in ALP activity in the secretory cells of the diseased mammary gland caused by *S. aureus* could lead to qualitative changes in the milk. Also the somatic cell count in milk was increased. All these changes can have negative effects on the further processing of milk, such as heating (pasteurisation efficiency, renneting, fermentation).

Key words: alkaline phosphatase; histochemistry; histology; mammary gland; *S. aureus*

INTRODUCTION

Numerous enzymes have been demonstrated in milk. The enzymes normally present include those that are constituents of the leukocytes and those that are transferred from the blood of the animal to its milk. Some additional enzymes have been detected in milk, but insufficiant work has been done to demonstrate conclusively their presence in milk as it comes from the cow. There are also enzymes in milk that result from microbial contamination or other foreign sources.

The distribution of various enzymes in the milk is rather specific. Some enzymes are associated with the casein micelles, fat globules or leukocytes. Batavani *et al.* (23) reported in native dairy ewes from 2 weeks after lambing until the 10th week postpartum, that the mean activity of alkaline phosphatase (ALP) was higher in milk from subclinical infected udders than in milk from healthy udders ($P < 0.01$). Also Babei *et al.* (1) reported that the mean activitiy of ALP was higher in the milk from udders with subclinical mastitis (SCM) than in the milk from healthy udders ($P < 0.05$). The maximum agreement rates between the results of the California Mastitis Test (CMT) and ALP values were seen according to biochemical investigations at the thresholds of > 180 IU.l^{-1} and > 40 IU.l^{-1} respectively (κ values 0.65 and 0.79, respectively). The results showed that the ALP test was a reliable parameter for the early diagnosis of subclinical mastitis.

ALP is the enzyme found primarily in the epithelial cell membranes of the bovine mammary gland where the active processes take place (15, 16). It was observed that the activity of ALP was significantly increased in cows affected with mastitis.

Mastitis is characterized by physical, chemical changes in the milk and by pathological changes in the glandular tissue (29). Although there is swelling, heat, pain and induration in the mammary gland in many cases, a large proportion of the mastitis glands is not readily detectable by manual palpation or by visual examination of the milk using a strip cup. Because of the very large numbers of such subclinical cases, the diagnosis of mastitis has come to depend largely on indirect tests which depend, in turn, on the leukocyte content in the milk from the affected glands.

Nowadays there are available various techniques, such as radiography, ultrasonography (5, 11, 22) and theloscopy (6, 7), which have mainly curative purposes and focus on treatment of teat injuries and stenosis in high-yielding dairy cows. Vangroenweghe

et al. (26) introduced the application of an endoscopic technique to investigate the teat and udder cisterns of the bovine mammary gland, and to biopsy tissues within the cisterns.

Still, there is little information about the histochemical analysis of alkaline phosphatase activity in the epithelial cells of glandular tissue as a response to the pathological process caused by different pathogenic bacteria. Many infectious agents have been implicated as causes of mastitis in cattle. Generally, in relation to control procedures, they can be divided to those causing contagious mastitis, particularly *Streptococcus agalactiae, Staphylococcus aureus,* agents of environmental mastitis, e.g. *E. coli, Streptococcus dysgalactiae, Streptococcus uberis,* and to normal teat flora, such as *Staphylococcus hyicus, Staphylococcus epidermidis,* coagulase-negative staphylococci and *Corynebacterium bovis* (22).

The aim of this study was to evaluate the application of a histochemical technique to demonstrate the activity of alkaline phosphatase located in the epithelial cells of the glandular tissue and endothelial cells of the capillaries of the udder parenchyma.

MATERIAL AND METHODS

Material for this study was obtained from slaughtered cows in Slovakia. A total of 80 samples were taken for the bacteriological investigations (40 samples of the milk before the cows were slaughtered and 40 samples of the mammary gland proper. In this study, we used only the mammary glands with positive bacteriological findings when examined for the presence of S*taphylococcus aureus* in the milk and in the parenchyma of the mammary gland. The samples of the mammary gland were used for the histological evaluation and for demonstration of alkaline phosphatase activity in the epithelial cells of the glandular tissue and endothelial cells of the capillaries of the udder parenchyma.

For bacteriological evaluation, samples of both the milk and mammary tissue were taken from the same site. They were collected aseptically, and then refrigerated and transported to the laboratory at 4 °C to await further examination. For *S. aureus* detection, 0.1 ml of homogenate with subsequent dilutions were inoculated onto the surface of Baird-Parker agar (Hi Media, India) and spread evenly with a sterile bent glass rod until the surface appeared dry. The plates were incubated at 37 °C for 24 to 48 h. Duplicate plates were prepared from the specimens. After incubation, *S. aureus* were determined as Gram-positive, catalase-positive, oxidase-negative, and tube coagulase test-positive cocci with rabbit plasma (Imuna Šarišské Michaľany, Slovak Republic), giving positive results for cytidine deaminase test as described by Krasuski *et al.* (14).

The haemolytic activity was determined on blood agar (defibrinated sheep blood) at 37 °C for 24 h. The type of haemolysis was recorded as α-, β-, or dual (α + β).

The methicilin susceptibility was determined by the standardized agar diffusion test on Muller-Hinton (Oxoid) using the discs of methicilin (10 μg). Isolates were categorized as susceptible, resistant and intermediate resistant based upon interpretative criteria developed by the National Committee of Clinical Laboratory Standards (18).

PCR amplification. The colonies of *S. aureus* were confirmed by PCR amplification of the species-specific 440-bp fragment. The multiplex methods used in this study were according to Sharma *et al.* (21), Strommenger *et al.* (24) and Tkáčiková *et al.* (25). The PCR product was analyzed on a 1.0 % agarose agar (Gibko, BRL, USA) diluted in 1X TAE buffer (20), stained with ethidium bromide (Amresco, USA) in concentration of 0.1 $\mu g.ml^{-1}$ and viewed under ultraviolet light. The 100 bp DNA ladder (Sigma) was used as a standard of the molecular weight.

Tissues (0.5 × 0.5 × 0.3 cm) for the microscopical evaluation were fixed in a solution of 4 % (v/v) formaldehyde, and stained with haematoxylin and eosin.

One centimetre cubes (1 × 1 × 0.5 cm) of mammary gland tissue (*pars glandularis sinus lactiferis*) were removed from cows post mortem within 10 minutes after slaughtering. The samples for histochemical evaluation were fixed in liquid nitrogen until processed. The blocks of the frozen tissue were cut to 7 μm sections of tissue on a cryostat at −21 °C using disposable steel blades. Tissue slices were transferred to glass slides and air dried. From each tissue segment, six sections were cut for alkaline phosphatase assay.

The demonstration of alkaline phosphatase activity was performed using the modified simultaneous azocoupling method (20). The incubation medium contained naphtol-AS-BI-phosphat (Sigma, Nemecko) and stabile diazone salt Fast Blue BB (Aldrich, Germany) in veronal acetate buffer (pH 9.2). The incubation was performed at 37 °C for 30 min. After incubation, sections were washed with distilled water to remove the incubation medium and to stop any reactions. Post fixation of the sections was performed in a solution of 4 % (v/v) formaldehyde for 10 h at 20 °C. The sections were rinsed in distilled water and mounted in glycerine jelly.

The enzyme activity was cytophotometrically analyzed with a Wickers M85 microdensitometer (USA). The measurements were performed using an × 40 objective, effectively scanning an area of 28.3 μm^2 and a scanning spot of 0.5 μm. The integrated absorbance was measured at a wavelength of 480 nm. ALP activity was calculated as the absorbance values recorded by the instrument in min/μm^3 epithelial cells ± S.E.M. The density of ALP investigated was determined in six sections of each sample at 10 sites corresponding to the epithelial cells in the mammary gland. Ten cows negative for *S. aureus* in the udder parenchyma served as controls (healthy).

The statistical evaluation of the results was carried out by the one-way analysis of variance (ANOVA). An unpaired Student *t*-test was used to determine the significance of the differences between the enzyme activity in positive and negative controls. In all cases $P < 0.05$ was selected as the minimal criterion for statistically significant differences.

RESULTS AND DISCUSSION

The bacteriological results of the udder parenchyma correlated with the microbiological results of the milk samples from the experimental animals (Table 1, 2). From the positive findings of confirmed *S. aureus,* both from the udder parenchyma and milk, 16 udders were used for the further testing.

Table 1. Bacteriological examination of the udder parenchyma (n = 40)

Isolates	Udder parenchyma	
	Total number	(%)
S. aureus	16	64
Total number	40	100

Totally, 240 colonies suspected of being *S. aureus* were confirmed by the PCR method. The numerical equality between milk and udder parenchyma samples was 98.25 % (Table 2).

Table 2. Numerical equality of microbiological examination of milk and udder parenchyma for *S. aureus* in experimental animals

	Samples			
	Raw milk		Udder parenchyma	
	Total number	%	Total number	%
Total number of animals	40	100	40	100
Positive	40	100	25	62.5
Negative	0	0	15	37.5
G^+, coagulase positive	40	100	16	64
S. aureus suspected colonies				
Isolates confirmed by PCR	44	100	232	96.67
Haemolysine α	29	55.77	184	79.31
Haemolysine β	23	44.23	48	20.69
Genes for staphylococcal enterotoxins production	0	0	0	0
mecA gene	0	0	0	0

The isolates of *S. aureus* from the udder parenchyma were mainly producers of haemolysin α (79.31 %). Haemolysine β was detected only in 20.69 % of the isolates of *S. aureus* from the parenchyma of the mammary gland of the experimental animals (Table 2).

It also follows from Table 2 that none of the strains of *S. aureus* isolated from the parenchyma of the mammary gland of the experimental animals were carriers of the *mecA* gene coding for methicillin resistance or carriers for the exotoxin genes, such as staphylococcal enterotoxins *sea, seb, sec, sed,* and *see.*

Staphylococcus aureus findings were equated with the histological picture of the investigated bovine glandular tissue. Microscopically, the *S. aureus* infected udder quarters of cows (28.57 %) showed an acute diffuse purulent mastitis characterised by the marked infiltration of neutrophils in the lumen of the acini. Examination of the inflamed glandular tissue revealed a large quantity of exudates and PMN in the lumen and interstitial tissues, whereas these signs were not observed in the noninflamed glands.

The atrophy of the udder parenchyma was detected in seven experimental animals. The mammary glands showed a chronic interstitial mastitis. Atrophied acini, proliferated fibrous connective tissue thickening of interlobular septa, chronic galactophoritis with squamous metaplasia of the lining epithelium and marked infiltration of lymphocytes were observed.

In comparison with the healthy tissue, diseased tissue exhibited more interalveolar stromal area, reduced alveolar luminal area, and more damaged and involuted alveolar epithelial area.

During the infection of the mammary glands, the tissue damage can initially be caused by bacteria and their products. Certain bacteria produce toxins that destroy cell membranes and damage milk-producing tissue, whereas other bacteria are able to invade and multiply within the bovine mammary epithelial cells before causing cell death (29).

Table 3 shows the values of ALP in the epithelial cells of bovine glandular tissue in the bovine udder (*Staphylococcus aureus* isolates) (diseased) and healthy bovine mammary glands (control). The data are means ± S.E.M. of 24 control cows and 16 diseased ones. ALP activity is given as the integrated absorbance in min/μm^3 epithelial cells at a wavelength of 480 nm. The alkaline phosphatase activity in the epithelial cells of the alveoli of the diseased glandular tissue was significantly increased in comparison with the controls. The alkaline phosphatase activity was stated in the epithelial cells from cows from the diseased udder in the value of 7.69 ± 0.49. In the group of animals with healthy mammary gland, the activity of ALP was significantly lower (5.11 ± 0.11) (Table 4).

Table 3. Microdenzitometric analysis of alkaline phosphatase in the epithelial cells of the bovine glandular tissue of experimental animals

Bovine glandular tissue	Alkaline phosphatase		
	Healthy n = 10	Diseased n = 10	Statistical significance
Epithelial cells	5.11 ± 0.11	7.69 ± 0.49	
minimum	4.95	6.85	P < 0.001 ***
median	5.10	7.91	
maximum	5.29	8.19	

The alkaline phosphatase activity in the endothelial cells of the capillaries in healthy animals was exhibited with a

moderate colouring of their structure, which was testified by microdensitometric analysis, in which the value of 4.17 ± 0.15 was detected (Table 4). There was not any significant difference in the activity of alkaline phosphatase in the endothelial cells of the control glandular tissue (4.39 ± 0.11).

Table 4. Microdenzitometric analysis of alkaline phosphatase in the endothelial cells of the bovine glandular tissue of experimental animals

Bovine glandular tissue	Alkaline phosphatase (AV/min/μm³) ± S.E.M.		
	Healthy n = 10	Diseased n = 10	Statistical significance
Endothelial cells	4.17 ± 0.15	4.39 ± 0.11	
minimum	3.95	4.25	ns
median	4.17	4.38	
maximum	4.37	4.59	

AV– absorbance value; ns – not significant

Elsayed *et al.* (4) reported, that the alkaline phosphatase enzyme was apparently on the outer surface of the alveolar secretory cells at the early and the mid stages of lactation, suggesting that this enzyme plays an important physiological role in the apical membrane of the alveolar epithelial cells during lactation.

The significant increase in alkaline phosphatase in the experimental mammary glands detected in our experiment is in agreement with earlier studies (12, 13). Moreover, our results revealed that the causative agents of mastitis (*Staphylococcus aureus*) did not stimulate a different ALP activity in the endothelial cells of the mammary capillaries. This is also in agreement with our previous study (16).

Our results also showed the close relationship between ALP activity and bovine chronic mastitis. Alkaline phosphatase is an enzyme which is found primarily in the cell membranes of the cow's glandular parenchyma. This is a very important part of the mammary epithelium, because of the active transport processes (19).

Wilson (27) reported, that the ALP increased gradually in the tissues of both udder halves in goats after inoculation of *Mycoplasma,* but the increase in this enzyme was more marked (about 2 times) in the right infected halves compared to the left halves (2). A histoenzymatic study also revealed an increase in the activity of ALP (also of ACP, LDH and SDH) in the infected right udder halves. The increased activity of the ALP enzyme in the udder might be due to marked aggregation of neutrophils (8, 9) and damage to the udder tissues (10).

Natural ALP in milk is very sensitive to heat treatment. That is why the detection of ALP in milk is used in the dairy industry for the evaluation of sufficient milk heat-treatment. On the other hand, ALP is also a constituent of the leukocytes which are present in milk in high quantities during mastitis. Neutrophils represent one part of the cell counts in bovine milk.

The physical tests carried out on milk in a mastitis examination are the individual cow cell count (ICCC) and its immediate development, the bulk milk cell count (BMCC). Indirect tests include tests such as the California mastitis test, Mastitis test-NK, the NAGASE test, a direct capture ELISA test and the Whiteside test which are dependent on the cell count. The methods of counting cells in milk include ICCC and BMCC both of which can be carried out by a direct microscopic examination of stained smears on a glass slide or the electronic somatic cell count done on a Fossomatic (28). The total count reflects the amount of the gland involved in the inflammatory process, whereas a high total count e.g. (10^6.ml^{-1}) and a high proportion of neutrophils (e.g. 90 %) indicate an acute inflammation affecting much of the quarter. A low total count (e.g. 500 000 ml^{-1}) and a low proportion of neutrophils (e.g. less than 40 %) indicate a small, chronic lesion. Attempts to carry out differential leukocyte counts on the automatic cell counting machines, using cell size as the differentiating criterion, have not provided greater accuracy in selecting infected quarters than the regular somatic cell count and are unlikely to be of practical value to determine the stage of udder inflammation. We supposed that the results of ALP activity determination in epithelial cells of bovine alveoli represents only the first step for the evaluation of ALP histochemical activity by a direct microscopic examination of stained milk smears on a glass slide. Thus these results should be helpful to producers of raw milk for human consumption to identify not only mastitis but also the stage of the inflammation. With regard to the present data, however, further research is required before definite conclusions concerning the causal relationship between increased alkaline phosphatase activity and the development of mastitis in lactating cows and its histochemical detection in milk can be drawn. Thus, these parameters might be suitable for use in the early diagnosis of mastitis in cows, including subclinical mastitis.

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ANALYSIS OF INCIDENCE OF THE CANINE STIFLE JOINT CRANIAL CRUCIATE LIGAMENT RUPTURE

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ABSTRACT

Cranial Cruciate Ligament Rupture (CCLR) is the most frequent adverse structural condition affecting the stifle joint in dogs. The exact cause of this rupture has not been determined; therefore, it is hard to talk about the prevention of this occurrence. Collecting more data on the possible breed predisposition, gender, age, and weight may facilitate the explanation of the etiology and pathogenesis of this disease. A retrospective study of the cases of CCL rupture in the canine stifle joint was carried out at the Small Animal Clinic of the University of Veterinary Medicine and Pharmacy in Košice for the time period between 2000 and 2009. The study included the clinical and radiological examinations of 195 dogs with the diagnosis of the CCL rupture. In addition, the dogs were divided into various groups by: breed; age; gender; weight category; and by the course of the CCLR formation. Most of the affected dogs were 5–8 years old (42 %) and in the weight category of above 30 kg (38 %). An increased predisposition for CCLR was observed in German Shepherds (7.7 %); Labrador Retrievers (7.7 %); and Rottweilers (6.7 %). The acute course of the CCLR was observed in 64 % of the dogs.

Key words: cranial cruciate ligament rupture; dog; etiopathogenesis; prevalence; stifle joint

INTRODUCTION

The cranial cruciate ligament (CCL), provides cranio-caudal stability, prevents hyperextension and constrains the medial rotation of the tibia in the canine stifle joint. The CCL rupture belongs to the most frequent causes of hind leg lameness; it is also the most frequent cause of the formation of degenerative disease of the canine stifle joint (1, 3, 5, 8, 11, 12, 15). The cause of rupture is often unknown and the optimal method of therapy remains disputable (7, 10). The CCL rupture can be the result of acute (traumatic) or chronic (degenerative) cause (6, 15). Most frequently it occurs among large dog breeds, active or overweight dogs. The function of the CCL is to stabilize the stifle joint, prevent movement of the tibia in a cranial direction in relation to the femoral condyles, and also to restrain the medial rotation of the leg distal from the stifle joint (1, 13, 14, 17). The clinical symptoms of the rupture of the cranial cruciate ligament (CCLR) depend on the course of the disease formation. Partial CCL rupture is connected with the short-lasting acute lameness which later develops into the 1st degree chronic lameness (18). The partial rupture over time leads to the total CCL rupture.

The total CCL rupture is clinically demonstrated by the 3rd degree acute lameness. It can be developed as a result of a chronic insult (crack) to the CCL, or can be connected with an acute trauma in which hyperextension and medial rotation of the knee occurs (18). Possible causes of the CCL rupture formation include the acute traumatic rupture of the CCL, the CCL rupture caused by ligament degeneration related to age or inactivity, or the CCL rupture in young dogs of large breeds (11). In the majority of cases, the ligament weakened by degeneration can be torn much easier than a healthy ligament (11, 18). The causes of the degeneration are various. More frequently it may be an age-related degeneration, growth abnormalities, or immune arthropathies; whereas more than one cause may be found in the same dog.

The aim of our analysis was to find out how the breed, age, gender, and weight of a dog relates to the CCL rupture prevalence.

Explanation of the disease pathogenesis would be facilitated by proving that the CCL rupture occurs within certain age range or with higher incidence in certain dog breeds, gender, or in dogs with extraordinary anatomy. The origin of the disease is thus multifactorial and until now the exact etiology of CCLR is not always defined for an individual.

MATERIAL AND METHODS

Results of the analysis of the CCL rupture in dogs treated at the Small Animal Clinic of the UVMaP are provided for the period 2000–2009 (10 years). Clinical data of the examined dogs with the CCL rupture were processed by our team on the basis of the retrospective trial. The CCL rupture diagnosis was determined on the basis of clinical examination of a patient by using the tibial compression test and cranial drawer movement. Radiological examination was carried out in two perpendicular projections; medial-to-lateral (ML) and caudal-to-cranial (CaCr), or cranial-to-caudal (CrCa), in order to assess the positions of femoral epicondyles in relation to the tibial plateau. Other radiological parameters noted were: the presence of subpatellar fat symptom; the presence of osteophytes on the femoral epicondyles; the patella in place of straight patellar ligament insertion, and also on the edges of articular cartilage of the proximal tibia.

The analysis includes all patients with the CCL rupture diagnosis. The evaluated group consisted of 195 dogs. The dogs were divided into groups by: breed; age (from 0–4, from 5–8, > 8 years); gender (female, male dog); weight (small breeds weighing up to 15 kg, medium-sized breeds 15–30 kg, large breeds above 30 kg); and by the course of rupture formation (acute, chronic). We also evaluated the body condition according to a 1–5 scale, where 1 – cachectic, 5 – highly obese. Our results were evaluated by percentage.

RESULTS

Evaluation of our group of 195 dogs showed that the highest number of dogs with the CCL rupture was among the crossbreeds. This group consisted of 53 dogs, representing 27.2 % of the whole group. The German Shepherd Breed, Labrador Retrievers and Rottweilers had the highest prevalence of CCL rupture of the purebreds represented. All dog breeds in our study with the CCL rupture are listed in Table 1.

In the gender category there was a slightly higher incidence of the CCL rupture in female dogs (105), which was 54 % (Table 2). A lower number of male dogs were affected by the CCL rupture, which amounted to 46 % of the total number in the group.

The evaluation of dogs with the CCL rupture showed the highest prevalence in the age range 5–8 years, including 81 dogs (42 %). In the age category over 8 years there were 55 dogs with the CCL rupture, which is 28 % of the whole group. The group of dogs with the CCL ruptures aged 0–4 years consisted of 59 dogs (30 %). Dogs aged between 5th and 8th year had the highest prevalence of the CCL rupture. Results are shown in Table 3.

Table 1. Incidence of cranial cruciate ligament rupture in dogs, by breed

Dog breed	Number	%
Crossbred dog	53	27.2
German Shepherd dog	15	7.7
Labrador Retriever	15	7.7
Rottweiler	13	6.7
German Spitz	10	5.1
Standard Poodle	9	4.6
Central Asian Shepherd Dog	7	3.6
Doberman Pinscher	6	3.1
American Staffordshire Terrier	5	2.6
Bichon Frise	4	2.1
Yorkshire Terrier	4	2.1
Boxer	4	2.1
Bullterrier	4	2.1
West Highland White Terrier	4	2.1
Chihuahua	3	1.5
Maltese	3	1.5
South Russian Shepherd dog	2	1.0
Schnauzer	2	1.0
Bavarian Mountain Hound	2	1.0
English Cocker Spaniel	2	1.0
Weimaraner	2	1.0
Bernese Mountain Dog	2	1.0
Siberian Husky	1	0.5
Tibetan Terrier	1	0.5
St. Bernard Dog	1	0.5
Mastiff	1	0.5
German Wire-Haired Pointer	1	0.5
Beagle	1	0.5
Hungarian Vizsla	1	0.5
Springer Spaniel	1	0.5
Giant Schnauzer	1	0.5
Great Dane	1	0.5
Dalmatian	1	0.5
Kerry Blue Terrier	1	0.5
Shar Pei	1	0.5
Bulldog	1	0.5
Bullmastiff	1	0.5
Bull Terrier	1	0.5
Rhodesian Ridgeback	1	0.5
Spanish Mastiff	1	0.5
Greater Swiss Mountain Dog	1	0.5
American Akita	1	0.5
French Mastiff	1	0.5
Dutch Shepherd Dog	1	0.5
Alpine Dachsbracke	1	0.5
Caucasian Shepherd Dog	1	0.5
TOTAL	**195**	**100.0**

Table 2. Incidence of cranial cruciate ligament rupture in dogs, by gender

Gender	Number	%
Females	105	54
Males	90	46
Total	195	100

Table 3. Incidence of cranial cruciate ligament rupture in dogs, by age

Age category	Number	%
0–4 years	59	30
5–8 years	81	42
Over 8 years	55	28
Total	195	100

Table 4. Incidence of cranial cruciate ligament rupture in dogs, by weight

Weight category	Number	%
Small breeds up to 15 kg	63	32
Medium-sized breeds 15–30 kg	58	30
Large breeds over 30 kg	74	38
Total	195	100

Table 5. Incidence of cranial cruciate ligament rupture in dogs, by course of rupture

Course	Number	%
Acute	124	64
Chronic	71	36
Total	195	100

There were 74 patients with the CCLR among large dog breeds weighing over 30 kg (38 %), 58 patients from the medium-size dog breeds weighing 15–30 kg (30 %), and 63 patients from small dog breeds weighing up to 15 kg (32 %).

