FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE AND
PHARMACY IN KOŠICE — The Slovak Republic

ISSN 0015-5748

4

LVI • 2012

EDITORIAL BOARD

FOLIA VETERINARIA is issued by the *University of Veterinary Medicine and Pharmacy in Košice (UVMP);* address: Komenského 73, 041 81 Košice, The Slovak Republic (tel.: +421 55 632 52 93, fax: +421 55 632 52 93, E-mail: vargovam@uvlf.sk).

The journal is published quarterly in English (numbers 1–4) and distributed worldwide.

Subscription rate for 1 year is 120 €. Orders are accepted by *The Department of The Scientific Information – The Library of The University of Veterinary Medicine and Pharmacy in Košice* (UVIK), E-mail: palencarova@uvlf.sk; the subscription is accepted by the State treasure.

Bank contact: State treasure, Radlinského 32, Bratislava 15, The Slovak Republic; **account number:** 7000072225/8180.

FOLIA VETERINARIA, vydáva *Univerzita veterinárskeho lekárstva a farmácie v Košiciach (UVLF),* Komenského 73, 041 81 Košice, Slovenská republika (tel.: 055/632 52 93, fax: 055/632 52 93, E-mail: vargovam@uvlf.sk).

Časopis vychádza kvartálne (č. 1–4) a je distribuovaný celosvetove.

Ročné predplatné 120 €. Objednávky prijíma *Ústav vedeckých informácií a knižnice Univerzity veterinárskeho lekárstva a farmácie v Košiciach* (UVIK), E-mail: palencarova@uvlf.sk; predplatné Štátna pokladnica (na nižšie uvedené číslo účtu).

Bankové spojenie: Štátna pokladnica, Radlinského 32, Bratislava 15; **číslo účtu:** 7000072225/8180.

Tlač: **Univerzita veterinárskeho lekárstva a farmácie** Komenského 73, 041 81 Košice
Sadzba: **Sapfo publishers**, Szakkayho 1, 040 01 Košice

EV 3485/09

For basic information about the journal see Internet home pages: www.uvm.sk; www.uvlf.sk

Indexed and abstracted in AGRIS, CAB, EBSCO

FOLIA VETERINARIA, 56, 4, 2012

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FOLIA VETERINARIA, 56, 4: 3–6, 2012

SALAMANDRA SALAMANDRA IN SLOVAKIA, DISTRIBUTION AND HABITAT

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ABSTRACT

The fire salamander (*Salamandra salamandra)* is one of the most common species of amphibians in Slovakia. It especially inhabits wooded areas such as old beech forests. Even though this salamander is ubiquitous, there has been no study summarizing the scattered data of its distribution in the Slovak Republic. We accumulated and studied data from 70 publications and other available web resources. The database also included unpublished data from ISTB (Information System on Taxa and Biotopes) from the State Nature Conservancy. The data were processed by Quantum GIS (1.7.0) and the final summarization confirmed that the fire salamander prefers a forest habitat. They are most frequently found at an elevation between 200 and 1000 m above sea level. We also noted the most frequent orographic units and the climatic regions where they are most prevalent. Detailed views of the salamander's habitat was obtained by the research conducted from October 2006 until April 2010 in the FMU (Forest Management Unit) Železná Studienka. The overall population density in the monitored area was 0.77 animals.ha^{-1}. We determined the size of the home range for individual salamanders based on the analysis of more than two retraps. Females showed a smaller mean distance to the nearest water area. We also investigated the character of shelters and the influence of slope ratings on the occurrence of the fire salamander.

Key words: distribution; extension; fire salamander; Quantum GIS

INTRODUCTION

The fire salamander (*Salamandra salamandra*) is found throughout central, western and southern Europe (3). The *S. salamandra* is widespread in Slovakia and it is the only representative of the genus *Salamandra* in our country. The typical salamander's habitat include deciduous or mixed forests (mainly beech) between the elevations of 200 and 1000 m; the highest documented incidence was under the top of Smrekov at an altitude of 1440 m (8). Some of the limiting factors of occurrence include; temperature, humidity and total precipitations (4). Although the *S. salamandra* is one of the most common amphibians in Slovakia, the knowledge of its biology and ecology remains insufficient. We also lack publications which would summarize the scattered fauna data in the Slovak part of the Carpathian mountains.

The aim of this study was to analyze the available data on the distribution of the fire salamander in Slovakia and to gain new knowledge on the salamander's habitat.

MATERIALS AND METHODS

We accumulated the available data from the published studies (70 publications, more than 420 bibliographic data) and various accessible internet resources. The database also contained 301 unpublished data from the Information System on Taxa and Biotopes

(ISTB) of the State Nature Conservancy (Banská Bystrica) and our own unpublished data. The locations of *S. salamandra* were processed by GIS in the JTSK coordinate system. By using Quantum GIS (1.7.0) software and vector maps, we assigned to each site: its corresponding cadastre, altitude, orographic unit, DFS (Databank of Slovakia Fauna) square, climatic region, area code and radiation on a horizontal surface. We also assigned: the date of record, the number of individuals seen, their developmental stage, type of habitat and author of legitimacy.