The acute course of the CCL rupture occurred in 124 dogs, which is 64 % of our group. The chronic course of the CCL rupture occurred in 71 dogs (36 %). In all crossbreeds diagnosed for the acute formation of CCLR, the dogs had 4th or 5th degree of shape (mild or strong obesity).

DISCUSSION

Many existing publications provide the information on the CCL rupture incidence in dogs in relation to their age, breed, or weight. This knowledge enables better understanding of the pathogenesis. Findings observed by Whitehair *et al.* (20) are important; they examined the etiology of the CCL rupture in dogs. They compared the data on 10,769 dogs with the CCL rupture with the data of the control specimen of the population consisting of 591,548 dogs. They were examining whether the age, breed, gender, or weight of a dog relates to the prevalence of the CCL rupture. Breeds with the number of individuals included in their study higher than 1,000 included Rottweilers, Newfoundlands, and American Staffordshire Terriers. Such high representation of these breeds was explained by their popularity. Particularly these breeds showed the highest prevalence of the CCL rupture. In our analysis, the highest prevalence was in the German Shepherds and Labrador Retrievers 7.7 %, as well as Rottweilers 6.7 %, which proves that Rottweilers have a predisposition to the CCL rupture. In our situation, these breeds are very popular, so their number in the population can correlate to a higher incidence of the CCL rupture in our group. Most frequently there were crossbreeds, representing more than 27 % of the total group of dogs with the CCLR. Frequency of CCLR incidence among these and other breeds did not appear to be statistically important. According to some authors, the incidence of the CCL rupture is the same in both genders; mostly, however, female dogs are more often reported to be affected. Neutered dogs (male and female) are usually affected more often (11). Female dogs have a higher prevalence of the CCL rupture than male dogs. Neutered dogs have a higher prevalence than non-neutered dogs (20). At the same time, a higher incidence was observed among sterilized female dogs than among sterilized male dogs. Whitehair *et al.* (20) explained this statement by consideration of the higher incidence of obesity among sterilized female dogs. In our study, the number of affected male dogs (46 %) and female dogs (54 %) with the CCL rupture was almost identical. There were no neutered dogs in our study.

Another factor which could have had a predisposing influence on the insult to the CCL in some breeds, might have been higher weight causing increased loading on the CCL. According to Nečas (11), the CCL rupture was described in all weight categories of dogs, from dwarf breeds to giant breeds. Overweight individuals are most likely to be the most susceptible to ligament rupture. Dogs with weights over 22 kg had a higher incidence of the rupture than dogs weighing less than 22 kg (20). Large dog breeds are affected by the CCLR in a younger age, when compared to dogs of smaller breeds. The highest proportion of dogs with the CCLR in our group was represented by dogs weighing more than 30 kg. This category mostly consisted of Rottweilers, German Shepherds, and Central Asian Shepherds. As for medium-sized breeds (15–30 kg), the more frequent incidence of the CCL rupture was among American Staffordshire terriers, Labrador retrievers, and also crossbreeds. As for small breeds weighing up to 15 kg, the incidence of the CCL rupture was among York-

shire terriers, Bichon Frises, and German Spitzs.

The CCL rupture can occur in any age (11). It seems that the CCLR appears in dogs with aging, especially in large breeds, which means that depending upon the age of the cranial cruciate ligament, it can break during normal activity with a sudden jump or when rotating the limb when falling. Whitehair *et al.* (20) came to the conclusion that the prevalence of the CCL rupture was increasing with age in cases when it appeared between 7 to 10 years of age. Studies with smaller numbers of cases, stated that the age related to the CCL rupture which occurred in dogs was an average age of 6 years. In spite of that, Whitehair *et al.* showed that there was a linear growth of the occurrence of CCL rupture until the age of 10 years. In our group we have also observed the growth of the CCL rupture incidence with increasing age of a dog; the highest incidence was among dogs aged 5–8 years.

Damage to the *ligamentum cruciatum craniale* can cause acute or chronic course of formation and the ligament can break completely or partially. The etiology of the CCL rupture can be traumatic and non-traumatic (degenerative). A partial rupture may later develop into a total rupture, as the inflammation of the joint, the gradual weakness of cranial cruciate ligament, and as disturbed biochemical factors develop into increase cranial tibial pressure. Acute rupture occurs after the trauma with hyperextension and medial rotation of the tibia in the stifle joint. Insufficient use of a leg and loading of a leg, or immobilization of a leg, reduce the strength of the CCL; at the same time, other stabilization elements from the soft tissue get weaker, and thus the stability of joint is reduced. In this state, the chronic insults to the CCL begin. In our study we evaluated the incidence of the CCL rupture from the formation point of view. The difference in the percentage of the CCLR is statistically extremely important. As much as 64 % (124 dogs) with the CCL rupture in our group was formed acutely, while 36 % (71 dogs) had a chronic course, which was demonstrated also in the radiological examination by the presence of osteophytes on the edge of the articular surface, or after arthrotomy of the stifle joint.

The results of our analysis of the incidence of the cranial cruciate ligament rupture show that this diagnosis occurs most frequently in dogs of large breeds with excessive weight. Rupture of the cranial cruciate ligament was diagnosed in the largest number among the crossbreeds; as for the pure breds, the highest representation was from German Shepherds, Labrador Retrievers, and Rottweilers. We have observed that gender has no effect on the incidence of the CCLR. The highest incidence of the CCLR was observed in dogs aged 5–8 years. We also state that the excessive weight of a dog leads to a higher incidence of the CCLR. Therefore, in dogs, it is necessary to monitor the body weight, especially after the 5th year of a dog's life. If a dog has an adequate weight, the CCLR may be avoided.

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THE EVALUATION OF SOME INFECTIOUS BURSAL DISEASE VACCINES ON THE HUMORAL IMMUNE RESPONSE OF CHICKENS VACCINATED WITH NEWCASTLE DISEASE VACCINE IN NIGERIA

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ABSTRACT

The effect of four commonly employed infectious bursal disease (IBD) vaccines on the immune response of chickens vaccinated with Newcastle disease (ND) vaccine were assessed in pullets using a pre-existing vaccination regimen, depicting field situations. The antibodies to IBD was evaluated by the qualitative and quantitative agar gel immunodiffusion method while the haemagglution inhibition (HI) test was used for the detection of ND antibodies. The immunosuppressive effect was assessed by: the suppression of the ND titres; the bursa of Fabricius (BF) index; the BF diameter; and the BF histopathological lesions. The results showed that the IBD vaccines caused a transient immunosuppression in the ND vaccinated chicks. This immunosuppressive effect was non existent when a booster dose of Newcastle disease – Komarov vaccine (NDV-K) was administered. Both the BF index and diameter were significantly higher ($P < 0.05$) in the vaccinated groups when compared with the control unvaccinated. Histological lesions in the BF were more pronounced in birds that received vaccine III. There were no significant differences between the BF index and histological lesions as well as the BF diameter and histological lesions in vaccines I, II and IV. This study revealed that the IBD vaccines employed in this study were: immunogenic; did not induce IBD death in vaccinated birds; and were not immunosuppressive when combined with ND vaccination, as they did not depress ND antibody titres.

Key words: immunosuppression; infectious bursal disease; Newcastle disease; vaccines

INTRODUCTION

Infectious bursa disease (IBD), also known as Gumboro, is an acute, highly contagious disease of young chickens caused by an avibirnavirus of the family *Birnaviridae.* The disease has long been identified with long-term immunosuppression in young chickens (14) thus making infected birds increasing susceptible to other pathogenic microorganisms such as secondaery bacterial, viral or protozoan infections (7, 13). The extensive destuction of the bursa and the bursal associated lymphocytes during infection with the virus leads to immune antibody suppression in chickens (11). This immunosuppressive phenomenon is also reflected in chickens vaccinated with the live attenuated IBD vaccines (1, 11).

The immunosuppressive effects of IBD virus has previously been reported to adversely affect vaccination against Newcastle disease (3, 5). A number of IBD vaccines are available in the Nigerian market today, whose immunosuppressive activities had not been studied before use in the field. This could be responsible for the vaccine failure usually observed in these chickens especially in young chicks following vaccination. Limited work has been done in Nigeria on the effect of the

IBD vaccine on Newcastle disease vaccination (2, 11). This study was designed to determine the extent of IBD vaccine induction of bursal lesions in vaccinated birds as well as the immunosuppressive effects of such vaccines to ND vaccinations programmes in Nigeria.

MATERIALS AND METHODS

Experimental chickens

Experimental 150 day-old pullet chicks obtained from a reputable hatchery were divided into six groups of 25 chicks: i. e. four experimental groups (A, B, C, D); one positive control group (E); and one negative control group (F). All the birds were fed with the same daily ration of chick mash and growers mash depending on the age of the birds as the experiment progressed. Birds were allowed access to adequate supplies of clean and fresh water daily.

Vaccines

Four different IBD vaccines were used: i.e. vaccine I from Nigeria; vaccine II from India; vaccine III from Israel; and vaccine IV from the USA. The ND vaccines used were the Lasota and Komarov strains produced by the National Veterinary Research Institute, Vom, Nigeria. The four IBD vaccines were titrated before use to determine their viral content. Both vaccines were reconstituted and administered based on the prexisting vaccination regimen in Nigeria and as recommended by the manufacturers.

Vaccination of chicks

Water was withheld from the birds for five hours before the vaccines were applied. The IBD and ND Lasota vaccines were given to birds in fresh drinking water at a sufficient dose (10 ml per bird) according to the manufacturer's instructions, while the NDV Komarov was given as an intramuscular injection at a dosage of 0.2 ml per bird. Groups A, B, C, D birds were vaccinated with the first dose of IBD vaccine at 7 day old and a second dose at 14 days old. The same birds were vaccinated with NDV Lasota at 21 day old and NDV-Komarov at 42 days old. Group A birds received IBD vaccine I, Group B received IBD vaccine II, Group C received IBD vaccine III while Group D received IBD vaccine IV within the said IBD vaccination enumerated above. Group E birds, which were the positive control birds, received NDV Lasota and Komarov only, while group F birds, which constituted the negative control group, were given ordinary water.

Experimental challenge

Birds in groups A–E and the negative control group F were challenged at 56 and 70 days of age with the challenge virus, ND virus Hertz 33/56, obtained from Viral Research Department, Virology Division, National Veterinary Research Institute Vom, Nigeria and diluted to contain Log 10^6 LD_{50} per ml. All the birds were given 0.2 ml of this challenge virus intramuscularly.

Serology

Blood samples were collected from the wing vein of groups A–F birds. Pre-vaccination screening for maternal antibodies against IBD and ND viruses (IBDV, NDV) were done by sacrificing 5 birds at one day old. Post vaccination sera were collected at ages of 7, 14, 21, 28, 35, 42, 49 and 56 days.

The haemagglutination (HA) test for NDV antigen quantitation and haemagglutination-inhibition (HI) test for the determination of the NDV antibody were used as described in the Office of International Epizootics (OIE) Manual of standard for diagnostic tests and vaccines protocol (10). The antibody titres were calculated as the dilution of the sera that inhibited haemagglutination of the chicken red blood cells. The sera obtained on the days enumerated above were used for the determination of NDV antibodies while those obtained on the day 7, 14, 21, 28 and 35 were used for the determination of IBD antibodies employing Agar Gel Immunodiffusion (AGID) test (10). Qualitative AGID was first used to determine the positive sera after which two fold serial dilution of such sera were tested in quantitative AGID (6) to determine the end point of the sera. The dilution at which no precipitin line was regarded as the end point of the sera.

Bursa to body weight ratio

The bursa to body weight ratio was determined as described by Morales *et al.* (9). Five birds from each group were weighed, sacrificed, and the bursa was immediately weighed. The bursa of Fabricius relative weight was calculated as BF weight (BW) and divided by the body weight (BBW) multiplied by 1000.

Histopathology

The bursa of Fabricius collected from each sacrified bird was fixed in 10 % formalin, and embedded in paraffin wax. Sections 5 µm thick were cut and stained with haematoxylin and eosin (H and E) and examined under light microscope.

Bursometry

Bursa diameters were measured pole-to-pole and border-to-border with a ruler.

Clinical observations

Birds in each group were observed daily for clinical signs of the NDV challenge. Mortality and morbidity were recorded in addition to the presented clinical signs.

Pathology

Following the necropsy of the birds that died following the viral challenge, the proventriculus, the digestive tract and other selective organs were particularly noted.

Statistical analysis

The data were analyzed by one way ANOVA.

RESULTS

The titre of the four IBD vaccines used for the vaccinations of the experimental birds were as follows: Vaccine I –

Log $10^{2.5}$; Vaccine II – Log $10^{2.5}$; Vaccine III – Log $10^{4.5;}$ and Vaccine IV – Log $10^{3.5}$.

IBD Maternal and Vaccine Induced antibody levels in the experimental groups

All the chickens tested had maternal antibodies against IBDV. At day 0, the Geometric Mean Titre (GMT) for IBDV of maternal antibodies was 5.70 which later dropped to 4.30 by day 7 and 2.6 on day 14 (Table 1).

All the birds in the experimental groups still had antibodies on days 21 and 28 following vaccination with the different IBD vaccines as enumerated above but the titres were low ranging between 1.4 and 3.0 in group A birds, 1.2 to 1.4 in group B birds, 1.3 to 4.6 in group C birds and 1.2 to 2.0 in group D birds. There was no relationship between the initial titre of the vaccine and the antibody level following vaccination. The vaccinal antibody was 0 on day 35 in all the groups of birds.

Table 1. Antibody profile showing maternal antibody decline between days 0 and 14

Age of birds (days)	Geometric mean titre
0	5.7 ± 0.24
7	4.30 ± 0.14
14	2.60 ± 0.23

ND Heamagglutination-Inhibition (HI) antibodies following ND vaccination (prior to IBD vaccination)

The geometric mean titres of antibodies to ND vaccine following the different regime of vaccination with four IBD vaccines are shown in Table 2. In the first week following IBD vaccination, the geometric mean titre (GMT) of birds in groups C–F was 52.0 while that of birds in A and B had GMT HI titre of 27.5 and 48.5 respectively. There was a decline in all the experimental groups two weeks afterward, with titres ranging from 8 to 21. At day 35, which is the second week following ND-Lasota vaccination, the titres had increased significantly in all the groups. However, at day 42 (the third week of ND-Lasota vaccination), the titres again dropped significantly in all the experimental groups with groups B and C

Table 2. Newcastle disease virus (NDV) heamaglutination-inhibition (HI) antibody level after vaccination with IBD, ND Lasota (ND-L) and ND Komarov (ND-K) vaccines

Age (days)	Days post vaccination	Experimental groups					
		GM Titre	GM Titre	GM Titre	GM Titre	GM Titre	GM Titre
	1 wk post 1st IBD	A	B	C	D	E	F
14		27.9 ± 0.24	48.5 ± 2.5	52.0 ± 2.5	52.0 ± 2.4	52.0 ± 3.0	52.0 ± 2.4
21	1 wk post 2nd IBD	11.3 ± 1.23	13.0 ± 1.5	12.1 ± 1.3	13.0 ± 1.4	21.1 ± 2.4	21.1 ± 1.5
28	1 wk post ND-L	11.3 ± 2.4	8.6 ± 1.4	8.0 ± 1.5	8.6 ± 1.4	9.8 ± 1.3	7.0 ± 0.4
35	2 wks post ND-L	64.0 ± 4.0	29.9 ± 2.5	68.6 ± 3.4	57.7 ± 3.4	39.4 ± 1.2	7.0 ± 0.4
42	3 wks post ND-L	39.4 ± 3.4	11.3 ± 1.5	12.1 ± 2.3	21.1 ± 2.4	59.7 ± 4.4	2.1 ± 0.2
49	1 wks post ND-K	42.2 ± 4.4	59.7 ± 3.5	97.0 ± 4.4	119.4 ± 5.5	90.5 ± 4.4	1.0 ± 0.4
56	2wks post ND-K	104.0 ± 3.4	194.0 ± 3.5	256.0 ± 4.4	194.0 ± 5.4	415.9 ± 6.5	0 ± 0

Table 3. Bursa and body weights days post IBD vaccination

Group	Bursa weight (g) days post vaccination				Body weight (g) days post vaccination			
	14	21	28	35	14	21	28	35
A	2.3 ± 0.2	2.7 ± 0.41	2.6 ± 0.3	3.0 ± 0.2	200 ± 5.0	275 ± 7.0	350 ± 7.0	375 ± 7.0
B	2.8 ± 0.1	6 ± 0.1	3.1 ± 0.2	3.0 ± 0.3	200 ± 5.0	275 ± 8.0	325 ± 7.0	450 ± 7.0
C	1.5 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	2.5 ± 0.3	200 ± 5.0	300 ± 7.0	350 ± 7.0	450 ± 7.0
D	2.1 ± 0.2	1.1 ± 0.1	1.6 ± 0.1	2.8 ± 0.2	200 ± 5.0	275 ± 7.0	325 ± 7.0	425 ± 7.0
F	1.6 ± 0.1	1.8 ± 0.2	2.0 ± 0.2	1.5 ± 0.2	200 ± 5.0	275 ± 9.0	300 ± 7.0	450 ± 7.0

having the lowest titres. At days 49 and 56 which correspond to the first and second week of ND Komarov vaccination, the titres had increased significantly many folds, with group C birds showing the highest titre followed by groups B, D and A in that order. Group E birds which received the ND vaccines alone had the highest titre of 415.9 while the negative control bird, that did not receive any vaccine, had dropped to zero.

Bursa to body weight ratio

This is shown in Tables 3 and 4. The BF relative weight in groups A, B, C and D was significantly higher when compared with the control group (F) at 14 days post 2nd IBD vaccination ($P<0.05$). However, at day 21, only group A birds showed a significant increase in the BF ratio ($P<0.05$). At 28 days, the BF relative weight in groups A and B were significantly high when compared with the other groups and the negative control group (F) ($P<0.05$). By day 35, all the birds in the experimental group had increased BF relative weight when compared with the control group ($P<0.05$).

Experimental challenge

No clinical signs were observed in any of the experimental groups. All of the negative control birds developed one or all of the following clinical symptoms consistent with Newcastle disease virus after 48 hours of the first and second challenge: torticollis; uncoordinated movement; circling; drooping wings; weakness; prostration; and partial paralysis. The death of all of the sick birds occurred between 3–7 days post challenge.

Post-mortem examination

Post-mortem examination of dead birds which were from the negative control group showed extensive congestion of the lungs, the trachea and pharynx. The spleen was slightly enlarged, while the proventriculus, small and large intestines and caecum showed petechial hemorrhages.

Bursometry

The diameter of the bursa of Fabricius of birds in all the groups is shown in Table 5. Birds in groups A, B, C and D that received IBD vaccines had bursa diameters that were slightly larger than those from the control group ($P<0.05$), especially at day 35.

Histopathological change scores of the bursa of Fabricius

The vaccinated group revealed varying degrees of pathological changes ranging from mild degeneration and depletion of lymphocytes. Vaccines I, II, III and IV induced mild degeneration and lymphoid depletion. The graded lesions are summarized in Table 6 in accordance to Rosales (12).

Table 4. Bursal to body weight ratios post IBD vaccination

Groups	Days post vaccination			
	14	21	28	35
A	*11.5 ± 2.3	*9.8 ± 0.5	*7.3 ± 0.3	*8.0 ± 1.1
B	*14.0 ± 2.5	5.9 ± 0.4	*9.5 ± 1.4	*6.6 ± 1.4
C	*7.5 ± 0.4	6.5 ± 0.3	6.3 ± 0.4	*5.6 ± 0.5
D	*10.5 ± 1.7	4.1 ± 0.5	4.8 ± 0.3	*6.7 ± 0.3
F	7.3 ± 0.4	6.6 ± 0.4	6.6 ± 0.5	3.2 ± 0.4

* – Significant P < 0.05

Table 5. The different diameters of the bursa of Fabricius post IBD vaccination

Group	Days post vaccination			
	14 (mm)	21 (mm)	28 (mm)	35 (mm)
A	5.1 ± 0.4	5.5 ± 0.4	*5.4 ± 0.4	*5.3 ± 0.3
B	5.7 ± 0.3	4.4 ± 0.4	*5.6 ± 0.3	*5.0 ± 0.2
C	4.5 ± 0.1	5.1 ± 0.3	4.7 ± 0.4	*5.3 ± 0.2
D	4.5 ± 0.4	3.8 ± 0.2	4.7 ± 0.3	*5.3 ± 0.1
F	4.1 ± 0.2	4.5 ± 0.3	4.8 ± 0.2	4.2 ± 0.1

* – Significant P < 0.05

DISCUSSION

The effects of IBD vaccines on the immune responsiveness of vaccinated chickens to vaccines have long been under investigation (2, 11). In an attempt to further understand the role of IBD vaccines on the immunosuppression of the im-

Table 6. Histopathological score of the bursa of Fabricius post IBD vaccination

Group	Days post vaccination			
	14	21	28	35
A	2	1	1	1
B	1	1	0	0
C	2	2	1	0
D	2	2	1	0
F	0	0	0	0

* – Significant P < 0.05

0 – Normal bursa
1 – Mild scattered cell depletion in a few follicles
2 – Mild to moderate atrophy or cell depletion in ⅓ to ½ of the follicles
3 – Moderate atrophy or cell depletion in ½ of the follicles and necrosis
4 – Severe atrophy of all follicle, necrosis and haemorrhage

mune responses of vaccinated chickens to ND, this study was conducted to determine the effect of four commonly used infectious bursal disease (Gumboro) vaccines in Nigeria. Our findings showed that the antibodies to ND virus in chickens vaccinated with IBD decrease sharply in the first 2–3 weeks post hatch. This may be connected with the bursa atrophy induced by the IBD vaccine during this same period. This is in agreement with the findings of Lukert and Mazariegos (8) who observed similar results when intermediate strains of IBD vaccines were used to vaccinate chickens.

In this study, the immunosuppressive effect of IBD was also observed at six weeks of age which corresponded to the 3-week post ND-Lasota vaccination. The effect was, however, transient as a slight recovery from the immunosuppressive effect was observed. A total recovery was observed in birds vaccinated with booster doses of ND-Komarov one week post vaccination. This was shown by the rise in the antibody level observed among those chickens vaccinated with the booster dose of ND-Komarov. From these findings it is evident that the immunosuppressive effect of the IBD vacines on ND-vaccinated birds was transient and non existent with a booster dose of ND-Komarov (3).

Our findings also showed that the immunosuppressive effect of the four IBD vaccines varied with birds in group B vaccinated with vaccine II recovering from the effect of immunosuppression after 4 weeks as compared to birds in the other three groups where recovery was observed to be about 3 weeks. This may be due to the degree of attenuation and inherent virulence of the IBD viruses contained in the vaccines. It is worth noting that vaccine II had a titre of Log $10^{2.5}$ as compared to vaccine III and IV with titres of Log $10^{4.5}$ and Log $10^{3.5}$, respectively.

The bursa of Fabricius is the antibody inducing organ of the chicken. Any damage to the organ is therefore reflected in the degree of unresponsiveness that may lead to low level production of antibodies. In this study, the bursae were found to be grossly enlarged in the vaccinated groups compared to the control groups, with the vaccinated birds demonstrating various degrees of pathological changes which was consistent with other reports (4, 9). From the lesion score, the vaccines could be said to induce mild pathological changes and such vaccines were graded as mild vaccines. This was translated in the first three weeks into lower antibody titres among birds vaccinated with IBD vaccines. Histopathological score seemed to be a sensitive and accurate method to evaluate the effect of IBD on vaccination. All the four vaccines compared in this study were found to be immunogenic and good for the routine immunization of chickens as there was no induced IBD death in vaccinated birds during the study. Results obtained from this study also suggested that NDV vaccination with Komarov as booster dose should still be given at 6 weeks of age if IBD vaccine is to be given at 7 and 14 days of age as a prophylactic in case of an outbreak.

In conclusion, this study revealed that the IBD vaccines employed in this study were immunogenic, did not induce IBD death in vaccinated birds and were not immunosuppressive when combined with ND vaccination as they did not depress the ND antibody titres.

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EVALUATION OF THE HEPATIC PATHOLOGY ASSOCIATED WITH LINEAGE 1 VARIANT OF PESTE DES PETIT RUMINANTS VIRUS IN GOATS

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ABSTRACT

The aim of this investigation, which was part of a larger study, was to evaluate the hepatic pathology associated with the lineage 1 variant of PPR (Peste des petit Ruminants) virus and the possible influence of *Mannheimia hemolytica* (MH). Gross, histopathology and serial serum aspartate amino transferase (AST) and alkaline phosphatase (ALP) in experimental PPR virus and combined PPR virus and MH infection in goats were evaluated with a view of understanding the hepatopathy in lineage 1 of PPR virus infection.

The animals were divided into groups A and B with 15 goats each, while 5 goats served as control. Group A goats were infected with 1ml of a pure culture of $10^{6.5}$ $TCID_{50}$ PPR virus grown in Baby hamster kidney cell lines, while group B was infected with 1ml of a pure culture of $10^{6.5}$ $TCID_{50}$ PPR virus grown in baby hamster kidney cell lines plus a week later infected with 1ml of MH A2. The blood samples for the analyses were collected for 6 weeks. Samples of the livers of two goats were also evaluated grossly and histopathologically weekly in each group for 6 weeks using standard techniques. In group A, there was an inconsistent pattern in the serum alkaline phosphatase (ALP) and the serum aspartate amino transferase (AST) values over the weeks while in group B, ALP values increased significantly in weeks 1, 2, 3, 4 with an inconsistent pattern in the AST values when compared to the control ($P < 0.05$). Grossly, the liver was yellow in colour in group A goats. Histopathologically, there was moderate generalised fatty degeneration in group A, mild hepatic degeneration in group B and no visible lesion in the control group. This study showed that in lineage 1 PPR virus infection, there was hepatic degeneration.

Key words: goats; hepatopathy; infection; *Mannheimia hemolytica;* Peste des petit Ruminants; serum aspartate amino transferase

INTRODUCTION

The investigations into the pathogenesis of Peste des petit Ruminants (PPR) virus, as reported in literature, focused on lineage 4 (14) and the pathogenicity of PPR virus strains and had only recorded observations of two weeks duration (4, 5) with very little information on the hepatic pathology. Hence, there is a need to study the pathogenesis of the disease for a longer duration in order to understand the hepatic pathogy associated with the disease.

Hepatic lesions have been associated with fatal cases of PPR caused by strain 4 PPR virus from Asian countries (13, 18). Alcigir *et al.* (1), originally described syncytial cells formed by hepatocytes and intranuclear inclusion bodies in the liver of lambs with PPR. Aruni *et al.* (2) also demonstrated intranuclear inclusion bodies in epithelial cells in the liver of infected goats. Some of these hepatic changes were later demonstrated with immunoperoxidase technique (13, 18). However, in strain 1 PPR virus, there had been no concrete evidence of hepatic involvement except for field reports. Ikede (11), reported mild hepatic necrosis while Rowland *et al.* (17) and Obi *et al.* (16) reported focal fatty degeneration and coagulative necrosis of the hepatocytes.

Although PPR has been found to affect some biochemical parameters, information on the sequential hepatic enzyme changes associated with this disease are also scanty in the literature (6).

In an attempt to fully understand the pathogenesis of PPR virus from the lineage 1 which are the circulating strains of PPR virus in Nigeria (4), there is a need to understand if this strain also results in hepatic necrosis as reported with lineage 4.

The liver has been shown to have a high activity of serum aspartate amino transferase (AST) and gamma glutamyl transferase (GGT) which are often determined if there is a suspicion of acute

and chronic liver disease. Hence, an increase in serum AST is a sensitive marker of liver damage, even if the damage is of a subclinical nature (12, 15).

The aim of this study, therefore, was to evaluate the hepatic pathological changes and also determine the activity of AST with ALP in goats during experimental infections with PPR virus and concurrent PPR virus and *Mannheimia hemolytica* (MH) in order to evaluate the hepatic pathology associated with lineage 1 PPR virus circulating in Nigeria and the possible effect of co-infection with MH.

MATERIALS AND METHODS

Animals

The experimental protocol and preparation of inoculum has been described earlier (8, 9, 10). Thirty five (35) apparently healthy West African dwarf (WAD) goats, averaged six kg weight and six months of age were used. Fifteen (15) West African dwarf goats were in each treatment group while five (5) uninfected goats served as controls. This investigation has been independently reviewed and approved by the ethical Committee of the faculty˝(*Certificate no: ethic/07/06)*. Adequate measures were taken to minimize pain or discomfort. Animals were infected intratracheally according to the method described by Davies *et al.* (7).

The animals were divided into groups A and B with 15 goats each while 5 goats served as control. Group A goats were infected with 1ml of a pure culture of $10^{6.5}$ $TCID_{50}$ (Tissue culture infective dose 50) PPR virus grown in baby hamster kidney cell lines while group B goats were infected with 1ml of pure culture of $10^{6.5}$ $TCID_{50}$ PPR virus grown in Baby hamster kidney cell lines and a week later infected with 1ml of *Mannheimia hemolytica* (MH) A2.

The clinical features including daily respiratory rate, temperature and weekly weight changes had been earlier described (8, 10). The serum alkaline phosphatase (ALP) and the serum aspartate amino transferase (AST) were determined using photoelectric colorimeter (Gallenkamp and sons Ltd., England) as described by Coles (3). Routine gross and histopathology were done on the liver tissue from goats euthanised weekly post inoculation and goats that died in the course of the infection. The histopathological changes were scored to be mild, moderate and marked as described by Kumar *et al.* (14).

Statistical analysis

The serum enzymic changes were analysed using ANOVA with multiple Duncan test.

RESULTS

From Table 1, in group A, there was a slight increase in ALP value in the third week and a sharp drop in the fouth and fifth week while in group B, the ALP values increased significantly in weeks 1, 2, 3 and 4 when compared to the control ($P < 0.05$). From table 2, in group A, the AST showed a slight increase in the second week post infection and a slight fluctuation in values from third to fifth week while there was no significant change in the group B despite a peak at week 3 ($P > 0.05$).

Grossly, the livers were yellow in colour in the group A goats while there was no visible gross lesion in group B or the control group. Histopathologically, the hepatic lesion was that of moderate generalised fatty degeneration in group A, mild hepatic degeneration in group B and no visible lesion in the control group.

Table 1. The weekly mean value (with standard error) of serum aspartate amino transferase (µkat.l^{-1}) changes

Groups	Week 1	Week 2	Week 3	Week 4	Week 5
A (PPRV)	95.0 ± 3.5	98.0 ± 4.5	94.5 ± 2.5	97.0 ± 4.5	67.0 ± 5.5
B (PPRV+MH)	55.0 ± 2.5	59.0 ± 4.5	73.5 ± 3.5	37.2 ± 5.5	31.6 ± 4.5
C (Control)	51.0 ± 2.5	51.7 ± 3.5	50.5 ± 3.7	52.4 ± 4.5	50.6 ± 4.5

Table 2. The weekly mean value (with standard error) of serum alkaline phosphatase (µkat.l^{-1}) changes

Groups	Week1	Week2	Week3	Week4	Week5
A (PPRV)	500 ± 4.5	430 ± 3.5	580 ± 3.6	380 ± 5.5	350 ± 4.5
B (PPRV+MH)	121 ± 3.6	363.2* ± 3.3	421* ± 3.2	392* ± 4.5	334* ± 5.5
C (Control)	120 ± 4.5	133.2 ± 3.5	134.2 ± 6.5	137.9 ± 2.5	135.4 ± 4.5

* – Significant ($P < 0.05$)

DISCUSSION

Although multifocal necrosis in the liver, inclusion bodies in hepatocytes and abomasal epithelial cells had been reported (18) with conspicuous syncytial cells and granular immunostaining pattern in goats naturally infected with strain 4 of PPR virus (13), the evidence for hepatic changes in lineage 1 of PPR virus is very scanty in literatures.

Although, immunolabelling of PPR viral antigens was essential to confirm the hepatic involvement, in areas where immunostaining facilities are not available, histopathology and the activity of aminotransferases in the blood could be helpful in evaluating liver damage. Aminotransferases act as a catalyst in connecting the metabolism of amino-acids and carbohydrates and changes in their activity in the blood can be a consequence of their increased activity or a reflection of structural damage of the hepatocytes. Althoughe gamma glutamyl transferase (GGT), ornithine carbamoyltransferase (OCT), sorbitol dehydrogenase are more specific for the evaluation of hepatic damage and where the kits of the enzymes are not available, AST, could be helpful where there is no obvious myopathies (12, 15) as observed in PPR virus infection.

In this investigation, it was observed that AST and ALP activities were inconsistent in group A over the weeks post innoculation while in group B, Alkaline phosphatase (ALP) values increased significantly in weeks 1, 2, 3 and 4 with inconsistent pattern in the AST values when compared to the control. ($P<0.05$). The increase in ALP may be associated with the pattern of inflammation affecting the lungs as observed in this infection (8, 9, 10). There was no significant and consistent change in the Aspartate aminotransferase (AST) values obtained despite a peak at week 2 ($P>0.05$), This may suggest a mild hepatic dysfunction as there was no obvious myopathies (12, 15).

At postmortem, the gross yellowish discolouration were observed in group A. Histopathologically, the hepatic fatty degeneration was mild in group A pre 28 dpi and moderate post 28 dpi while in group B, the hepatic degeneration was mild throughout the course of the infection. The result of the enzyme assay and pathological changes suggests that the Nigerian strain of PPRV employed in this investigation may not be associated with hepatic necrosis as observed with the lineage 4 (14, 18) but with fatty degeneration as reported by field worker in earlier reports (16, 17).

Seasons, circadian changes, age of the animal and energy status could influence the result but in this study, we tried to avoid the impact of these factors as much as possible. Blood was taken always at the same time (morning) and the animals were of the same age and the same feed was given to all the animals. As regards the sex, fairly equal numbers of females and males were used.

In conclusion, the results of this study showed that the lineage 1 of PPR virus employed in this investigation may not be associated with hepatic necrosis as reported for lineage 4 PPR virus infection (13). The hepatic involvement in combined infection of PPR virus and *Mannheimia haemolytica* was not significantly influenced by the co infection with MH. This showed that the hepatic injury observed in some natural cases of PPRV infection in Nigerian goats (10, 15, 16) may not be as a result of circulating PPR virus and the bacterial complication as earlier speculated.

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A PRELIMINARY REPORT ON TIME DEPENDENT CHANGES OF SOME IMMUNOPHENOTYPIC CHARACTERISTICS OF ADULT RAT BONE MARROW DERIVED STEM/PROGENITOR CELLS

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ABSTRACT

Stem/progenitor cells (S/PCs) derived from the adult bone marrow represent a potentially promising source of precursors for transplantation therapy. The aim of this study was to evaluate the stability of some immunophenotypic characteristics of adult rat bone marrow derived S/PCs harvested from femora of 6 SD rats. Following 4 passages in alpha-MEM medium supplemented with 10 % ES-FBS, 2 mM L-glutamine, 10 ng.ml^{-1} rat LIF and 10 ng.ml^{-1} human LIF, cultures were split into two parts. The first culture continued with the original medium, whereas the second one received additional supplementation with human FGF-b and EGF. The expression of CD45, CD90 and cyNestin by the cells was analysed by flow cytometry at different points of time. After the initial 14 days, a heterogeneous culture of adherent cells was obtained. Twenty-five per cent (range 21.2–30.3 %) of the cells displayed phenotype (CD45–, CD90+++, cyNestin++) compatible with that of mesenchymal stem/progenitor cells (MS/PCs). During subsequent cultivation, cells started to gradually lose the expression of both, CD90 and cyNestin, but retained their viability. This decrease of MS/PCs marker expression was even more pronounced when cells were cultured in the medium containing human FGF-ß and EGF. The results of the study showed, that cultivation of rat bone marrow cells in appropriate culture media lead to the generation of cells with phenotype characteristic of MS/PCs. However, expression of this phenotype was gradually reduced with time. The observations suggest that the quality of rat bone marrow-derived MS/PCs and their suitability for replacement therapy is highest between days 12 to 15 of the cultivation (passages P4-P5) and then diminishes.

Key words: immunophenotype changes; rat bone marrow; stem/progenitor cells; time factor

INTRODUCTION

Stem/progenitor cells (S/PCs) show enormous promise for many areas of medicine and have the potential to revolutionize it as fundamentally as vaccinations, antibiotics and organ transplantations (2–6, 8, 9, 11, 12, 19). Due to their well known capacity to differentiate into any cell type *in vivo*, pluripotent embryonic stem cells (ESCs) have been taken into consideration as the first choice (20). However, utilisation of ESCs is restricted by serious ethical and technical problems. As a suitable alternative, pluripotent adult mesenchymal stem/progenitor cells (MS/PCs) derived from bone marrow or subcutaneous fat, have acquired increasing popularity (4, 8, 11, 13, 16). Since it is possible to obtain them without undue risk from the intended recipient, to expand them *in vitro*, to

modulate their characteristics by composition of culture media and/or by culture micro-environment, then to administer them in the form of an autograft, the transplantation of adult S/PCs is considered a very convenient option for the treatment of neurodegenerative diseases, stroke, brain and spinal cord injuries, myocardial infarction, intervertebral disc disease, etc. (2–4, 8, 11, 13, 15–19). Following the elaboration of a successful method of isolation and harvesting of MS/PCs (expressing nestin and CD90) derived from the bone marrow of adult rats (14), we decided to continue our experiments with an aim to evaluate the stability of their immunophenotypic characteristics.

MATERIAL AND METHODS

The experimental protocols were elaborated in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 and approved by the State Veterinary and Food Administration of the Slovak Republic in Bratislava (decision No. SK P 10552/03-220), as well as the Ethical Commission of the P. J. Šafárik University, Faculty of Medicine in Košice. All surgical procedures were performed under general anaesthesia, and the painful sensations following the experimental operations were suppressed by analgesics administered intramuscularly (2 mg of *Tramadoli hydrochloricum* – "Tramadol AL 100", Aliud Pharma GmbH Co, KG, Leichingen, Germany), as was described in detail in our previous publications (14).

The bone marrow (BM), collected from both femora of six adult Sprague-Dawley (S-D) rats weighing 480–690 g, was dissected into small pieces, homogenised, filtered (to remove possible bone fragments) and diluted 1 : 1 with Hank's balanced salt solution (HBSS, Gibco Co, UK). Mononuclear cells were isolated by 30 min centrifugation (Ficoll-Urographin density centrifuge, Sigma, Schering, Germany) at 1 600 r. p. m. and collected from the interface. Isolated BM cells were washed two times in DMEM (Dulbecco's Modified Eagle Medium – Stem Cell Technologies, USA), suspended at 10^6 cells.ml^{-1} and cultured in alpha-MEM (Minimum Essential Medium – Stem Cell Technologies, USA), supplemented with 10 % ES-FBS (Embryonic Stem-cell-qualified Foetal Bovine Serum – Invitrogen, USA), 2 mM L-glutamine (Invitrogen, USA), 10 ng.ml^{-1} rat LIF (Leukemia Inhibitory Factor – Chemicon, USA) and 10 ng.ml^{-1} human LIF (Chemicon, USA), 100 i. u./ml^{-1} PNC (*Benzylpenicillinum kalicum*) and 100 µg. ml^{-1} STM (*Streptomycinum sulphate*).

At passage 4, cultures were divided into two parts. The first culture continued with the same medium, whereas the second one received additional supplementation with rh FGF-ß (recombinant human basic Fibroblast Growth Factor – BD Biosciences, USA) and rh EGF (recombinant human Epidermal Growth Factor – BD Biosciences, USA).

The cells were cultured for 45 days, with half-volume of the corresponding culture medium being replaced with a fresh medium every third day. Following 14 days of culturing, then at 21st, 36th and 45th day of cultivation (passages P7, P12, P15) samples of cells were stained with a combination of monoclonal antibodies (FITC/PE/PE-Cy5) and the mean fluorescence intensities (MFIs) for CD 90 and cyNestin were evaluated by three-colour flow cytometry (10). The results were expressed as mean fluorescence intensities of CD 90 and cyNestin (14).

We avoided statistical analyses due to an apprehension of possible distortion of the results in such a small studied group.

RESULTS

The light-microscopic analyses of cells isolated from the material obtained by the aspiration and flushing of bone marrow from both femora of adult S-D male rats showed that

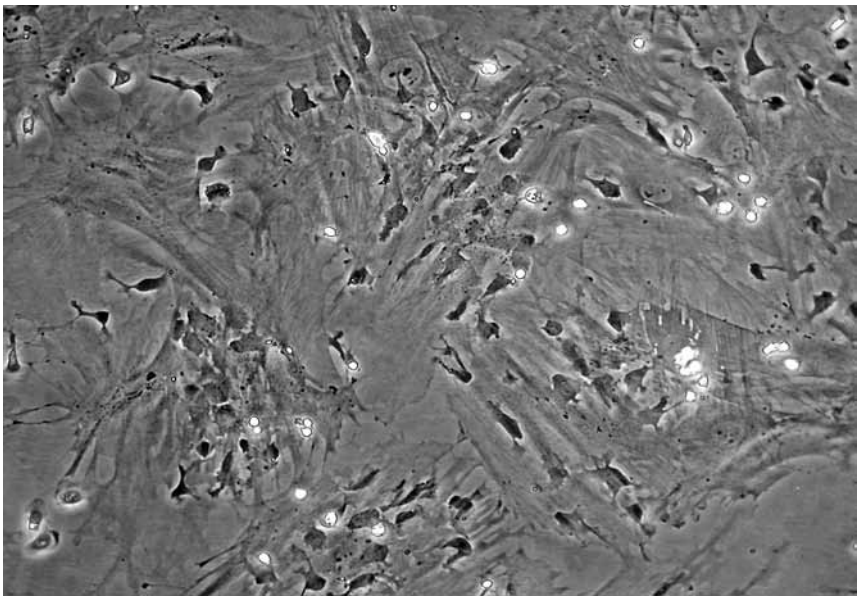

Fig. 1. A photomicrograph of the culture of large adherent cells with MS/PCs-like morphology obtained after the initial 14 days of cultivation of adult male S-D rat bone marrow derived cells stained by a combination of monoclonal antibodies (FITC/PE/PE-cy5). Original magn. × 50

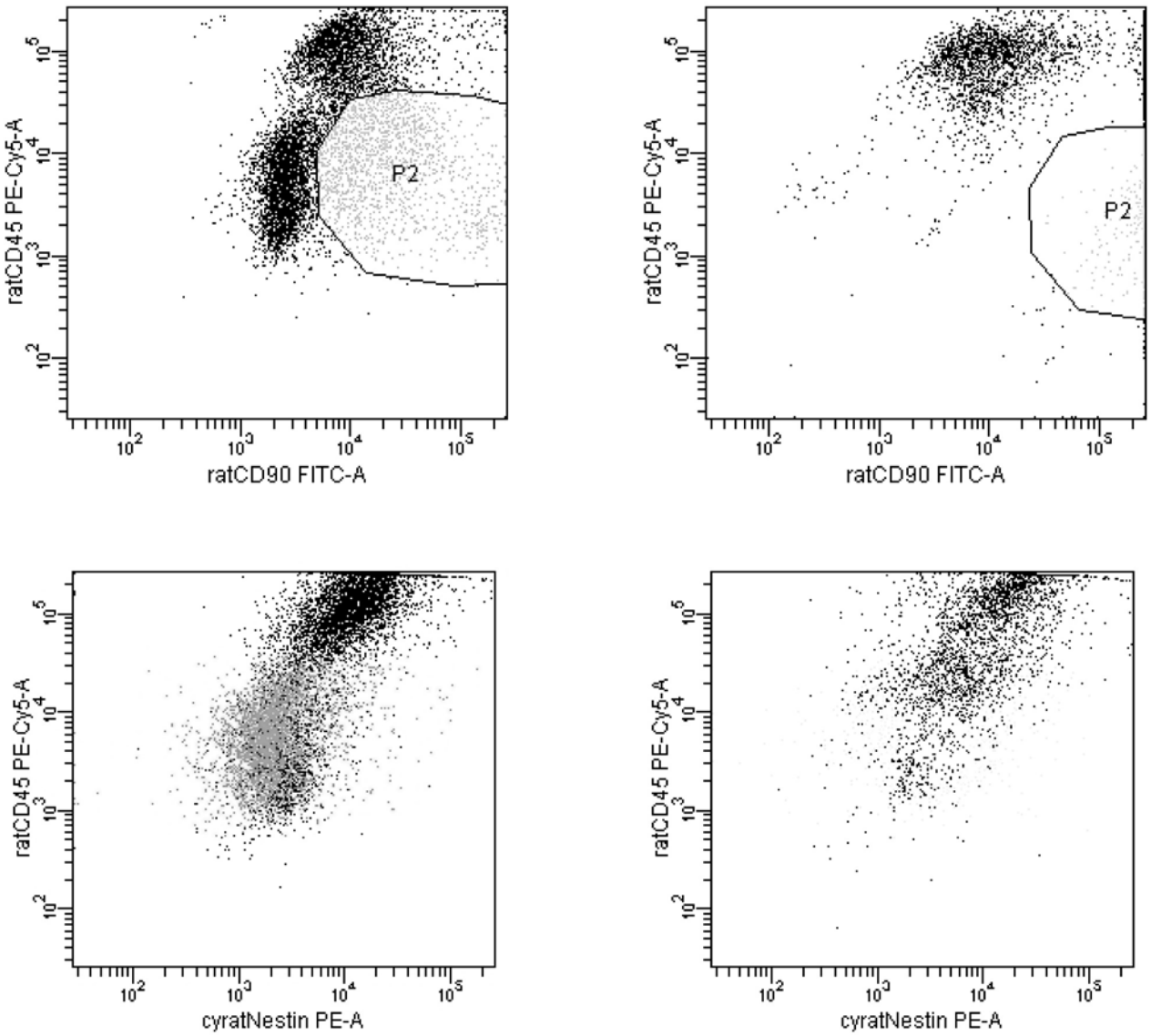

Fig. 2. Results of flow cytometric analyses of adult male S-D rat bone marrow derived stem cells. Illustrative bivariate dot plots show the expression of CD90 and cyNestin by the cells at the 15th (left) and at the 36th day (right) of cultivation

a total number of mononuclear cells was 1.5–4.0 million per sample and the proportion of stem/progenitor cells expressing CD 90/cyNestin was less than 1.0 %.

After an initial 14 days *in vitro* cultivation in a medium described in the previous section (Material and Methods), a heterogeneous mixture of adherent cells with similar diameters and with the morphology of mesenchymal stem/progenitor cells (Fig. 1) was obtained. Approximately twenty five per cent (range 21.2–30.3 %) of cells displayed a phenotype CD 45–, CD 90 +++ and cyNestin ++, compatible with the basic phenotypic characteristics of MS/PCs (Fig. 2). During subsequent cultivation, cells started to lose the expression of CD 90 and cyNestin, when the mean fluorescence intensities of 9320, 9356, 9336 and 9250 for CD 90, and 3620, 3614, 2681, 1628 for cyNestin, were observed at passages taking 14, 21, 36 and 45 days. This decrease of MS/PCs markers expression was even clearer when the adult male S-D rat bone marrow-derived cells were passed in the second medium (with additional supplementation – rh FGF-ß and rh EGF). In this situation the MFI displayed by cells were 9552, 8772, and 6671 for CD 90, and 3605, 1757, and 541 for cyNestin.

DISCUSSION

Due to their assumed ability to replace lost cells, reconstitute damaged organs, produce growth factors and cytokines, especially in systems with limited regenerative potential (e.g. myocardium, *nucleus pulposus* of intervertebral disc, brain or spinal cord), stem cell therapy has attracted an increasing interest of scientists and health workers around the world during the past two decades (2–4, 8, 11–13, 15–19). At first, the

utilisation of embryonic stem cells (ESCs), was considered (9, 20). However, the production of ESCs from vital morula or blastocyst is considered unethical (and even is prohibited by law) in many countries. The direct implantation of ESCs into another organism can induce a rejection reaction and pluripotent ESCs require specific signals for correct differentiation, otherwise their transplantation may lead to the development of a teratoma in the host (11, 20).

The above mentioned reasons has inspired scientists to search for alternative sources of precursors. Several experimental works have shown that cells recovered with similar properties, such as ESCs, can be found in the tissues of developed organisms, including umbilical cord blood, bone marrow, fat, subventricular zone of the brain, and the endothelium, as well (7, 8, 11, 13, 16). Since these cells are lineage restricted (multipotent, not pluripotent), the term adult stem/progenitor cells has become popular (1, 17, 18). Originally it was believed that neural progenitors derived from brain tissue would be the best choice, while MS/PCs derived from adult bone marrow were supposed to be able to differentiate only along osteoblastic, adipocytic, or chondrotic lineages (1, 5, 7, 20). However, further research demonstrated that MS/PCs can also acquire the phenotype of neural stem/progenitor cells (4, 8, 10, 11, 13, 16, 19).

Preliminary results of our experiments were promising in this respects, as well (14). The numbers of S/PCs harvested from adult rat bone marrow increased substantially during first several passages. The cells were viable and expressed markers characteristic for MS/PCs; even possibly compatible with neural stem/progenitor cells (10, 14). As a next step in our research, we decided to try to find out if MS/PCs derived from adult bone marrow will maintain their original properties, i. e. the expression of cyNestin (a marker predominantly expressed by stem/progenitor cells developing along the neural lineage), CD 90 (cell surface marker expressed mainly by mesenchymal stem cells) and, as a control, CD 45 (marker expressed in all hematopoietic lineages).

The study showed that our technique of bone marrow stromal cells harvesting, culturing and isolation of CD 45–, CD 90+++, and cyNestin++ expressing MS/PCs was a quite straightforward procedure. Simultaneously, the CD45 negativity excluded a possible tendency of cultured cells to develop along the hematopoietic lineage. During an initial 14 day's passage in an appropriate culture media, the proportion of cells with phenotype characteristic for MS/PCs increased from about 1 % to approximately 25 % (which would be sufficient for transplantation). According to our findings, the observation of the gradual decrease of CD 90 and cyNestin expression during medium-term cultivation (confirmed by the declining of the mean fluorescence intensities of CD 90 and cyNestin at the 21st, 36th, and 45th day of passage) implies, that adult rat bone marrow derived stem/precursor cells are most suitable for replacement therapy between 12th to 15th day of cultivation. However, additional experimental work involving the detection of properties and therapeutic potentials of adult bone marrow derived mesenchymal stem/progenitor cells is necessary.

CONCLUSIONS

Cultivation of adult rat bone marrow-derived cells in appropriate media led to the generation of cells with a phenotype and MFI characteristics of MS/PCs. However, this phenotype, as well as MFI characteristics were gradually reduced with elapsing time during passage. The results of the study imply that the quality of rat bone marrow-derived S/PCs and their suitability for replacement therapy is highest between 12th to 15th day of cultivation (passages P4–P5), and then the quality subsequently decreases.

ACKNOWLEDGEMENT

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DIFFERENT TREATMENT EFFECTS ON THE HYDROXYMETHYLFURFURAL CONTENT IN THE HONEY

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ABSTRACT

The aim of this study was to determine the impact of the different treatment of honey on the production of hydroxymethylfurfural. Two types of honey (nectar and honeydew) were treated by different means (heating in a water bath, microwave oven and ultrasonic bath) for different time periods. Depending on its quantity and exposure time, the temperature of honey heating was monitored in the microwave. Hydroxymethylfurfural content was determined by high performance liquid chromatography with UV detection. All treatment methods caused an increase in the hydroxymethylfurfural content; however the most significant increase in hydroxymethylfurfural content was produced by the heat treatment in a water bath. All treatments caused significant changes ($P > 0.05$) in hydroxymethylfurfural content and the threshold level of 40 mg.kg^{-1} was exceeded after treatment in a water bath at 95 °C for 45 min. The temperature of 95 °C increased the hydroxymethylfurfural content by 650 % (in honeydew honey, 90 minutes) compared to the initial level. The treatment in an ultrasonic bath resulted in a slight increase in hydroxymethylfurfural content depending on the time of exposure; the total increase was more than 34 % in nectar honey and up to 86 % in honeydew honey. The experiments showed that the formation of hydroxymethylfurfural in honey is significantly influenced by temperature (especially heating in a water bath). The hydroxymethylfurfural content in honeydew increased faster than in floral honey in all methods of treatment.

Key words: heating; honey; HPLC; ultrasonic bath

INTRODUCTION

Honey is the natural sweet substance produced by Apis mellifera bees: from the nectar of plants; or from secretions of living parts of plants; or from excretions of plant-sucking insects that live on parts of plants. After the bees collect the honey, it is transformed by combining it with specific substances of their own, which is then deposited, dehydrated and stored in honeycombs to ripen and mature (6). Appearance plays a key role in the commercial success of honey, as consumers demand a fluid, non-crystallized product. Recently harvested raw honey is in a liquid state, but it crystallizes with greater or lesser speed depending on numerous factors such as origin (botanical and geographical), temperature, moisture content, and sugar content (10). Thermal treatment, applied to honey, may destroy vitamins and bionutrients, and produce a simultaneous decrease in diastase activity and an increase in hydroxymethylfurfural (HMF) content. Honey thermal temperature and time must be limited (13). The shelf life limitation of a maximum of three years is legitimate because the HMF content increases during storage (9).

Hydroxymethylfurfural (HMF, 5-hydroxymethyl-2-furaldehyde) and congered compounds are spontaneously formed in carbohydrate-containing foods by the Maillard reaction (the nonenzymatic browning) or the acid-catalysed dehydratation of hexoses (1). HMF is a recognized indicator of reduced quality in numerous foods that contain carbohydrates in an acid medium (11, 15). HMF is the substance, which originate by reaction of the acids present with sugars under specific conditions. The heating of the honey accelerates HMF formation. Long-term storage at inappropriate temperature (e.g. higher than 30 °C) or heating increase the HMF concentration very quickly. The higher content of HMF can also be caused by the

addition of invert sugar to honey (12). Although some questions still remain about the reactions that take place in food as a result of heat treatment (mainly Maillard reactions), it is known that they are related to transformations in flavour, aroma, taste and colour and are closely associated with temperature, time, pH, and the nature of reactants, (i.e. the type of sugar and amino acids, or proteins), etc. (10). The HMF limit in honey should be related to the initial honey pH; and also the HMF concentration in honey is also related to honey composition (pH, acidity) at heating temperature below 50 °C (7). The presence of HMF deteriorates organoleptic properties and nutritional value of food (3). The maximum value of HMF is 40 mg.kg^{-1} with the exception of honey coming from countries or regions where tropical ambient temperatures prevail (max. 80 mg.kg^{-1}) (5, 6).

The aim of this study was to evaluate the effect of three different treatments (heating at different temperatures in a water bath, microwave oven or ultrasonic bath for different time intervals) on HMF concentration in the honey.

MATERIALS AND METHODS

Two kinds of honey (nectar from multifloral and honeydew) with different initial HMF content were used in the experiments. The samples of honey were harvested from beekeepers in East Slovakia in 2008. The honey samples were stored in glass vessels at 4 °C until analysis. The honey samples (50 g) were transferred into

Table 1. HMF levels in honey exposed to various temperatures for different time periods and their increase in per cent

Heating temperature	40 °C		50 °C		60 °C		70°C		80°C		95°C	
	HMF											
	mg.kg^{-1}	Increase %	mg.kg^{-1}	Increase %	mg.kg^{-1}	Increase %	mg.kg^{-1}	Increase %	mg.kg^{-1}	Increase %	mg.kg^{-1}	Increase %
Nectar honey												
Initial level	20.57		20.57		20.57		20.57		20.57		20.57	
1min	21.00	2.09	22.00	6.95	22.65	10.11	22.32	8.51	30.5	48.27	31.35	52.40
5 min	21.04	2.28	22.30	8.41	22.73	10.50	22.54	9.58	33.24	61.59	33.56	63.20
15 min	21.09	2.53	22.80	10.84	22.85	11.08	22.9	11.33	34.92	69.76	34.97	70.00
30 min	21.15	2.82	22.93	11.47	23.44	13.95	23.56	14.53	35.37	71.95	36.82	79.00
45 min	21.25	3.31	23.16	12.59	23.50	14.24	23.78	15.61	36.34	76.66	51.63	151.00
60 min	21.36	3.84	23.75	15.45	23.67	15.07	23.57	14.58	37.93	84.39	55.71	170.80
90 min	21.39	4.00	23.87	16.04	24.7	20.08	24.76	20.37	37.97	84.59	83.72	307.00
Honeydew honey												
Initial level	13:46		13.46		13.46		13.46		13.46		13.46	
1min	13.68	1.63	14.00	4.01	14.20	5.50	15.00	11.44	20.40	51.56	24.60	79.49
5 min	13.75	2.15	14.60	8.47	14.32	6.39	15.80	17.38	24.32	80.68	25.34	88.26
15 min	13.84	2.82	15.20	7.43	14.46	7.43	16.40	21.84	25.34	88.26	26.28	98.74
30 min	13.97	3.79	15.40	14.41	15.50	15.16	17.10	27.04	28.12	108.90	35.52	163.89
45 min	14.05	4.38	15.60	15.90	16.60	23.33	17.50	30.00	28.52	111.89	40.16	198.36
60 min	14.18	5.35	15.68	16.49	16.87	25.33	18.56	37.89	30.83	129.05	59.10	339.00
90 min	14.22	5.65	16.00	18.80	17.40	29.27	19.56	45.32	33.96	152.30	101.24	652.15

Table 2. Effect of the time of microwave heating and honey weight on the honey temperature (°C)

	Time (min)			
Weight (g)	**1**	**2**	**3**	**4**
5	82.3	100	100	100
10	78.5	100	100	100
15	76	100	100	100
20	70	100	100	100
30	65	100	100	100
50	60	100	100	100
80	52.8	80	86	94
100	48.3	70	79.2	84.3
150	41.6	61	65	70
350	34.5	44	46.4	48.5
500	31	38	41	42.3
700	28	34	37.4	39.6

the sample tubes, enclosed and treated by three different methods: i.e. heating in a thermostatic water bath at different temperature; heating in a microwave oven; and treatment in an ultrasonic bath. After the treatment, the samples were cooled and the HMF content was determined.

The individual samples were analysed in duplicate. The samples were heated in a water bath at 40, 50, 60, 70, 80 and 95 °C for different time (1, 5, 15, 30, 45, 60 a 95 min.); in a microwave oven (at 90 W) for 5, 10, 15, 30 seconds, one, two and three minutes; and in an ultrasonic bath for 5, 10, 15, 30, 60, 120 minutes. The honey temperature in relation to honey quantity (5, 10, 15, 20, 30, 50, 80, 100, 150, 350, 500, 700 g) and time (1, 2, 3, 4 min.) of microwave treatment were observed.

The HMF content was determined by the high performance liquid chromatography (HPLC) method (8). Chromatograph HP 1050 S Hewlett Packard was used. The chromatograph consisted of a quarter pump, auto sampler, UV detector Agillent 1500 and 3396 Integrator. The honey sample was accurately weighed (5 g) into a 25 ml beaker, dissolved in approximately 10 ml of HPLC quality water, quantitatively transferred into a 25 ml volumetric flask and made up with water to 25 ml. Then the sample was filtered through a 0.45 μm membrane filter into a vial and analysed (quantity injected 10 μl) on Hypersil BDS (100 × 4 mm 3 μm) colon, with mobile phase, water – acetonitrile (90 : 10), flow 1 ml.min^{-1}. Detection by UV at 285 nm was used and the signal was compared with that of the standard (5-(hydroxymethyl-furan-2-carbaldehyde (HMF), Merck No. 820678). The solution used was prepared fresh daily.

GraphPad Prism was used to perform statistical analyses of the data obtained. ANOVA (one-way analysis of variance) was performed to study the effect of heating at different temperatures on HMF. The differences between the means were considered significant at the 5 % level using Dunnett's test.

Table 3. Changes in HMF content (mg.kg^{-1}) after treatment in a microwave oven

	HMF level mg.kg^{-1}	Increase in HMF %
Initial value	20.57	0
5 sec	21.13	2.70
10 sec	21.49	4.97
15 sec	22.83	10.99
30 sec	23.56	14.54
1 min	26.35	28.10
2 min	26.75	30.04
3 min	27.71	34.71

RESULTS

The values of HMF content in the honey samples after heating in a water bath are presented in the Table 1 and Fig. 1. They showed increasing HMF content with treated samples (mg.kg^{-1}) in comparison with the original samples. HMF content in the honey samples gradually increased depending up the temperature and time. The heating at 40, 50, 60, 70 °C within 1 minute caused only a gentle increase in the HMF content, but heating at temperatures higher than 80 °C increased the HMF content by approximately 50 %. Heating for 90 min at temperatures up to 70 °C did not increase the HMF content markedly. After heating at 50, 60, 70 °C the HMF content increased by 20 % (nectar honey) or by 45 % (honeydew honey), but after heating at higher temperatures the HMF values were manifold higher (80 °C by 84 % – nectar honey and by 152 % – honeydew honey; 95 °C by 307 % – nectar honey and by 652 % – honeydew honey) in comparison with the initial values. The results showed that HMF content increased more rapidly in honeydew honey than in nectar honey.

The results of heating the honey samples in the microwave oven (at 90 W), dependent upon the quantity and time of treatment, are showed in Table 2. The honey temperature after microwave treatment increased depending on the sample weight. Microwave treatment (1 min) of honey samples (5 g) increased the honey temperature up to a maximum of 82.3 °C. But two minute treatments of the samples with the weight lower than 50 g increased the honey temperature up to 100 °C.

Table 3 shows the change in HMF content in the honey samples after treatment in a microwave oven depending on the

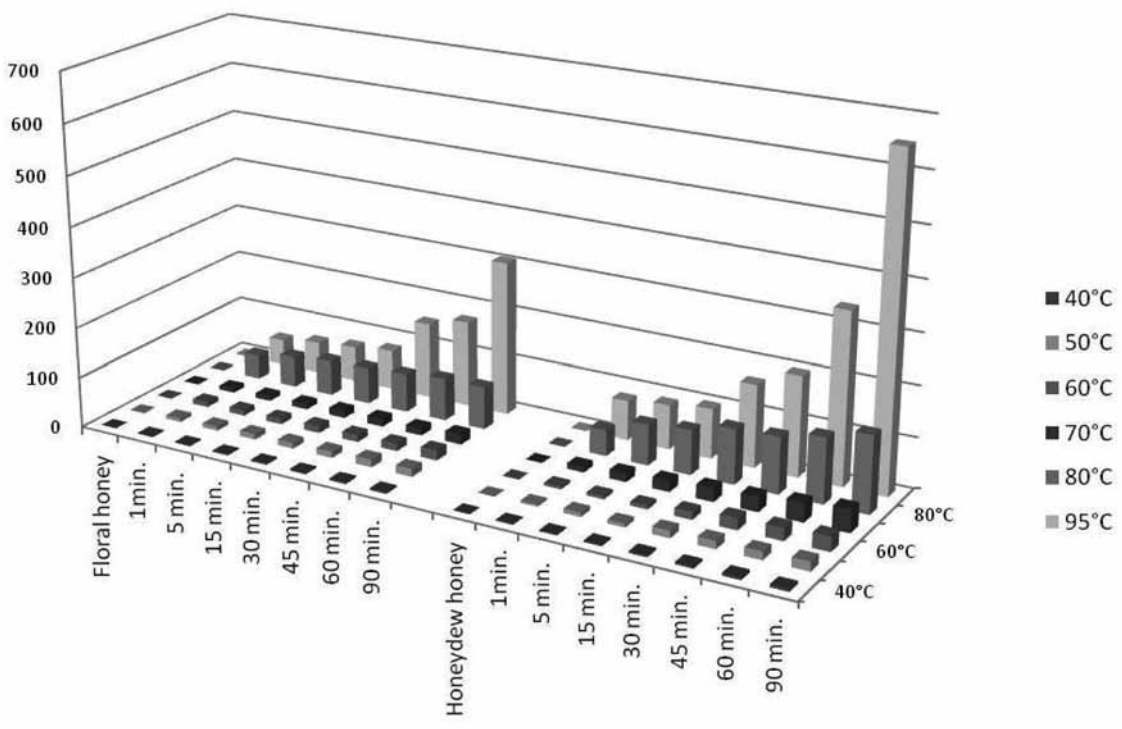

Fig. 1. Comparison of HMF (%) content in the honey samples after heating in a water bath

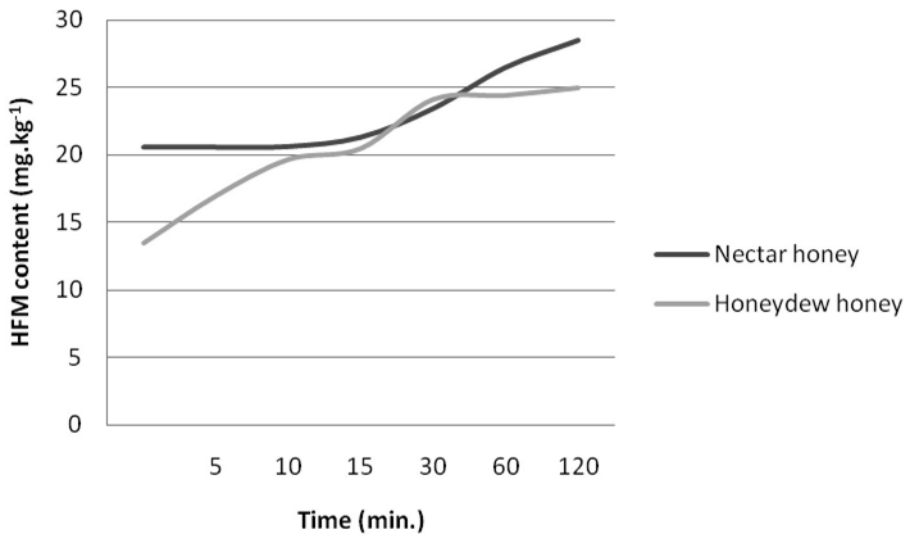

Fig. 2. Changes in HMF content (mg.kg^{-1}) in the honey after treatment in an ultrasonic bath

time of treatment. Three minutes treatment (sample weight 50 g) increased the HMF content by 34.71 %, but marked increase (by more than 20 %) in the HMF content was observed already after a 1 minute treatment. The HMF content in honey samples increased depending on the honey temperature.

The values of HMF content in the honey samples after treatment in an ultrasonic bath are presented in Fig. 2. The HMF content increased after ultrasonic treatment in both kinds of honey. The five minute treatment caused a marked increase in the HMF content (by 26.08 %) in honeydew honey. However, a marked increase in the HMF content in nectar honey was observed only after 60 minutes (26.49 %). The ten minute treatment did not increase the HMF content in nectar honey (only by 0.24 %). Long time treatment (120 min) increased the HMF content by 38.5 % in nectar honey and by 85.6 % in honeydew honey.

The heating of unprocessed honey obtained directly from beekeepers caused a significant increase ($P < 0.01$) in HMF

content. Heating the honeydew honey at 40 °C for 1 minute caused a significant increase ($P < 0.05$). The microwave heating of honey caused a significant increase ($P < 0.01$) in the HMF content. A significant increase ($P < 0.01$) in HMF content in nectar and honeydew honey was also caused by treatment in an ultrasonic bath with the exception of nectar honey treated for 5 and 10 min ($P > 0.05$).

DISCUSSION

Turhan *et al.* (14) studied the effects of thermal treatment on the HMF content of honeydew and nectar honey during the isothermal heating process at different temperatures. Heating at 90 °C for up to 90 min of nectar honeys and up 75 min of honeydew honeys did not cause a significant increase in HMF and did not exceed the threshold level of 40 $mg.kg^{-1}$. Their results showed that the excessive HMF content might be related to primitive storage conditions rather than to overheating. HMF formation was very different according to the origin of honey, nectar honeys contained low HMF content at 100 °C for up to 60 min while the honeydew honeys exceeded the 40 $mg.kg^{-1}$. Transient heating stage effect is less harmful, but HMF increases reach significantly higher values at high temperatures and longer time of heating (13). Formation of HMF increased with the increase in microwave power levels and duration of heating. However, microwave power levels showed a greater effect (4).

Heating unprocessed honey at 85 °C for 2 min caused a significant ($P \leq 0.05$) increment in HMF contents. The results revealed also that heating was not the only factor influencing HMF formation in honey, but also honey composition, pH value and floral source can contribute to these variations. Consequently, the amount of HMF may be an insufficient sole indicator of honey quality (1).

The results obtained indicate that manipulation with honey should comply with the requirements of the Codex Alimentarius of SR (5), i.e. the honey should not be subjected to microwave heating or ultrasound treatment.

CONCLUSION

The treatment of honey (water bath, microwave oven, and ultrasonic bath) increased the HMF content in the honey. The treatments accompanied by temperature increase resulted in higher amounts of the HMF in the honey samples in comparison with the treatment in ultrasonic bath. The initial HMF content was lower in the honeydew honey samples in comparison with nectar honey samples, but after the treatments used, the increase in HMF content was higher in honeydew honey.

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EVALUATING THE PRO-ARRHYTMIC POTENTIAL OF PARENTERALLY ADMINISTERED DIMINAZENE ACETURATE IN NIGERIAN LOCAL DOGS

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ABSTRACT

The pro-arrhythmic property of some non-cardiac drugs has been widely reported. This work is intended to investigate the cardiotoxic potential of diminazene aceturate in the light of current safety requirements. Six healthy Nigerian local puppies aged between 3–4 months were used for this study. The technique of electrocardiography was used in this study. Standard bipolar leads were recorded in anaesthetized animals serially before and at 30 minutes intervals for 3 hours after the administration of the drug. Diminazene aceturate was found to significantly affect the QT_C in Lead III. The mean QT_C at 120 minutes (0.28 ± 0.04), 150 minutes (0.29 ± 0.03) and 180 minutes (0.28 ± 0.04) post drug administration were significantly lower than the pre-treatment control value of 0.32 ± 0.037 in Lead III. The drug also significantly affected the heart rate ($P < 0.01$). There was a significant reduction in the heart rate at 60 min (78.00 ± 6.00) and 150 min (80.00 ± 3.46) when compared to the control value of 104 ± 13.85. Other ECG indices, like the PR interval, QRS interval, R wave amplitude, T wave amplitude and the mean electrical axis were not affected by the drug. From the findings of this study we hypothesised that the Ca^{2+} ATP_{ASE} inhibition and the subsequent increase in the intracellular calcium could possibly be responsible for the shortened QT_C and the likely pro-arrhythmic effect of diminazene aceturate.

Key words: cardiotoxicity; diminazene aceturate; dogs; ECG

INTRODUCTION

Diminazene aceturate (variously referred to as Berenil[R] and diminazene) is an aromatic diamidine (29) compound chemically described as 4-4'-diamidinodiazoaminobenzene diaceturate (24, 38). It has a molecular weight of 515.5 and decomposes at 217 ^{0}C (24). It is commonly used in tropical countries at a dose rate of between 3.5–8.0 $mg.kg^{-1}$ intramuscularly for the treatment of animal trypanosomiasis and babesiosis (37).

Generally, very little is known about the drug's mechanism of action (1, 2), and the toxicity profile (37). What is known, however, is that the drug is capable of exerting an hypotensive effect either through a parasympathomimetic action (38) or by inhibiting the $Ca^{2+}ATP_{ASE}$ pump on the plasma membrane (1).

Although there had been few reports on the toxicity of the drug, there have been sporadic cases of hepatoxicity (20) and more commonly, neurotoxicity in dogs. Most cases of neurotoxicity in dogs have been associated with multiple rather than single doses of the drug. It has been reported that healthy dogs given intramuscular diminazene aceturate in multiple doses showed severe clinical signs associated with damage to the central nervous system (CNS) and then died (19). The evidence that brain damage could be associated with enormous autonomic imbalances and subsequently myocardial injury, arrhythmias, and sudden death (31) called for a more critical look at the drug.

As of now, there is only a single published report on the acute cardiotoxicity of diminazene aceturate in dogs. Experience with pentamidine (another anti-protozoan drug used in third world countries) has shown that the it is capable of inducing QT-interval prolongation, leading to the onset of ventricular arrhythmias (18, 36). This knowledge of pentamidine and the acute mortality occasionally experienced during single diminazene aceturate therapy leaves more to be desired. In line with the current thinking that some non-cardiac drugs could affect cardiac repolarisation and cause lethal arrhythmias by prolonging the QT-interval, of the electrocardiogram (ECG), regulatory bodies have recommended that newer drugs should be subjected to preclinical evaluation in dogs to ascertain their pro-arrhythmic tendencies (8, 9).

Since most death associated with single diminazene aceturate administration in dogs cannot be readily rationalised and because the drug, as far as we know, has not been previously evaluated by the joint FAO/WHO expert committee on feed additives (37), for its pro-arrhythmic potentials, we are strongly compelled to investigate the cardiotoxic potential of the drug in the light of current safety requirements of regulatory bodies.

In this study, electrocardiographic changes associated with a single diminazene aceturate administration was investigated in Nigerian local puppies. The ECG was evaluated for rate, rhythm and wave form morphology.

MATERIALS AND METHODS

The protocols for this experiment were approved by the local ethical committee of the University of Agriculture, Abeokuta, Nigeria.

Six Nigerian local puppies of mixed sexes, aged between 3–4 months and weighing 3–4 kg were used for this study. Only dogs vaccinated against common viral diseases and free from both heartworm and *Babesia* infection qualify for this study. The dogs were deemed healthy on the basis of physical examination, thoracic auscultation and chest radiographs. Anaesthesia was achieved by administering a combination of xylazine ($3\,mg.kg^{-1}$) and ketamine ($10\,mg.kg^{-1}$) intramuscularly to each dog. Patency of the respiratory tract was ensured by endotracheal intubation. The dogs were kept at a temperature of 39 °C, throughout the experiment using a blanket. A 7 % solution of diminazene aceturate (Berenil, Jubali Inc.) was prepared by dissolving 2.35 grams of the powder in 12.5 ml of water. The drug was administered at a dose rate of $3.5\,mg.kg^{-1}$ intramuscularly to individual dogs.

Standard bipolar limb leads (Leads I, II, and III) were recorded serially before (0 min) and at 30 min intervals for three hours after the administration of the drug. The mean electrical axis was determined using the sum of the vectors in Lead I and III and then using the hexa-axial reference guide. The duration of the PR, QRS and QT intervals and the amplitude of the Q, R, S, and T amplitude were manually determined using a calliper. All these ECG indices were measured and averaged from 5 non consecutive RR intervals. The QT le ngth was measured from the beginning of Q to the end of T. The corrected QT interval ($QT_{C,}$) was obtained using Bazett's formula; $QTC = QT/\sqrt{RR}$ (3). The heart rate was determined by counting the number of cycles (RR-intervals) in 6 seconds and multiplying by a factor of 10.

Limb leads were constructed using plate electrodes. Adequate electrical contact between both the proximal metatarsal and metacarpal region was established by applying electrode gel. At a paper speed of $25\,mm.sec^{-1}$ and amplitude calibration of 1 mV = 10 mm ECG waves were recorded for 30 seconds per trial with the filter on. The recordings were made on a 4-channel oscillograph (Harvard apparatus, UK).

The ECG tracings were analysed and interpreted by a panel of cardiologist who were not part of the studies. Cardiac rhythm was scored on a numerical rating scale as follows; Normal sinus rhythm = 5, Sinus arrhythmia = 4, Supraventricular arrhythmia = 3, Ventricular arrhythmia = 2, Atrioventricular block = 1.

Statistical analysis

ECG data are presented as the mean ± standard deviation (SD). Differences within parameters were evaluated by one way repeated measures analysis of variance (ANOVA). When a $P < 0.05$, the drug was judged as having affected the parameter. Statistical significance between the pre-drug control and a value at a particular time point after the drug administration was determined by paired *t*-test with Bonferoni correction. The prevalence of the occurrence of the S-wave across the leads was compared using Man-Whitney test. Arrhythmia scores were compared using the Kruskal-Wallis test. In all cases, $P < 0.05$ was declared significant. The SPSS version 12 was used.

RESULTS

Throughout the course of the experiment none of the dogs showed any clinical signs of acute cardiopathy. All the dogs survived the study. One dog each had biphasic T-wave at 90 minutes, inverted T-wave at 180 minutes and R-wave alternant at 180 minutes.

Figures 1, 2, 3 and 4 show a time variation in the T wave amplitude, $QT_{C,}$ R wave voltage and the heart rate, respectively, across the three limb leads used in this experiment.

The R-wave voltages in Lead I, II, and III after the administration of the drug did not differ significantly from the pre-treatment values of (0.50 ± 0.29, P = 0.74; 0.71 ± 0.31, P = 0.79; and 0.77 ± 0.30, P = 0.09), respectively. In Lead III, the pre-treatment value of the R-wave voltage (0.77 ± 0.30) was reduced by 44 % to 0.43 ± 0.17 at 180 minutes after the drug administration (P = 0.1).

The Q-wave voltage in Lead II showed an almost significant increase (P = 0.05) in amplitude at 30 minutes (0.33 ± 0.05) post treatment, when compared to controls (0.19 ± 0.00).

In Lead I, the S-wave was recorded at 60 min, 90 min, 120 min, and 180 min in 66 % of the dogs. Only 33 % of the dogs showed S-wave in controls. None of the dogs displayed an S wave at 150 min. In Lead II, S-wave was recorded in 66 % of the dogs at 60 min and 90 min and in only 33 % of the dogs at 30 min, 120 min, 150 min, and 180 min. No S-wave was recorded at 0 min. In Lead III, none of the dog showed an S-wave at 0 and 30 min. Only 33 % of the dogs however showed an S-wave at 60 min, 90 min, 120 min, 150 min, and at 180 min. The incidence of the occurrence of S-wave used in this study differed significantly between lead I and III (P = 0.01).

Table 1. ECG parameters in Leads I, II, and III during the experiment

	0 min	30 min	60 min	90 min	120 min	150 min	180 min
Heart axis (^{0})	58.5 ± 13.48	38.33± 32.74	48.00 ± 15.39	64.33 ± 36.00	62.33 ± 11.59	66.66 ± 16.50	63.33 ± 12.85
Heart rate.	104.00 ± 13.85	90.00 ± 6.00	78.00 ± 6.00*	76.00 ±3.46	74.00 ± 9.16	80.00 ±3.46*	71.33 ± 7.02
PR I (sec)	0.09 ± 0.02	0.08 ± 0.00	0.09 ± 0.02	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.02
PR II (sec)	0.08 ± 0.01	0.10 ± 0.00	0.10 ± 0.02	0.09 ± 0.01	0.09±0.01	0.09 ± 0.01	0.08 ± 0.00
PR III (sec)	0.10 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.11 ± 0.01	0.09 ± 0.02
QRS I (sec)	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
QRS II (sec)	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
QRS III (sec)	0.04 ± 0.00	0.04 ± 0.015	0.03 ± 0.005	0.04 ± 0.00	0.03 ± 0.005	0.04 ± 0.00	0.04 ± 0.00
QT_C I (sec)	0.32 ± 0.05	0.31 ± 0.02	0.31 ± 0.04	0.30 ± 0.05	0.30 ± 0.04	0.30 ± 0.05	0.29 ± 0.07
QT_C II (sec)	0.33 ± 0.07	0.33 ± 0.01	0.30 ± 0.05	0.31 ± 0.03	0.30 ± 0.05	0.29 ± 0.03	0.29 ± 0.02
QT_C III (sec)	0.32 ± 0.037	0.30 ± 0.06	0.30 ± 0.05	0.29 ± 0.07	0.28 ± 0.04*	0.29 ± 0.03**	0.28 ± 0.04**
R I (mV)	0.50 ± 0.29	0.33 ± 0.21	0.50 ± 0.19	0.36 ± 0.12	0.52 ± 0.14	0.53 ± 0.26	0.61 ± 0.43
R II (mV)	0.71 ± 0.07	0.70 ± 0.08	0.62 ± 0.21	0.73 ± 0.25	0.58 ± 0.07	0.61 ± 0.11	0.62 ± 0.08
RIII (mV)	0.77 ± 0.30	0.90 ± 0.28	0.64 ± 0.44	0.60 ± 0.20	0.72 ± 0.23	0.78 ± 0.16	0.43 ± 0.17
T I (mV)	0.21 ± 0.10	0.11 ± 0.08	0.15 ± 0.08	0.12 ± 0.03	0.17 ± 0.13	0.16 ± 0.04	0.17 ± 0.08
TII (mV)	0.25 ± 0.14	0.21 ± 0.14	0.18 ± 0.02	0.18 ± 0.07	0.25 ± 0.12	0.25 ± 0.06	0.13 ± 0.06
TIII (mV)	0.25 ± 0.17	0.30 ± 0.13	0.12 ± 0.04	0.21 ± 0.16	0.24 ± 0.06	0.44 ± 0.35	0.17 ± 0.06
QII (mV)	0.19 ± 0.00	0.33 ± 0.05*	0.13 ± 0.04	0.24 ± 0.13	0.16 ± 0.03	0.17 ± 0.06	0.23 ± 0.07
QIII (mV)	0.33 ± 0.23	0.28 ± 0.01	0.17 ± 0.13	0.15 ± 0.12	0.21 ± 0.15	0.35 ± 0.20	0.18 ± 0.09
T_I/R_I	0.43 ± 0.08	0.35 ± 0.08	0.34 ± 0.18	0.38 ± 0.23	0.32 ± 0.18	0.29 ± 0.13	0.29 ± 0.03
T_{II}/R_{II}	0.35 ± 0.20	0.30 ± 0.17	0.32 ± 0.12	0.27 ± 0.14	0.45 ± 0.27	0.42 ± 0.12	0.21 ± 0.11
T_{III}/R_{III}	0.33 ± 0.17	0.35 ± 0.17	0.23 ± 0.15	0.32 ± 0.12	0.34 ± 0.07	0.53 ± 0.35	0.42 ± 0.21
TI (sec)	0.13 ± 0.04	0.10 ± 0.02	0.16 ± 0.04	0.16 ± 0.08	0.16 ± 0.03	0.16 ± 0.04	0.17 ± 0.08
TII (sec)	0.13 ± 0.02	0.16 ± 0.00	0.15 ± 0.05	0.16 ± 0.04	0.16 ± 0.06	0.14 ± 0.02	0.14 ± 0.04
TIII (sec)	0.16 ± 0.06	0.16 ± 0.04	0.16 ± 0.06	0.13 ± 0.06	0.12 ± 0.04	0.13 ± 0.02	0.14 ± 0.06
SI (mV)	0.11 ± 0.00		0.10 ± 0.05	0.10 ± 0.03	0.12 ± 0.06		0.13 ± 0.04
SII (mV)		0.12 ± 0.00	0.16 ± 0.03	0.18 ± 0.09	0.07 ± 0.00	0.12 ± 0.00	0.16 ± 0.00
SIII (mV)			0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	0.15 ± 0.00

*−P<0.05, **−P<0.01 compared to baseline

PR I−PR interval in Lead I, PRII−PR interval in Lead II, PRIII−PR interval in Lead III, QRSI−QRS interval in Lead I, QRSII−QRS interval in Lead II, QRS III−QRS interval in Lead III, QTc I−Corrected QT interval in Lead I, QTc II−Corrected QT interval in Lead II, QTc III−Corrected QT interval in Lead III, R I−R-wave in Lead I, R II−R- wave in Lead II, R III−R-wave in Lead III, T I−T-wave amplitude in Lead I, T II−T-wave amplitude in Lead II, T III−T-wave amplitude in Lead III, Q I−Q-wave amplitude in Lead I, Q II−Q-wave amplitude in Lead II, Q III−Q-wave amplitude in Lead III, Q II−Q-wave amplitude in Lead II, Q III−Q-wave amplitude in Lead III, T_I/R_I−T amplitude to R amplitude ratio in lead I, T_{II}/R_{II}−T amplitude to R amplitude ratio in Lead II, T_{III}/R_{III}−T amplitude to R amplitude ratio in Lead III, TI−T interval in Lead I, TII−T interval in Lead II, TIII−T interval in Lead III, SI−S amplitude in Lead I, SII−S amplitude in Lead II, SIII−S amplitude in Lead III

A significant reduction in the mean QT_C interval at 120 min (0.28 ± 0.04; P = 0.02) 150 min (0.29 ± 0.03; P = 0.008) and at 180 min (0.28 ± 0.04; P = 0.005) compared to controls (0.32 ± 0.37) was noticed in Lead III. In lead I, there was no significant difference between the pre-treatment and post treatments mean QT_C (P = 0.36). The QT_C at 90 minutes post-treatment (0.30 ± 0.05) was however almost significantly lower than the pre-treatment value. (0.32 ± 0.05) at P = 0.07. In Lead II, there was no significant difference in the QT_C value between groups (P = 0.67). Although the mean QT_C interval decreased transiently from a pre-treatment value of 0.33 ± 0.07 to 0.29 ± 0.02 at 180 minutes, the difference was not significant.

From Table 1 it is clear that the drug affected the heart rate significantly (P = 0.004). The mean heart rate of the pre-treatment control of 104 ± 13.85 differed significantly from the heart rate at 60 minutes (78.00 ± 6.00; P = 0.03) and 150 minutes (80.00 ± 3.46; P = 0.04).

Table 2. Time variations (min) in the arrhythmia scores. The median score is not affected by the drug (P > 0.05)

Score/ Time	0	30	60	90	120	150	180
	5	4	5	4	5	5	5
	5	4	4	4	4	4	4
	5	4	4	4	4	4	4
	4	4	4	4	4	4	4
	4	5	4	4	4	4	4
	1	5	4	4	4	4	4
Median Score	4 1/2	4	4	4	4	4	4

The post treatment values of T voltage did not differ significantly from the pre-treatment values in any lead (Lead I, P = 0.93; Lead II, P = 0.5; Lead III, P = 0.5). In Lead I, the pre-treatment value of T voltage (0.21 ± 0.10), however decreased almost significantly by 34 % to 0.18 ± 0.12 at 180 min (P = 0.09). Also in Lead II, the T amplitude was reduced by 10 % from a control value of 0.25 ± 0.14 to an almost significant level of 0.13 ± 0.06 at 180 min (P = 0.14).

Diminazene aceturate's administration did not affect the QRS, PR and T-wave width significantly in all the three leads. As shown in Table 1, there was no statistically significant difference in the changes observed in these indices when post treatment groups were compared with the pre-treatment controls (Lead I, P < 0.09; Lead II, P < 0.9; Lead III, P < 0.65). There was however, an almost significant increase in the T-wavelength of controls (0.13 ± 0.02) when compared with that of 30 min (0.16 ± 0.00; P = 0.18) and 90 min (0.16 ± 0.04; P = 0.18) post-treatment in Lead II.

The T/R ratio in all the leads did not show a statistically significant difference between the pre-treatment and the post treatment groups (Lead I, P = 0.50; Lead II, P = 0.51; Lead III, P = 0.55). In Lead I, the T/R ratio showed a transient decrease from a pre-treatment value of 0.43 ± 0.08 to 0.29 ± 0.03 at 180 min. The result in Lead II was also similar, as the T/R ratio also decreased from a control value of 0.35 ± 0.20 to 0.21 ± 0.11 at 180 min.

Table 1 also showed that the mean electrical axis (MEA) of the heart was not affected by a single intramuscular administration of a clinical relevant dose of the drug. The MEA of the pre-treatment group (58.50 ± 13.48) did not differ significantly from the post treatment groups (P = 0.12). At 120 minutes post treatment, the heart axis (62.33 ± 11.59) increased by 6.8 % from a pre-treatment value of 58.5 ± 13.48 (P = 0.15). The MEA also increased to 66.00 ± 16.50 at 150 minutes from a baseline value of 58.5 ± 13.48 (P = 0.2).

The median arrhythmia score for each of the group was shown in Table 2. There is no significant difference (P = 0.8) between the median arrhythmia scores of the pre-treatment groups and the post treatment groups.

DISCUSSION

This study showed that a single administration of diminazene aceturate at a clinical relevant dose of 3.5 $mg.kg^{-1}$ may be pro-arrhythmic. Quite unlike pentamidine, another diamidine and a sister antiprotozoal drug (11, 26) whose proarrhythmic tendency has been attributed to its QT_C prolonging effect, an effect which promotes the risk of lethal ventricular arrhythmia (36) diminazene aceturate's pro-arrhythmic tendency could be due to its QT shortening effect. The QT interval provides a measure of ventricular repolarisation and is determined by the balance of the repolarising inward sodium and calcium current and the outward potassium and chloride current (30).

The shortened QT_C normally results from a reduced repolarisation and hence the action potential duration. This could also reflect the heterogeneity of the intra-myocardial action potential. This abnormality can induce a pro-arrhythmic effect similar to that characterised by a long QT_C syndromes. The genetic basis of the shortened QT_C, just like that of the congenital long QT_C has been elucidated. Shortened QT_C increases the likelihood of re-entrant pathways which ultimately could lead to ventricular fibrillation (23). Hyperkalemia, hypocalcaemia, (28) and hypercalcaemia (11) and increased sympathetic activity are among the possible causes of shortened QT_C.

Abnormalities involving the membrane L-calcium channels, $Ca^{2+}ATP_{ASE}$ could affect the trans-membrane gradient for calcium ion fluxes across the membrane. An increase in intracellular Ca^{2+}, as a result of the inhibition of the $Ca^{2+}ATP_{ASE}$ is expected to affect the calcium current by decreasing the trans-membrane gradient. It may also have an indirect effect on K^+ channel functions (16). Increasing intracellular calcium has been reported to activate the time dependent delayed rectifier K^+ channel (35). There is evidence that the inactivation of L-type calcium channels is enhanced by the increased intracellular calcium ion thereby accelerating the decay of calcium currents and contributing to the shortening of the action potential (32). Diminazene has been

reported to inhibit the membrane $Ca^{2+}ATP_{ASE}$ pump (1). This no doubt will affect the trans-membrane calcium gradient, the L-calcium channel and the intracellular level of calcium. This could ultimately lead to the reduced duration of action potential and a shortened QT_C interval.

Another major finding of this work was the bradycardiac effect of the drug. Diminazene was shown to reduce the heart rate of the post-treatment groups when compared to controls. Although, the drug has been previously reported to exert a parasympathomimetic action on blood vessels (38), an effect which will most probably brings about a vasodilation and hypotension; the effect noticed in this study could have resulted from the indirect action of the drug on the calcium channels through the muscarinic receptors of the myocytes or directly on the calcium channels of the sinoatrial node. We are of the opinion that the intraventricular conduction which is largely mediated by Na^+ channel activity (27) may not be involved in the effects seen in this study. Again, this may be the reason why the QRS which represents the duration of ventricular depolarization, and the PR interval which represents the atrioventricular conduction time and the mean electrical axis of the heart was not affected in this study. MEA values determine the site, size and direction of the conduction system (33).

The R-wave amplitude is a sum of the simultaneous fibre to fibre conduction through the ventricular myocardium (15). In this study, low voltage R-amplitude was generally reported. This could be as a result of the small sizes of dogs used in the study (25). R-wave alternans was noticed in one dog, this also could be attributable to the size of the dogs used for the study. Although Berenil did not produce a consistent effect on the R- wave form, it produced a nearly significant reduction in its amplitude at 180 minutes post treatment. Because a decreased intracellular calcium within the myocardial cell results in slowing of the conduction and decreased contractility, the reduction in R-amplitude noticed in this study could result from a reduced concentration of calcium in the cytosol of the myocytes as a consequence of the $Ca^{2+}ATP_{ASE}$ pump inhibition.

There is no set standard for the dog's Q and S-wave in the canine ECG reference ranges (34). In the dog, an S-wave is not normally seen in Lead I but it is often present in Lead III. Our current study revealed a higher incidence of S-wave in Lead I when compared to Lead III. This finding, though curious, might not be of serious importance since the heart axis was not affected in this study. Our finding that the Q-wave voltage at 30 minutes post treatment was almost significantly increased in Lead II is in agreement with what has been previously reported. The amplitude of the Q-wave has been found to vary with the depth of the chest in dogs and a high voltage Q-wave is a physiological finding in dogs with deep chests (33) and even normal dogs have been found to have Q-wave (17).

The T-wave amplitude expressed directly or indirectly as T/R ratio was higher than 0.25, the reference value for dogs (6), but it is not affected by diminazene's administration. Although T-wave voltage abnormalities are considered of little diagnostic significance in dogs (14), nevertheless, tall T-waves have been reported in myocardial infarction in dogs (10, 34). The occurrence of inverted and biphasic T-wave occurring separately in two dogs during the course of this study lacked consistency and can therefore be regarded as inconsequential. The reduction in the T-volt at 90 and 180 minutes after the drug's administration at a near significance level could be attributable to increased calcium level within the cell which can trigger the inwardly rectifying potassium current, thus affecting the height of the T-wave.

Generally, as shown in Figures 1, 2, 3 and 4, there is a time variation in the T wave amplitude, $QT_{C,}$ R wave voltage and the heart rate, respectively, across the three limb leads used in this experiment.

As shown in Table 2, diminazene aceturate did not affect the cardiac rhythm score. In this study there was no incidence of supraventricular or ventricular arrhythmia as a result of reentrant or triggered activity. The incidence of secondary atrioventricular block recorded in one dog could also be accidental as this finding was not consistent. The median score of 4.5 showed that the heart rhythm of most of the dogs fluctuated between normal sinus rhythm and sinus bradycardia. Normally arrhythmias could result from either abnormal impulse generation or propagation. In this study, diminazene appeared to affect impulse generation without substantially interfering with the impulse conduction. We believe that the bradyarrhythmias after the administration of the drug is peripherally and not centrally mediated. This is because neurogenically mediated arrhythmias (the type that could result from neurotoxicity) normally produce significant changes in the ST-T and QT_C prolongation (4, 5); the type not experienced in our study.

Considering the various effects noticed in this study, it is our belief within reasonable limits that the pro-arrhythmic tendency of diminazene aceturate appears to be a function of the calcium ATP_{ASE} inhibition, and its primary effect on intracellular calcium, which will ultimately affect the repolarising potassium currents and the duration of the action potential. We are aware of the severe limitations which the use of anaesthesia is likely to pose to this study and this to a large extent influenced our choice of ketamine, a known sympathomimetic and xylazine, an α2 adrenoceptor agonist (12) with considerable muscle relaxant effects (21). It is assumed that the pressor effect of ketamine will be compensated for by the depressor effect of xylazine and a moderate influence on the cardiovascular system is thus expected (22). Ketamine-xylazine anaesthesia has however, been reported to cause bradycardia in dogs (7). The bradycardia thus reported in this study might be due to the effect of the anaesthesia and not that of diminazene aceturate.

There are several limitations to this study, some of which include: first, unavailability of reference data on the ECG of Nigerian dogs; secondly, the possibility of the anaesthesia affecting the heart functions and; thirdly the absence of data on serial electrolyte and blood pressure monitoring. In spite of these perceived limitations, this work is an attempt at evaluating the cardio toxicity of diminazene aceturate in the light of the safety requirements of regulatory bodies. It also represents the first documented report on the ECG of Nigerian local dogs.

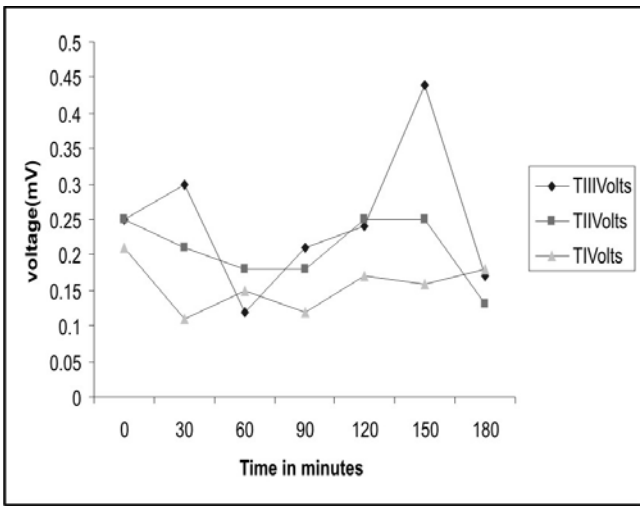

Fig. 1. The time course of the T-wave amplitude (mV) in Lead I (triangles), Lead II (square) and Lead III (diamond)

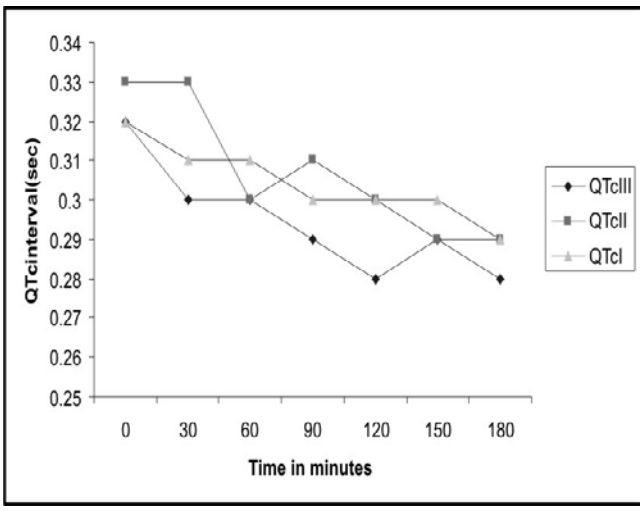

Fig. 2. The time course of the corrected QT interval (QTC) in Lead I, (triangles) Lead II (squares) and Lead III (diamond)

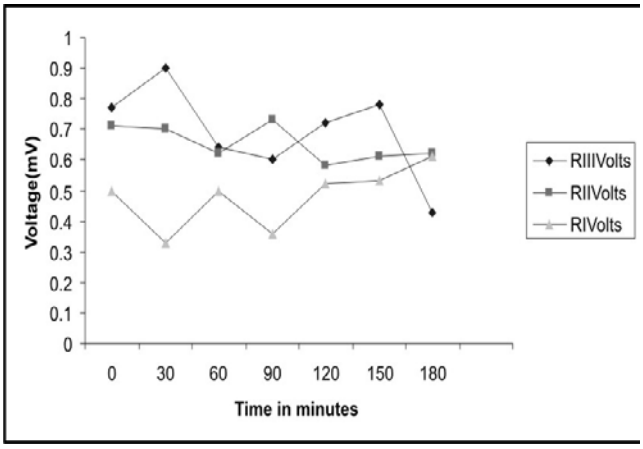

Fig. 3. The time course of the R amplitude (mV) in Lead I (triangle), Lead II (square) and Lead III (diamond)

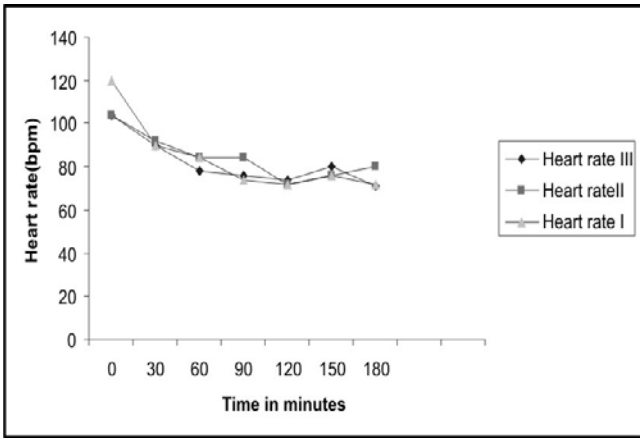

Fig. 4. The time course of the heart rate (bpm) in Lead I (triangle), Lead II (squares) and Lead III (diamond)

In future we shall not only attempt to address the identified limitations in this study, we shall deploy in-vitro electrophysiological technique to further validate the claims we made in this work.

CONCLUSION

The shortened QT_C reported in this study could be a marker of cardiotoxicity and an indication that diminazene aceturate is pro-arrhythmic. We therefore hypothesise that the pro-arrhythmic property of the drug could have resulted from calcium ATP_{ASE} inhibition.

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ACTIVITY OF CERTAIN TRANSAMINASES AND HISTOMORPHOLOGY OF THE LIVER OF MALE WEST AFRICAN DWARF GOATS EXPOSED TO 2,4-DICHLOROPHENOXYACETIC ACID (2,4,-D)

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ABSTRACT

Animals and humans are exposed to myriads of environmental toxicants such as herbicides of different chemical classes. Some of these herbicides may be ingested or absorbed through the skin or respiratory system. We previously found that exposure of 2,4-diclorophenoxyacetic acid (2,4-D) herbicide to male West African Dwarf (WAD) goats resulted in a hypochromic microcytic type of anaemia. In this study, we further investigated the effect of the oral administration of 2,4-D every 72 hours for 112 days of 75, 100 and 125 mg.kg^{-1} body weight, on the activity of certain transaminases and the histomorphology of the liver of male WAD goats. The oral administration of 2,4-D for 112 days, in WAD goats significantly ($P < 0.05$) increased the mean Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) values. Liver sections from goats treated with the graded levels of 2,4-D showed: a mild to moderate exudation (fibrinous) into peripheral areas; hepatocytes degeneration/necrosis; and fibrous connective tissue. The infolding of the ductular mucosae and hyperplasia of the epithelial linning were very evident. The sinusoids were hyperaemic, with kupffer cell proliferations and erythrophagocytosis. Our results demonstrated that exposure of 2,4-D to WAD goats can result in liver toxicity which may lead to serious consequences considering the importance of liver function to the life and health of animals and humans.

Key words: alanine aminotransferase; alkaline phosphatase; aspartate aminotransferase; liver; 2,4-dichlorophenoxyacetic acid; West African Dwarf goats

INTRODUCTION

2,4-dichlorophenoxyacetic acid is a herbicide that has been commercially available throughout the world (18). Crop farmers in Nigeria use these herbicides and other agro-chemicals extensively on their farms to control farm weeds without considering the environmental and animal/human health problems often associated with the extensive use of these chemicals. Environmental contamination by herbicides/pesticide has been documented in biotic and abiotic components (14). The majority of ruminants species living in the ecological habitats which are similar to the ones in which they evolved, often fall victim to some of these deleterious herbicides/pesticides that contaminate the environment (11). These animals are predominantly grazers and browsers which live off natural vegetation. Man has domesticated a few of these species and their numbers have increased enormously during their husbandry. Despite their domestication, many if not most of the ruminants (goats) which are owned by man still get a large proportion of their food by walking in vegetated areas of the environment and eating at will (11). Goats and to a lesser extent other ruminants, are enthusiastic grazers/browsers (11). This tendency to browse and forage on farm lands where herbicides/pesticides have been spread, often expose them to the untold dangers of these chemicals.

Furthermore, animal health problems that result from exposure to herbicides/pesticides are also due to the improper use or careless disposal of containers after application of their contents on the farm. The run-off of herbicides from agricultural applications and their entrance into the drinking water supply (15, 16) and municipal

dumps where our domestic animals (sheep and goats) scavenge is of great veterinary concern. Haematologic, hepatic, and renal toxicity have been demonstrated in Fischer rats (strain 344) exposed to 2,4-D treatment (17). There are documentations on the teratogenic, neurotoxic, immunosuppressive, cytotoxic and hepatotoxic effects of 2,4-D (5, 31, 3, 30, 34, 9, 23, 26, 33). There are other reports on the oxidant effects of 2,4-D, indicating the potential for cytotoxicity (7).

For our domestic small ruminant species (such as the West African Dwarf goats) that are always on free range wandering into farms where these herbicides might have been spread, there is no doubt that they are exposed to untold health hazards following their exposure to these chemicals. The present study was therefore designed to investigate the health implications of exposing male West African Dwarf (WAD) goats to graded levels of 2,4-dichlorophenoxyacetic acid.

MATERIALS AND METHODS

Care of the experimental goats and feeding

Twenty male West African Dwarf goats aged between seven and twelve months of age that weighed between 5.5–11.5 kg were used for this study. The goats were procured from different flocks, belonging to traditional goat keepers in Nsukka area of Enugu State, Nigeria.

The animals, on arrival to our Animal House Unit (AHU), were weighed using a standard weighing balance (Model Number H89 DK BLUE, Hanson, England). Faecal samples were collected for routine parasitological analysis for gastrointestinal (GI) helminth infections. In addition, blood samples were collected through jugular venipuncture and screened for the presence of haemoparasites, some of which are known to be enzootic in goats in this area (13). Levamisol^R and Asuntol^R (Bayer Leverkussen, Germany) were routinely used at the recommended doses of 7.5 mg.kg^{-1} and 8 mg.kg^{-1} respectively, to control endoparasites and ectoparasites in the experimental goats. Fifty dose vial of Peste des petites ruminants (PPR) vaccine, obtained from National Veterinary Research Institute (NVRI), Vom, Plateau State, Nigeria, was administered subcutaneously in the neck region of the goats at 1 ml/goat. Feeding was by daily provision of freshly cut *Panicum maximum, Stylosanthes gracilis,* and *Penisetum purpureum*. These forages were usually tied in a bundle before offering them to the animals. Grasses were suspended in each pen using a nylon rope material to make for a good ground clearance. Handling, management, and use of animals for experimentation were in conformity with laboratory Animal Rights Regulation of the University of Nigeria, Nsukka.

2,4-Dichlorophenoxyacetic acid

The 2,4-dichlorophenoxyacetic acid (Delmin Forte^R) used for this study was obtained from an Agro-allied venture at Onitsha, Anambra State, Nigeria. The concentration of 2,4-D in Delmin Forte^R was 720 gm per litre.

Experimental design and sample collection

The 20 male WAD goats were randomly divided into four groups (A, B, C, and D) of five goats per group after three weeks of acclimatization. The group D goats served as the control and received no 2,4-D treatment. Goats in groups A, B, and C received low (75 mg.kg^{-1} body weight), medium (100 mg.kg^{-1} body weight), and high (125 mg.kg^{-1} body weight) doses of 2,4-D orally through drenching (6) every 72 hours for a period of 112 days.

Blood for serum samples were collected from all the goats in groups A to D on day zero, before the commencement of the experiment, via venipuncture, using sterile needles and syringes. The serum samples were used for assessment of baseline values for the following enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)

On commencement of the experiment, goats in treatment groups A, B, and C and the control group D goats were bled every 28 day for serum samples which were used for enzyme assay. On day 112 of study, all goats in the four groups (A, B, C, and D), were sacrificed; and the liver carefully dissected out. Sections of the liver were transferred into clean bottles containing formol saline and used for histopathology.

ENZYME ASSAYS

Aspartate aminotransferase (AST)

AST was determined using the standard colorimetric method (29) for the *in vitro* determination of AST in serum or plasma using the Quimica Clinica Applicada (QCA) test kit (Quimica Clinica Applicada, Spain).

Alanine aminotranferase (ALT)

ALT determination was done by the Reitman-Frankel colorimetric method (29) for *in vitro* assessment of ALT in serum or plasma using the Quimica Clinica Applicada (QCA) test kit (Quimica Clinica Applicada, Spain).

Alkaline phosphatase (ALP)

ALP was determined using the phenolphthalein monophosphate method (1, 20) for the *in vitro* determination of alkaline phosphatase in serum or plasma, using Quimica Clinica Applicada (QCA) test kit (QCA, Spain).

Histopathological preparation

Tissue sections of the liver from 2,4-D exposed and control WAD goats were fixed in 10% formosaline and dehydrated in ascending grades of ethanol. Thereafter, the tissue sections were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5–6 microns. The sections were deparaffinized in xylene, taken to water, and subsequently stained with haematoxylin and eosin (H and E) for light microscopy (2).

Statistical analysis

The data collected from this study were summarized as means with standard deviations and comparison of means was done by one-way ANOVA with least significant difference (LSD) at the probability of 5% (10). Cross checking of analysis was done using the SPSS software (Statistical Package for Social Science, version 12.0, of 1999, New Jersey, USA).

RESULTS

Aspartate aminotransferase (AST)

The mean (± SD) AST levels for treatment groups A, B, and C and the control group D WAD goats are presented in Table 1. By day 28, the group B goats recorded a higher ($P < 0.05$) mean AST value than the control group D goats but the value was not significantly ($P > 0.05$) different from the mean value of the group C goats. Day 56 of the study did not reveal any significant variation in the mean AST levels of the four different goat groups. By day 84, the mean AST values for the groups B and C goats increased significantly ($P < 0.05$) relative to the mean value of the control group D goats. There was a significant increase in the mean AST value of the group C goats when compared to group A goats on day 84, but the mean value of the group A goats did not differ significantly ($P > 0.05$) from the value of the control group D goats. On day 112, the mean AST values for goats in groups B and C differed from the mean value of the control group D goats. The recorded mean AST value for the group A goats did not differ ($P > 0.05$) from the mean value of the control group D goat on day 112.

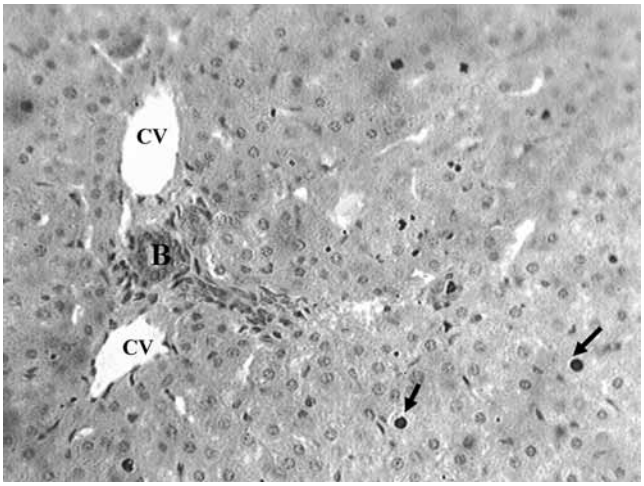

Fig. 1. Liver section of untreated (control) goat showing bile duct (B) and central veins (CV). Note apoptotic hepatocytes (arrows). H & E stain. Magn. × 400

Alanine aminotransferase (ALT)

The mean (± SD) ALT levels for treatment groups A, B, and C and the control group D WAD goats are presented in Table 2. By day 28, the mean ALT values in treatment groups A and B increased significantly ($P < 0.05$) relative to the mean value of the control group D goats. There was no significant ($P > 0.05$) variation in the mean ALT values of the group C and the control group D goats on day 28 of the study. By day 56, the mean ALT values in all the treatment groups, continued to increase ($P < 0.05$) relative to the control. The increase in mean ALT was more pronounced in groups B and C goats on day 84. On day 112, the mean ALT value in group A goats did not vary from the mean value of the control group D goats. Groups B and C goats on day 112, recorded significantly ($P < 0.05$) higher mean ALT values than the control group D goats.

Alkaline phosphatase (ALP)

Presented in Table 3 are the mean (± SD) ALP values for treatment groups A, B, C and the control group D WAD goats. All through the first 28 day period of the study, the mean ALP values of the 2,4-D exposed goats in treatment groups A, B and C differed significantly ($P < 0.05$) from the mean value of the control group D goats. In between the treatment groups on day 28, it was only the mean ALP of the group B goats that showed a significant ($P < 0.05$) variation from the mean value of the group C goats, but no significant ($P > 0.05$) variation was found to exist between the mean ALP value of the group B goats and that of the group A goats. The observed significant increase in the mean ALP values in all the treatment groups continued up to days 56 and 84 respectively, relative to the mean value of the control;

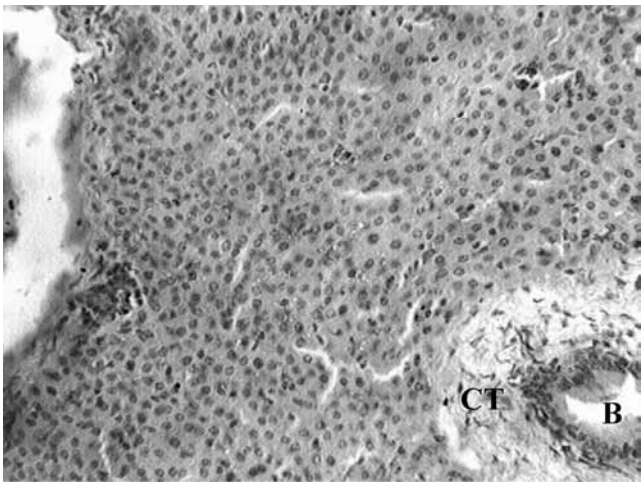

Fig. 2. Liver section of goat treated with 2,4-D (100 mg.kg^{-1} b.w.) showing bile duct (B) with hyperplasia and atrophy of mucosal epithelium. Note area of connective tissue (CT) replacement of necrotic hepatocytes. H & E. Magn. × 400

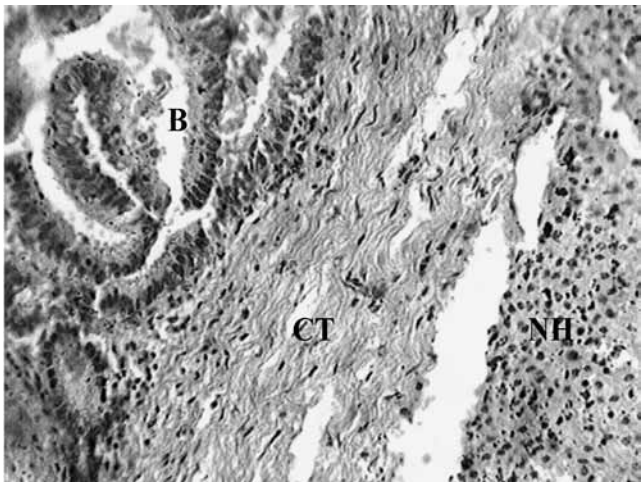

Fig. 3. Liver section of goat treated with 2,4-D (125 mg.kg^{-1} b.w.) showing infolding, hyperplasia and hypertrophy of bile duct (B) mucosa. Note fibrous connective tissue (CT) replacement of necrotic hepatocytes (NH). H & E. Magn. × 400

Table 1. Serum aspartate aminotransferase (AST) level in the treatment groups A, B, and C and the control group D goats ($\bar{x} \pm SD$)

Experimental period (day)	Serum aspartate aminotransferase level ($IU.l^{-1}$)			
	Group A (75 $mg.kg^{-1}$ b.w.)	**Group B** (100 $mg.kg^{-1}$ b.w.)	**Group C** (125 $mg.kg^{-1}$ b.w.)	**Group D** (control)
0	43.16 ± 0.87^{a}	41.89 ± 7.45^{a}	40.08 ± 4.58^{a}	42.08 ± 8.17^{a}
28	53.62 ± 7.09^{a}	50.63 ± 8.83^{b}	54.05 ± 8.80^{b}	41.66 ± 2.05^{a}
56	47.67 ± 3.19^{a}	47.49 ± 5.25^{a}	46.77 ± 7.09^{a}	40.97 ± 2.08^{a}
84	52.70 ± 5.60^{ab}	62.43 ± 10.62^{bc}	72.78 ± 9.72^{c}	47.29 ± 4.72^{a}
112	48.01 ± 6.86^{ab}	52.29 ± 4.88^{b}	55.32 ± 7.11^{b}	43.96 ± 3.31^{a}

Values in the same row with different superscripts differ significantly ($P < 0.05$)
^{a} – mean ± SD at $P < 0.05$

Table 2. Serum alanine aminotransferase (ALT) in the treatment groups A, B, and C and the control group D goats ($\bar{x} \pm SD$)

Experimental period (day)	Serum alanine aminotransferase level ($IU.l^{-1}$)			
	Group A (75 $mg.kg^{-1}$ b.w.)	**Group B** (100 $mg.kg^{-1}$ b.w.)	**Group C** (125 $mg.kg^{-1}$ b.w.)	**Group D** (control)
0	9.32 ± 1.49^{a}	9.93 ± 2.07^{a}	9.45 ± 1.41^{a}	9.45 ± 1.26^{a}
28	9.30 ± 2.42^{a}	9.52 ± 1.67^{b}	8.60 ± 2.13^{a}	6.81 ± 0.77^{a}
56	7.51 ± 0.60^{ab}	8.81 ± 1.96^{b}	8.29 ± 1.51^{ac}	6.46 ± 1.14^{d}
84	8.82 ± 1.70^{ab}	10.25 ± 2.62^{ab}	10.87 ± 2.25^{b}	7.62 ± 2.31^{a}
112	7.34 ± 1.64^{a}	9.63 ± 0.78^{b}	10.07 ± 1.96^{b}	7.16 ± 0.45^{a}

Values in the same row with different superscripts differ significantly ($P < 0.05$)
a – mean ± SD at $P < 0.05$

Table 3. Serum alkaline phosphatase levels (ALP) of the treatment groups A, B, and C and the control group D goats ($\bar{x} \pm SD$)

Experimental period (day)	Serum alkaline phosphatase level ($IU.l^{-1}$)			
	Group A (75 $mg.kg^{-1}$ b.w.)	**Group B** (100 $mg.kg^{-1}$ b.w.)	**Group C** (125 $mg.kg^{-1}$ b.w.)	**Group D** (control)
0	63.84 ± 7.14^{a}	54.20 ± 6.80^{a}	49.07 ± 9.71^{a}	51.97 ± 15.37^{a}
28	146.70 ± 14.04^{bc}	148.77 ± 10.66^{c}	134.54 ± 4.44^{b}	59.56 ± 8.35^{a}
56	132.48 ± 21.20^{b}	139.33 ± 16.10^{b}	190.53 ± 24.46^{c}	66.07 ± 16.00^{a}
84	101.34 ± 12.14^{b}	170.51 ± 11.68^{c}	195.16 ± 21.98^{d}	72.99 ± 9.31^{a}
112	194.06 ± 11.74^{b}	168.29 ± 47.73^{b}	176.76 ± 36.55^{b}	72.33 ± 5.00^{a}

Values in the same row with different superscripts differ significantly ($P < 0.05$)
a – mean ± SD at $P < 0.05$

with the group C goats that received the highest dose level of 2,4-D recording 190.53 ± 24.46 and 195.16 ± 21.96 IU.l^{-1} of ALP mean values on the said days respectively. On day 112 of the study, the significant increase in the mean ALP values of the three treatment groups was still outstanding, except that the group A goats recorded the highest mean ALP value.

Histopathology

The liver sections collected from untreated (control) goats showed well delineated hepatic lobules, with cords of normal hepatocytes; normal blood vessels and ducts (Fig. 1). The sections from goats treated with graded levels of 2,4-D showed mild to moderate exudation (fibrinous) into the periportal areas; hepatocytes degeneration/necrosis and fibrous connective tissue deposition. There was infolding of the ductular mucosae and hyperplasia of the epithelial lining. Sinusoids were hyperaemic, with kupffer cell proliferations and erythrophagocytosis (Figs. 2 and 3). The severity of the lesions correlated with exposure to increasing concentrations of 2,4-D.

DISCUSSION

Evaluation of some of the liver functions was conducted in order to determine the hepatotoxic effects of 2,4-D on the liver indicated by the activity of AST and ALT.

Serum AST activity is more useful in assessing the histological severity of the liver disease. Probably this cytosolic and mitochondrial enzyme (AST) is present in higher quantities in the liver compared to cytosolic ALT and thus more is released when the tissue damage is severe (25). On the other hand, ALT is more specific in the liver than AST (24). The significant increase in the mean values of the AST and ALT in the treatment groups B and C goats in this study, signified that exposing goats to 2,4-D at the dose levels of 100 and 125 mg. kg^{-1} body weight affected the activity of these transaminases. Enzymes are protein catalysts present mostly in living cells and are constantly and rapidly degraded although, renewed by new synthesis (12). The normal enzyme levels in the serum is a reflection of a balance between their synthesis and release, as a result of the different physiological processes in the body (36). Transaminase enzymes are those mostly responsible for the synthesis of non-essential amino acids through the process known as transamination (8). In this study, the increase in the mean levels of the transaminases in the 2,4-D exposed goats in groups B and C could be an indicator that the herbicide toxicant had an effect on the enzyme secretion mechanism.

Serum levels of AST are significantly higher under disease and morbid conditions involving injuries to large numbers of metabolically active cells (19). ALT activity in animals is not specific for the liver, in order to have a diagnostic significance (21). ALT activity in the blood is influenced by age and muscle activity (35). The result of this study shows that 2,4-D herbicide in the exposed goats necessitated the release of cellular enzymes (AST and ALT), possibly from injured cell (hepatocytes and other cells), which ultimately led to the rise in the mean serum enzyme concentration (4) as evidenced in this experiment.

The periportal lesions in the liver sections (hepatocytes necrosis and fibrosis; and infolding/hyperplasia of the bile duct mucosa) strongly suggest biliary stasis; which is either intra-or extra-hepatic. The situation shall, with time, cause more degeneration and necrosis of hepatocytes; with the tendency to cause hepatic dysfunction, especially in more severely affected situations. Typically, liver necrosis results in the elevation of serum transaminases such as alanine transaminase (ALT), aspartate transaminases (AST) and sorbitol dehydrogenase (SDH) (37, 32). The observed hepatocytes necrosis in the medium and high dose levels of 2,4-D treatment probably led to the increased levels of AST and ALT in the exposed goats. The observed possible liver toxicity of this herbicide may lead to serious consequences considering the importance of liver function to the life and health of the animal. The liver is directly involved in nutrient homeostasis, including glucose regulation (32, 27); cholesterol synthesis and uptake (32); synthesis of proteins (32, 28) etc. The finding of other workers (18) who observed some changes in the levels of transaminases (AST and ALT) in rats exposed to high doses of 2,4-D, lend credence to the observed effects in this study.

The significant increase in the mean ALP values of goats exposed to 2,4-D continued to days 56 and 84 with the group C goats that received the highest oral dose level of 2,4-D treatment recording 190.53 ± 24.46 and 195.16 ± 21.96 IU.l^{-1} of ALP mean values on the said days respectively. ALP level in the blood is usually a good indicator of bone formation since osteoblasts secrete large quantities of this enzyme. It can be deduced from this study that the 2,4-D in the system of the exposed goats may have led to the disruption in the activity of these osteoblast, thus leading to increase in their mean ALP values.

This study has established that exposing male West African Dwarf goats to varied concentrations of 2,4-D can lead to liver injury with changes in the levels of transaminases (AST, ALT) and ALP. The possible liver toxicity of this herbicide (2,4-D) in the exposed goats may lead to serious consequences considering the importance of liver function to the life and health of the animal. Crop farmers who use these chemicals to control weeds on their farms should be meant to understand the potential catastrophic health hazards (environmental health risk) for our animals and human beings that may run the risk of being exposed to these chemical toxicants when they are applied on farmlands.

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IN VIVO EFFECTS OF LEAD ON HAEMOGRAM AND HEPATIC ENZYMES

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ABBSTRACT

In order to investigate the haematological and hepatotoxicity of lead, twenty male Wistar albino rats were randomly assigned to four experimental groups (A, B, C and D) with five animals per group. Group A served as the control and was given distilled water free of lead, while groups B, C, and D were orally exposed to 200, 300 and 400 ppm of lead (Pb^{2+}) as lead acetate respectively for a period of two weeks. The results showed that lead induced significant alterations in the haematological parameters, plasma lipids, proteins and aminotransferases of rats exposed to varying concentrations of lead compared to the control group. It was concluded that exposure to lead can result in a significant decrease in the haematological parameters predisposing the affected animals to anaemia with concomitant increases in aminotransferases that can also predispose them to liver dysfunction.

Key words: aminotransferase; anaemia; haematological parameters; hepatotoxicity; lead

INTRODUCTION

Environmental lead poisoning is increasingly becoming a health burden and chronic exposure to high levels of lead, leads to adverse effects on liver function and the haematopoietic system in both humans and other animals (14, 22). Although lead has been eliminated from petrol in many countries, it may have other origins, such as industrial pollution. Exposure to lead may occur during the manufacture of batteries, printing, pottery glazing and lead smelting processes (11). It is a common cause of poisoning of domestic animals throughout the world. Cattle are the most susceptible livestock, with calves the most likely victims. However, lead poisoning can occur in all domestic animals including horses, birds/poultry and dogs. Pigs are the least susceptible (18). Lead can cause a wide range of biological effects depending upon the level and duration of exposure. It causes adverse effects in several organs and systems including nervous, renal, cardiovascular, reproductive, haematological and the immune system (1, 22, 24). Lead toxicity is closely related to its accumulation in certain tissues and its interference with the bioelements, whose role is critical for several physiological processes.

Studies have reported that the negative effects of lead ranged from slight biochemical or physiological disorders to serious pathological conditions in which some organs and systems can be damaged or have their functions altered according to the degree of exposure (6). Many enzymes, membrane and biochemical processes have been shown to be affected by lead, but none of these have been shown to be both sensitive and of key importance in explaining the manifestations of the toxicity. Among the well-established effects of lead is anaemia due to the inhibition of haeme synthesizing enzymes. Altered haeme synthesis is an early effect associated with increasing lead concentration in the soft tissues. Inhibition of delta-aminolevulinic acid dehydratase (ALAD) and elevation of protoporphyrins in erythrocytes (zinc protoporphyrin – ZPP) are the earliest effects, followed increased delta-aminolevulinic acid (ALA) and coproporphyrin (CP) excretion in the urine (19, 26). Lead is known to reduce erythrocyte membrane stability, while the formation of intranuclear inclusion bodies is one of the earliest manifestations of the exposure to lead. Lead affects the metabolism of other minerals and has an affinity for bone, where it acts by replacing calcium; thus the highest concentrations of lead are usually found in the bone, kidney and liver (7, 12).

Lead toxicity has been extensively studied in animals and both occupational and environmental exposures remain a serious problem in many developing and industrialized countries. Heavy metal poisoning like lead cause adverse effects to hepatic cells because the liver is one of the major organs involved in the storage, biotransformation and detoxification. There are only a limited number of studies on the effects of exposure to different concentrations of lead, hence the current study aimed to access lead toxicity by evaluating various biochemical and haematological parameters in male Wistar rats exposed to different concentrations of lead as lead acetate.

MATERIALS AND METHODS

Experimental design

Twenty male Wistar rats were used in this investigation. The average weight of the rats was 150 ± 4.56 g. The animals were maintained under standard laboratory conditions (temperature 28 °C, 14 hours light). They were fed dry ration ad libitum (fat/oil 6 %, crude fibre 5 %, calcium 1 %, available phosphorus 0.4 %, lysine 0.85 %, methionine 0.35 %, salt 0.3 %, crude protein 18 %, metabolisable energy 2900 kcal.kg^{-1}, manufactured by TOPFEEDS®, Lagos, Nigeria). The animals were divided equally into four groups as follows:

Group A: served as the control and was given distilled water.

Group B: was given 200 ppm lead as lead acetate in the drinking water.

Group C: was given 300 ppm lead as lead acetate in the drinking water.

Group D: was given 400 ppm lead as lead acetate in the drinking water.

The animals were exposed to different concentrations of lead as lead acetate ($(CH_3.COO)_2Pb.3H_2O$, assay (ex Pb) 99 – 103 %, maximum limits of impurities, chloride 0.005 %, copper 0.002 %, a product of Cartivalues, England) for two weeks. At the end of the exposure time, each animal of all groups was anaesthesized with ether and then blood samples were collected by the heparinated capillary tubes from the eye vein into heparin containing tubes.

Haematological analyses

A cell counter was used to analyze haematological indices. The packed cell volume (PCV) was estimated by the microhaematocrit method, while the red blood cell (RBC) and white blood cell (WBC) counts were determined using haemocytometer as described by Schalm *et al.* (27).

Haemoglobin (Hb) concentrations were estimated by the method of VanKampen and Zijlstra (35), as described in the haemoglobin kit manual supplied by Randox® Laboratories Limited, United Kingdom.

The plasma was separated by centrifugation and biochemical analyses were done on fresh plasma samples.

Biochemical analyses

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed by the method of Schmidt and Schmidt (28), as described in ALT and AST Randox® diagnostic kits manual respectively.

The total plasma triglycerides were determined spectrophotometrically by the method of Tietz (33), as described in triglyceride kits manual supplied by Randox® Laboratories Limited, United Kingdom.

The total plasma cholesterol was determined spectrophotometrically according to the method of Richmond (25), as described in the cholesterol Randox® diagnostic kit manual.

The total plasma protein was determined using the method of Tietz (34), as described in the total protein kit manual supplied by the Randox® Laboratories Limited, United Kingdom.

Statistical analysis

The results were evaluated statistically using one-way analyses of variance (ANOVA) with the Duncan test of the Statistical Package for the Social Sciences (SPSS v. 16). The significance of the differences among treatment groups to all statements of significance were based on the probability of $P < 0.05$.

RESULTS

The haematological parameters of rats exposed to different concentrations of lead as lead acetate are shown in Table 1.

There were significant reductions ($P < 0.05$) in both the PCV and haemoglobin concentrations of rats exposed to different concentration of lead compared to the control group. The reduction in both the PCV and haemoglobin concentrations was more prominent in group D which was about 24.42 % and 39.38 %, respectively, compared to the control group.

There were also significant reductions ($P < 0.05$) in both the red and white blood cell counts as the concentrations of lead exposure increased compared to the control group. The reduction in both the red and white blood cell counts was about 40.59 % and 45.90 % respectively in group D compared to the control group.

Table 1. Haematological parameters of rats exposed to different concentrations of lead as lead acetate

Groups	PCV (%)	Hb (g.dl^{-1})	RBC (×10^6.mm^{-3})	WBC (×10^3.mm^{-3})
A	43.40 ± 1.14^c	9.04 ± 0.94^c	8.77 ± 0.14^d	9.63 ± 0.12^c
B	38.80 ± 1.64^b	8.12 ± 0.96^{ab}	7.23 ± 0.21^c	7.42 ± 0.15^b
C	37.80 ± 1.30^b	7.36 ± 0.83^b	5.70 ± 0.49^b	5.72 ± 0.60^a
D	32.80 ± 2.59^a	5.48 ± 1.13^a	5.21 ± 0.70^a	5.21 ± 0.70^a

Values in the same column with different superscript differ significantly ($P < 0.05$)

Table 2 shows the plasma alanine aminotransferase and aspartate aminotransferase of rats exposed to different concentrations of lead as lead acetate. In both ALT and AST, there was a significant increase in both of the activities of

these two enzymes ($P < 0.05$) compared to the control group. The activity of AST was higher than that of the ALT. The activity of these enzymes increased with an increase in the concentration of lead exposure. The activity of ALT and AST was found to be about 1.55 and 1.25 times that of the control group respectively.

Table 2. Plasma alanine aminotransferase and aspartate aminotransferase of rats exposed to different concentrations of lead as lead acetate

Groups	ALT (UL^{-1})	AST (UL^{-1})
A	62.25 ± 1.19^{a}	91.70 ± 0.87^{a}
B	75.95 ± 1.78^{b}	97.38 ± 2.34^{ab}
C	83.71 ± 5.64^{c}	100.28 ± 2.03^{b}
D	96.51 ± 2.06^{d}	114.17 ± 9.38^{c}

Values in the same column with different superscript differ significantly ($P < 0.05$)

Table 3 shows the plasma triglyceride, cholesterol and total protein of rats exposed to different concentration of lead as lead acetate. There was a significant increase in both the total plasma triglyceride and cholesterol with a progressive decrease in the total plasma protein of rats exposed to different concentration of lead as lead acetate compared to the control group ($P < 0.05$).

Table 3. Plasma triglyceride, cholesterol and total protein of rats exposed to different concentration of lead as lead acetate

Groups	Triglyceride ($mg.dl^{-1}$)	Cholesterol ($mmol.l^{-1}$)	Protein ($g.dl^{-1}$)
A	38.48 ± 4.39^{a}	0.68 ± 0.06^{a}	70.91 ± 8.10^{c}
B	59.54 ± 4.08^{b}	0.76 ± 0.06^{a}	61.62 ± 2.31^{b}
C	65.79 ± 3.61^{c}	1.01 ± 0.17^{a}	54.65 ± 2.94^{a}
D	72.57 ± 2.08^{d}	1.47 ± 0.44^{b}	51.36 ± 1.24^{a}

Values in the same column with different superscript differ significantly ($P < 0.05$)

DISCUSSION

In the present study, the biochemical and haematological changes following exposure to different concentrations of lead was investigated in male Wistar rats. The concentrations of lead acetate utilized were based on reports in the literature (15). The lead exposure was of the subchronic type and the oral route was chosen because of it convenience and it was less stressful to the rats.

From our results, we noticed a decrease in packed cell volume, haemoglobin, red and white blood cells owing to the fact that lead intoxication causes a documented defect in haeme synthesis. The result obtained agreed with several authors (16, 20). Several reports have indicated that lead can cause neurological, haematological, gastrointestinal, reproductive, circulatory and immunological pathologies, with all of them related to the dose and the amount of time of lead exposure (3, 23).

About 99 % of the lead present in the blood is bound to the erythrocytes. They have a high affinity for lead and contain the majority of the lead found in the blood stream, which make them more vulnerable to oxidative damage than many other cells. Moreover, erythrocytes can distribute the lead to different organs of the body (31). Anaemia, reticulocytosis and basophilic stripling have been recognized in lead poisoning since the start of century (3, 9, 21). Lead binds avidly to the red cells with up to fifty times as much as that found in the bone (2, 22, 36). The abnormalities are confined to the erythroid series with ineffective erythroid hyperplasia being prominent.

Liver is one of the organs involved in the storage, biotransformation and detoxification of toxic substances and it is of interest in heavy metal poisoning (13). The activities of both aspartate and alanine aminotransferases are good indicators of hepatotoxicity (32, 37). In this study, we noticed a significant increase in both the activities of aspartate and alanine aminotransferases in rats exposed to different concentrations of lead compared to the control group. On the other hand, the result also showed a significant increase in both the total plasma triglyceride and cholesterol compared to the control group. The increased levels of lipids with increased activities of both the aspartate and alanine aminotransferases may indicate liver dysfunction (17). These results showed that exposure to lead affects the hepatic tissue, which is consistent with other reports (4, 30).

Aminotransferase (ALT and AST) are an important class of enzymes linking carbohydrate and amino acid metabolism, and the relationship between the intermediates of the citric acid cycle is well established. These enzymes are regarded as markers of liver injury (32). ALT was elevated significantly more than AST on lead exposure which is an indication of liver damage (29) and development of liver fibrosis (5).

Lead binds to plasma proteins where it can cause alteration to a high number of enzymes. It can also perturb protein synthesis in hepatocytes (10). The observed decrease in the protein content of the plasma of rats exposed to different concentrations of lead may be due to decreased hepatic deoxyribonucleic acid and ribonucleic acid (29).

El-Zayat *et al.* (8) also reported a decrease in hepatic total protein content in response to lead intoxication which was attributed to decreased utilization of free amino acids for protein synthesis.

In conclusion, this study has demonstrated that exposure to lead could have generated alterations in both the haematological and biochemical parameters. These alterations are dose dependent resulting in decreased haematological and protein synthesis followed by the elevation of the plasma lipids and activities of AST and ALT.

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AZOLE DERIVATIVES AND THEIR USE IN THE THERAPY OF MYCOSES

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ABSTRACT

This paper provides the brief characteristics of individual representatives of azole derivatives used for the treatment of mycotic infections in animals. It describes the pharmacological properties of the respective substances with a focus on the indication spectrum, way of administration, and dosage.

INTRODUCTION

Mycoses are serious infections of animals and humans. Agents of mycoses – microscopic fungi – are extremely resistant to adverse environmental conditions (9). The development of mycotic infections is affected by a number of factors, such as: decreased resistance (due to serious diseases, *diabetes mellitus*, thyroid gland disorders, and malnutrition); treatment with antibiotics; corticosteroids; skin microtraumas; and increased environmental humidity (27).

According to the localisation of the disease process, mycotic infections are divided into topical (dermatomycoses) and systemic (organ mycoses). The most frequent agents of dermatomycoses are dermatophytes – imperfect fibrous fungi, which specialise during their development on the utilization of one of the least digestible protein – keratin (44). Animal dermatophytoses are caused by the genera *Microsporum* and *Trichophyton* (50). The dominant pathogens of systemic mycoses are *Cryptoccocus neoformans* var. *neoformans, Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Aspergillus* spp. (16).

Mycotic infections are treated with highly specific antimycotics. Substances with antifungal activity may be divided into natural (polyenic antibiotics), semi-synthetic (echinocandines), or synthetic (azole derivatives, alyl amines and pyrimidines). The most frequently used and at the same time most numerous group of antimycotics are synthetic azole derivatives (13).

Mechanism of effect of azole antimycotics

Azoles block the synthesis of ergosterol, the most important structural sterol of the cellular membrane of micromycetes. Ergosterol, as an equivalent of cholesterol, is an essential component of cytoplasmic membrane of yeasts and moulds (30). Sterols are an important group of membrane lipids of eukaryotic cells. Mammals, plants and mushrooms synthesize sterols of very similar composition differing essentially only by the number and localisation of double bonds and methyl side chains. Ergosterol is a final product of sterol biosynthesis and is the most important sterol of cellular membranes. It is produced from squalene through lanosterol, zymosterol, fekosterol, episterol, ergosta-5,7-dienol and ergosta-5,7,22,24-tetraene triol. Similar to cholesterol in mammalian cells, ergosterol is responsible for fluidity and permeability of the cytoplasmic membrane (21).

Azoles prevent conversion of lanosterol (precursor of ergosterol) to ergosterol. This reaction is ensured by the enzyme C-14-alfa-demethylase, the catalytic activity of which depends on the mediator action of cytochrome P-450, responsible for demethylation of lanosterol to ergosterol (27). At the molecular level, the N4 atom of azole compounds binds to the atom of haeme iron in the molecule of cytochrome P-450. This limits the bonds Fe^{3+}-superoxide complexes, necessary for hydroxylation of methyl sterols (20). Inhibition of the enzyme C-14-alfa-demethylase causes the accumulation of methylated sterols (for example lanosterol) which cannot be integrated into the membrane (13).

Azole derivatives have a broad spectrum of action against dermatophytes, yeasts and fungi. They are divided into the derivatives

of imidazoles (klotrimazole, econazole, miconazole, ketoconazole, enilconazole) and triazoles (fluconazole, itraconazole, posaconazole, voriconazole) (13).

IMIDAZOLES

Klotrimazole

Klotrimazole is a synthetic derivative of imidazole (3). It is applied locally, principally in dermatology (13).

The indication spectrum of klotrimazole is relatively wide. It includes mycotic infections caused by *Trichophyton (T.) rubrum, T. mentagrophytes, Epidermophyton floccosum*, *Microsporum canis, Malassezia furfur, Aspergillus fumigatus, Candida albicans,* but also by the bacteria *Staphylococcus aureus, Streptococcus pyogenes* and *Proteus vulgaris* (34, 39).

In dogs and cats it is used mostly for the treatment of mycotic infections caused by *Microsporum canis* and *T. mentagrophytes* (20), and to treat candidal stomatitis (3). In birds it acts on *Aspergillus* spp., *Mucor* spp. and *Penicillium* spp. (24)

In mamals klotrimazole is used in the form of a topical preparations (unguents, ointments, solutions) applied to the affected area (44, 26). In birds, klotrimazole can be applied directly to the nasal cavity in the form of isotonic solution, or by inhalation of saline solution (1: 50) (24); under our conditions such way of application is used rarely.

Klotrimazole should not be administered to patients showing increased sensitivity. As it is applied topically, one should observe its skin reaction (34). More rapid recovery of patients was observed when administered in parallel with hydrocortisone or other glucocorticoids (39).

Econazole

Therapeutic properties were reported for econazole nitrate, a substance with low solubility in water. Its action is fungistatic, at higher concentration even fungicidal, particularly with respect to some of the more sensitive species of micromycetes (34).

The mechanism of action is the same as with other azole antimycotics. Its high concentrations result in a reduced permeability of the cellular membranes and it can even cause complete lysis of cell organelles. After the local application to skin, econazole penetrates well through *stratum corneum* and approx. 1 % of the dose is resorbed into the blood circulation (34).

When administered *in vitro*, it is effective against *T. mentagrophytes, T. rubrum, T. tonsurans, T. verrucosum, T. violaceum, Epidermophyton floccosum., Microsporum (M.) canis, M. gypseum, Malassezia furfur*, *Cladosporium* spp., *Aspergillus* spp., and *Sporothrix* spp. The growth of the more sensitive strains of *Candida albicans* was inhibited at concentration of 4 $\mu g.ml^{-1}$ and its effect against bacteria *Staphylococcus* (*S.*) *aureus, S. epidermidis, Streptococcus pyogenes* and *Corynebacterium diphteriae* has also been proven. Cross-resistance with klotrimazole was observed with respect to mikonazol-resistant strains of *Candida albicans* (34).

It has been used to treat skin and oral forms of candidosis (48) and as local therapeutics in the treatment of dermatophytoses, demodicosis, mycoplasmosis and local infections caused by Gram-positive bacteria. When used to treat the dermatophytoses of dogs and cats, it is applied topically 2–3 times daily for two weeks (26).

Similar to klotrimazole, hypersensitivity may occur during econazole therapy in some individuals, and they should not be treated with the substance (34). Interactions with glucocorticoids were also described, with the warning that their parallel administration may decrease the antifungal action of econazole against *Saccharomyces cerevisiae* and *Candida albicans* (39).

Miconazole

This econazole-like derivative is insoluble in water. Miconazole was the first imidazole derivative with systemic effects that could be administered intravenously. So far, no significant manifestations of the development of resistance has been recorded (34). The spectrum of effects includes genera *Candida, Cryptococcus, Aspergillus, Coccidioides, Paracoccidioides* and *Histoplasma* (7), species *Malassezia furfur, Sporothrix schenckii* and *Microsporum canis* (34).

After the oral administration, approximately 50 % of the dose is absorbed, while the absorption after dermal administration is lower than 1 %. Approximately 91–93 % is bound to the plasma proteins. It is metabolised in the liver. When administered orally, 45–50 % of miconazole is eliminated by the gallbladder in the faeces in an unchanged form and 10–14 % is eliminated by the kidneys as an inactive metabolite (5, 34).

Rabbits can be administered intravenously 90 mg miconazole *pro toto*, and parrots 20 $mg.kg^{-1}$ during 14 days (24).

Miconazole should be used very carefully in animals with liver function disorders or with thrombocytopenia (34). The action of miconazole with amphotericin B is antagonistic. Miconazole increases the effect of coumarin anticoagulants administered in parallel, and in combination with norfloxacin, it increases its antimycotic activity (39).

Ketoconazole

The structure of ketoconazole resembles that of miconazole and klotrimazole. The substance is insoluble in water. Ketoconazole is the first suitable alternative to amphotericin B because it is less toxic and has a wide spectrum of antifungal effects. In recommended concentrations it has a fungistatic effect and at higher dosage it acts as a fungicide (34).

Ketoconazole is effective against *Blastomyces, Coccididoides, Cryptococcus, Histoplasma*, yeast infections caused by *Candida* spp. and against dermatophytes (41). Additional sensitive micromycetes include: *T. mentagrophytes*, *M. canis*, *Penicillium* spp., and *Malassezia* spp. (26). However, resistance appeared in some *Candida albicans* strains (37). Less sensitive micromycetes are *Aspegillus* spp., *Sporohtrix* spp. and *Mucor* spp. (22).

After peroral administration, ketoconazole is absorbed well and rapidly in the stomach as a result of its good solubility in an acidic environment. Up to 84–99 % of the absorbed antimycotic is bound to plasma proteins, particularly to the albumins (34). In the liver it is transformed into a number of inactive metabolites. It is eliminated with faeces by means of the gallbladder (37). After dermal application, ketoconazole is retained between the *stratum corneum* and *stratum granulosum*. It penetrates well into the skin structures (hairs, claws, hoofs) (39).

Ketoconazole is administered to dogs, cats, horses, birds and reptiles to treat local and systemic mycoses and yeasts infections. The dosage is animal specific and dependent on the dosage unit (Table 1).

Table 1. Dosage of ketoconazole for some animal species

Species Disease	Administration	Ketoconazole dose
Cat		
General dose	p.o.	10–30 mg.kg^{-1} 1–3× daily, 2–6 months (26)
dermatophytoses	p.o.	10 mg.kg^{-1} 1–2× daily up to neg. result (35)
coccidioidomycosis	p.o.	10 mg.kg^{-1} 1–2× daily, 6 months (37)
– systemic form	p.o.	5–10 mg.kg^{-1} 2× daily (37)
– nervous form	p.o.	15–20 mg.kg^{-1} 2× daily, 3–6 months (37)
blastomycosis	p.o.	5 mg.kg^{-1} 2× daily, 2–6 months (28)
– nervous and ocular form		20–40 mg.kg^{-1} 2× daily (37)
histoplasmosis	p.o.	10 mg.kg^{-1} 2× daily, 2–6 months (28)
kryptococcosis	p.o.	10 mg.kg^{-1} 1–2× daily, 3 months (37)
aspergillosis	p.o.	20 mg.kg^{-1} 1× daily, 6 weeks (37)
kandidosis	p.o.	10 mg.kg^{-1} 1× daily, 8 weeks (28)
Dog		
general dose	p.o.	10–30 mg.kg^{-1} 1–3× daily, 2–6 months (26)
dermatophytoses	p.o.	10 mg.kg^{-1} 1–2× daily up to neg. result (3)
coccidioidomycosis	p.o.	10 mg.kg^{-1} 3× daily, 6 months (28)
– systemic form	p.o.	5–10 mg.kg^{-1} 2× daily (37)
– nervous form	p.o.	15–20 mg.kg^{-1} 2× daily, 3–6 months (37)
blastomycosis	p.o.	20 mg.kg^{-1} 2× daily, 6 months (28)
– nervous and ocular form	p.o.	20–40 mg.kg^{-1} 2× daily, 2–3 months (37)
histoplasmosis	p.o.	10 mg.kg^{-1} 2× daily, 2–6 months (28)
aspergillosis	p.o.	20 mg.kg^{-1} 1× daily, 2–18 weeks (42)
candidosis	p.o.	10 mg.kg^{-1} 1× daily, 8 weeks (28)
malasseziosis	p.o.	5–10 mg.kg^{-1} 2× daily, 30 days (37)
Horse		
mycoses	p.o.	30 mg.kg^{-1} 1–2× daily (3)
Rabbit		
dermatophytoses	p.o.	5–10 mg.kg^{-1} 1× daily, 5 weeks (43) 10–15 mg.kg^{-1} 1× daily, 3–4 weeks (2)
Guinea pig and hamster		
systemic mycoses, candidosis, dermatomycoses	p.o.	5–20 mg.kg^{-1}1× daily, 3–6 weeks (49) 10–40 mg.kg^{-1} 1× daily, 14 days (1)
Turtle		
systemic mycoses	p.o.	20–30 mg.kg^{-1} 1× daily, 2 weeks (40)
dermatomycoses	p.o.	20–30 mg.kg^{-1} 1× daily, 2 weeks (40), or 25 mg.kg^{-1} 1× daily, 2 weeks (2)
aspergillosis	p.o.	50 mg.kg^{-1} 2× daily, 14–28 days (23)
shell mycoses	p.o.	20–30 mg.kg^{-1} 1× daily, 2 weeks (40), or 25 mg.kg^{-1} 1× daily, 2–4 weeks (37)
Parrots		
aspergillosis	p.o.	20–30 mg.kg^{-1} 2× daily (18)
candidosis	p.o.	20–30 mg.kg^{-1} 2× daily (32)
inf. *Mucor* and *Penicillium*	p.o.	20–30 mg.kg^{-1} 2× daily (24)
Songbirds		
candidosis	p.o.	20–50 mg.kg^{-1} 1× daily, 21 days (46)
aspergillosis	pot.	20–50 mg.kg^{-1} 1× daily, 21 days (46), or 30–40 mg.kg^{-1} 1× daily, several weeks (14)
Predatory birds		
candidosis and aspergillosis	p.o.	20–50 mg.kg^{-1} 1× daily, 21 days (46)

Increased attention should be paid to animals with liver disorders, reduced renal function and to dogs with leishmaniosis (37). Antacids administered in parallel (cimetidine, ranitidine) decrease resorption of ketoconazole and phenytoin has an antagonistic effect. A synergistic effect with aciklovir was observed in the therapy of herpes-simplex infection and ketoconazole prolonged the effect of prednizolone, methylprednizolone and coumarin anticoagulants. Combination with norfloxacin extended the antifungal spectrum of ketoconazole (3, 34, 37).

Enilconazole

This imidazole is a synthetic, wide-spectrum antimycotic with high effectiveness against the majority of common fungi and yeasts (*Microsporum, Aspergillus, Trichophyton, and Mucor*). The systemic availability after local application in animals is very low. After peroral administration it is metabolized slowly. Biological half-time of enilconazole in tissues and the plasma of cattle is approximately12 to 16 hours. The principal excretion pathways are the urine and faeces. The currently available veterinary preparations are used for the local treatment of dermatomycoses. The animals are treated with an emulsion, in the concentration of 0.2 % which is applied best with a sprayer; in dogs and horses as it is necessary to treat the whole body. The therapy includes 4 applications in intervals of 3–4 days (14). In addition to horses, dogs and cattle, the 0.2 % solution was successfully applied also to domestic rodents, rabbits, hedgehogs, weasels, martens and some reptiles. Parrots, songbirds and predatory birds can be treated with enilconazole by inhalation or aerodispersion (18). The development of side effects in the treated animals is improbable because absorption through the skin is very low. Accidental *per os* uptake does not cause intoxication because its bioavailability within the organism is low (14).

TRIAZOLES

Fluconazole

Fluconazole is a synthetic fluoridated antimycotic. It is very soluble in water and depends on the pH. Fluconazole acts as a fungistatics with systemic affect and high antifungal activity. It appeared effective in the treatment of infections caused by candida, cryptococci and in some cases also by aspergillus and trichophytes. Other sensitive species are *Blastomyces* spp., *Histoplasma* spp. and *Coccidioides* spp. (26). Studies on animals showed that fluconazole is 5–10 times more effective against surface candidoses and dermatophytic fungi than ketoconazole and 100-times more effective in the treatment of systemic candidoses. A disadvantage of this triazole is its high percentage of development of primary resistance, particularly in candida and cryptococci. Owing to good solubility in water, it is rapidly absorbed from the gastrointestinal tract after peroral administration (up to 90 %) (26). Due to its very low ability to be metabolized, up to 80 % of the active ingredient is eliminated in the urine (48).

The indication spectrum of fluconazole is diverse. In cats it is used to treat infections caused by *Cryptococcus neoformans* and candidoses (7). In dogs it is used for the treatment of nasal aspergillosis and penicillosis, infections induced by *Cryptococcus neoformans,* and candidosis (30), and in those horses afflicted by ocular fusarium infections, candidosis and other systemic mycoses (5). The preparation is administered perorally or by injection. Table 2 shows the dosage of fluconazole for selected animal species.

Despite the fact that fluconazole is generally less toxic than ketoconazole it is contraindicated in patients with liver diseases (20). The parallel administration of amphotericin B has an additive effect (against candida and cryptococci). Fluconazole increases serum concentrations of orally administered antidiabetics (chlorpropamid, glypizid) and prolongs prothrombin time in patients treated with warfarin (3, 37).

Table 2. Dosage of fluconazole for selected animal species

Species Disease	**Administration**	**Fluconazole dose**
Cat		
cryptococcosis	p.o.	2.5–10 mg.kg^{-1} 2× daily (26)
	p.o.	2.5–5 mg.kg^{-1} 1–2× daily for 8 weeks (29)
candidosis	p.o.	2.5–5 mg.kg^{-1} 1–2× daily (39)
Dog		
aspergillosis and	p.o.	2.5–5 mg.kg^{-1} daily, 8 weeks (7)
penicillosis	p.o.	2.5–5 mg.kg^{-1} daily (39)
candidosis		
Horse		
general dose	p.o.	at first 14 mg.kg^{-1}, then daily, 5 mg.kg^{-1} (5)
Rabbit		
mycoses	i.v.	25–43 mg.kg^{-1} 2× daily (51)
candidal pyelonephritis	p.o.	80 mg.kg^{-1} 1× daily (51)
Turtle		
aspergillosis	s.c.	21 mg.kg^{-1}, then 10 mg.kg^{-1} every 5 days (36)
Reptiles, snakes and crocodiles general dose	p.o.	2–5 mg.kg^{-1} 1× daily 5 days (15)
Parrots		
candidosis	i.v., s.c.	5–15 mg.kg^{-1} 2× daily 14–60 days (10)
	p.o.	5–15 mg.kg^{-1} 2× daily 14–60 days (10, 12)
cryptococcosis	i.v., s.c.	5–15 mg.kg^{-1} 2× daily 14–60 days (10)
	p.o.	5–15 mg.kg^{-1} 2× daily 14–60 days (10)
aspergillosis	i.v.	20 mg.kg^{-1} every other day (31)
	p.o.	5–15 mg.kg^{-1} 2× daily (12)
Songbirds		
aspergilllosis	p.o.	10–25 mg.kg^{-1} 2× daily, 4 weeks (33)

Itraconazole

Itraconazole is a synthetic antimycotic of lipophylic character with systemic effects, high antifungal specificity and fungistatic activity (27). It shows a broad spectrum of effectiveness against dermatophytes, *Candida* spp., *Cryptococcus neoformans, Aspegillus* spp., *Cladosporium* spp. and *Penicillium* spp. Very high effectiveness was recorded also against blastomycosis (8) and histoplasmosis (17). Other susceptible pathogens are *Microsporum* spp., *Trichophyton* spp., *Sporothrix* spp. and *Coccidioides* spp. (20).

Itraconazole shows the best absorption and widest distribution after peroral administration. Its absorption depends on the pH of the

Table 3. Dosage of itraconazole for some animal species

Species Disease	Administration	Itraconazole dose
Cat		
cryptococcosis	p.o.	10 mg.kg^{-1} daily, 8 weeks (51)
dermatophytoses	p.o.	10 mg.kg^{-1} 1–2× daily (37)
mycrosporosis	p.o.	5 mg.kg^{-1} 7 days, then pause for 7 days, treatment repeated 2× (11)
		5–10 mg.kg^{-1} 1–2× daily (26, 37)
systemic mycoses	p.o.	
Dog		
systemic mycoses	p.o.	5 mg.kg^{-1} 1–2× daily (37)
dermatomycoses	p.o.	10 mg.kg^{-1} daily (6)
aspergillosis	p.o.	10 mg.kg^{-1} daily (6, 51)
blastomycosis		
– systemic form	p.o.	5 mg.kg^{-1} every 24 hours, 60 days (4)
– ocular form	p.o.	5 mg.kg^{-1} 2× daily, 60 days (20)
Horse		
aspergillosis	p.o.	3 mg.kg^{-1} 2× daily, 2 months (3, 37)
coccidioidomycosis	p.o.	2,6 mg.kg^{-1} 2× daily, 3 months (20)
Rabbit		
dermatomycoses	p.o.	5–10 mg.kg^{-1} 1× daily, 3–4 weeks (2)
Guinea pig		
Systemic mycoses	p.o.	5 mg.kg^{-1} 1× daily (1)
Reptiles and crocodiles		
Systemic mycoses	p.o.	25 mg.kg^{-1} daily (55)
Turtles		
candidosis	p.o.	5 mg.kg^{-1} daily (32) or
	p.o.	15 mg.kg^{-1} every 72 hours (32)
cryptococcosis	p.o.	5 mg.kg^{-1} daily (32) or
	p.o.	15 mg.kg^{-1} every 72 hours (32)
Parrots		
candidosis	p.o.	5–10 mg.kg^{-1}, 1–2× daily (23) or
	p.o.	10 mg.kg^{-1} 2× daily, 4–5 weeks (10, 45)
aspergillosis	p.o.	5–10 mg.kg^{-1} 1–2× daily (2)
		5–10 mg.kg^{-1} daily, 14 days (24)
Mucor and *Penicillium*	p.o.	5–10 mg.kg^{-1} daily, 14 days (24)
Songbirds		
candidosis	p.o.	10 mg.kg^{-1} 2× daily, 4–5 weeks (18, 46)
aspergillosis	p.o.	10 mg.kg^{-1} 2× daily, 4–5 weeks (18) or
	p.o.	5–10 mg.kg^{-1} daily, 14 days (24)
infection *Mucor*	p.o.	5–10 mg.kg^{-1} daily, 14 days (24)
Predatory birds		
candidosis	p.o.	5–10 mg.kg^{-1}, 1–2× daily (2) or
	p.o.	10 mg.kg^{-1} 2× daily, 4–5 weeks (46)
aspergillosis	p.o.	5–10 mg.kg^{-1}, 1–2× daily (2) or
	p.o.	10 mg.kg^{-1} 2× daily (19, 46)

stomach contents (it is better absorbed in the acidic environment). The preferred administration is together with food which facilitates its absorption and eliminates the potential undesirable effects associated with stomach irritation. The maximum concentration of itraconazole is reached at 2–5 hours after administration with feed. In the blood, approximately 95 % of itraconazole is bound to plasma proteins and 5 % to blood corpuscles. The levels in keratin-containing tissue (skin, claws) are about 4-times higher than in the plasma and after several week treatment itraconazole persists in the skin for 2–4 weeks, and in the claw for 6–9 months. Itraconazole is metabolised in the liver to a large number of metabolites and is eliminated in the gallbladder secretions and up to 40 % in the urine (11, 17, 37).

Itraconazole is a medication that is of use in mammals, birds and reptiles. The dosage recommended for domestic and companion animals is presented in Table 3. It is contraindicated in animals with liver diseases or those with achlorhydria or hypochlorhydria. The parallel administration of antacids or H2-blockers results in a marked decrease in its resorption. Itraconazole increases the concentration of parallel

administered medications which are metabolised through cytochrome P-450, for example digoxin, cyclosporin and fenytoin (37).

Voriconazole

Voriconazole is an antifungal triazole substance which is used predominantly in the treatment of invasive mycoses. After peroral administration, it is absorbed rapidly and almost completely and its resorption is not affected by changes in the stomach pH. The maximum plasma concentrations are reached within 1–2 hours. The absolute biological availability of voriconazole is as high as 96 %. It is metabolized by hepatic izoenzymes of cytochrome P-450. It is eliminated in the urine and less than 2 % of this substance is eliminated in an unchanged form (54).

Voriconazole exhibits wide antimycotic activity *in vitro* – it has fungistatic effects against *Candida* spp. (including *Candida krusei* and *C. glabrata* which show primary resistance to fluconazole) and fungicidal effect against all *Aspergillus* and *Fusarium* spp. Its spectrum of activity includes *Cryptococcus neoformans*, *Coccidioides immitis* and *Penicillium* spp. (22). Additional susceptible species are *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp. and *Absidia* spp. (52).

The administration of voriconazole is contraindicated with some other medications (for example, sirolimus, rifampicin, rifabutin and ergot alkaloids). They can be safely administered with cimetidin, ranitidin, indinavir, makrolide antibiotics and prednizolone (54).

It is less important in veterinary medicine.

Posaconazole

Posaconazole is a substance used mostly in the treatment of infections caused by *Candida albicans* and *Aspergillus* spp. (38). *In vitro* it is effective against a wide spectrum of yeasts and is very effective against *Aspergillus* spp. Its *in vitro* effect against zygomycetes is supposed to be low, however, 60 % effectiveness was described at the clinical level (47).

The antifungal activity of posaconazole against dermatophytes is comparable to that of itraconazole, and higher than the activity of fluconazole. Posaconazole is more effective against *Cryptococcus* spp. than fluconazole and acts also against *Fusarium* spp. After peroral administration it is absorbed slowly (ideally with feed with high proportion of fat) and its distribution is slow also. It binds markedly to proteins, particularly to serum albumin. It is eliminated gradually, mostly in faeces (almost in 80 %), and less by the kidneys (up to 15 %) (25, 47, 53).

Posaconazole is used in the treatment of invasive aspergillosis in the case of refractory infection or in individuals intolerant of antimycotics and for the treatment of some rare invasive mycoses (fusariosis, koccidiomycosis, chromoblastomycosis). The most frequent side effects are weak manifestations of hepatotoxicity (25).

It is rarely used in veterinary medicine.

CONCLUSIONS

Despite the use of various immunization programmes aimed at the prevention and therapy of mycotic diseases, antimycotics still play an important role in veterinary practice. The successful management of mycotic infections requires a good knowledge of the spectrum of the effects and correct dosage of antifungal substances because their frequent, incorrect and inadequate use can result in the development of resistance. One should also pay sufficient attention to hygiene conditions and nutrition. The immunological status of patients must be maintained on an appropriate level so their organs can cope with the infection. Antimycotics as well as other antimicrobial substances do not eliminate the infection completely; they only support conditions which allow the patient to recover. The combined action of the defensive mechanisms of the body and antifungal substances increases the successfulness of the treatment.

Azole derivatives belong among the most frequently used antimycotics owing to their intensive action against a wide range of pathogenic micromycetes and low occurrence of resistance. The treatment of mycotic infections is frequently protracted and financially demanding so the animal owner should focus first of all on preventive measures.

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