Additional information was obtained by conducting research in a forest management unit (FMU) Železná Studienka during the period from October 2006 until April 2010 (total area 475 ha). We performed 58 inspections with 43 positive findings (1 or more found individuals). In the research, we used the method of field observation and capturing of individuals at selected locations. For each captured individual we recorded: the time of finding, exact location using GPS Garmin 60 CSx, and the biometric data. For the identification of individuals, we used the photodocumentation of the dorsal side (a method of individual marking modified by Opatrný (5)). We also observed the character of the place of occurrence and other ecological factors (activity of the individual, character of the surrounding vegetation, slope rating, and meteorological characteristics). The coordinates of each identified individual were transferred to the Map Source ver. 6.15, which allowed a special view of an orthophotomap of the monitored site. The calculations of the density and all measured distances in retraps were

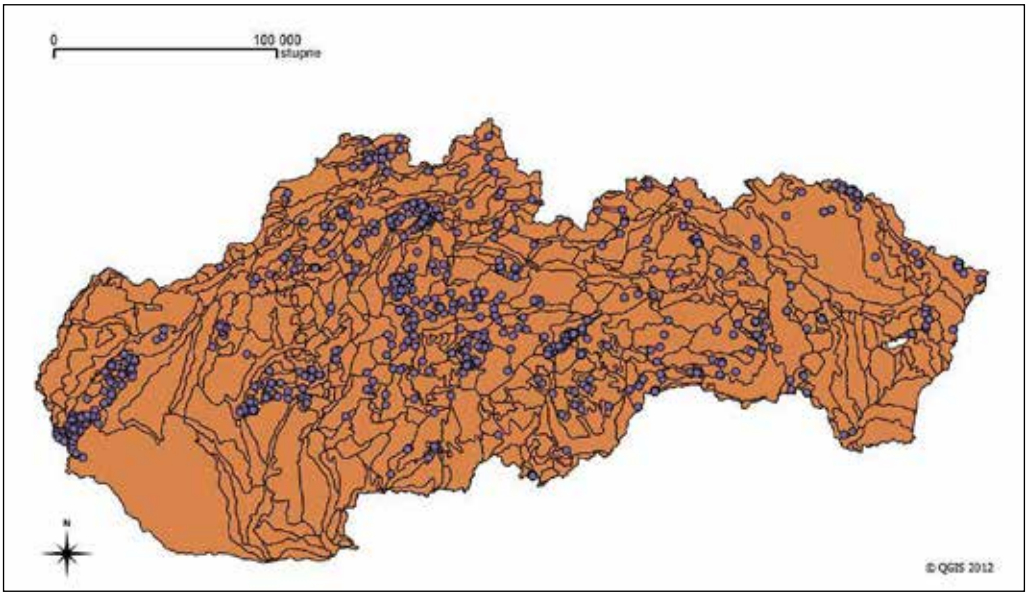

Fig. 1. Record of excerpted localities in Slovakia (1956–2012)

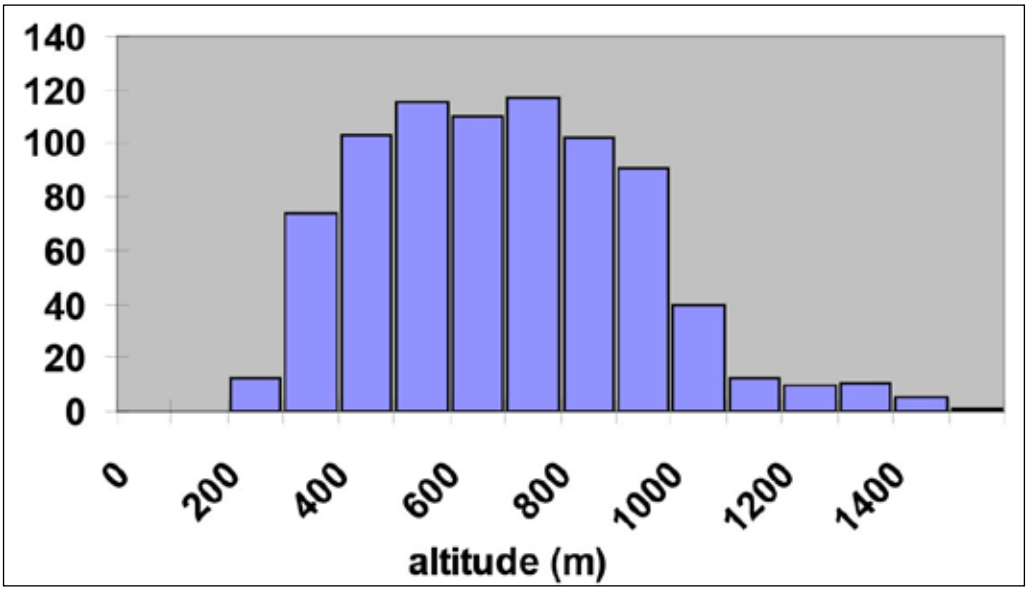

Fig. 2. Altitudinal range of salamander's occurrence

calculated using Google Earth 2010 software. The central axis for the categories of slopes was determined by regression analysis, employing a polynomial and an exponential model.

RESULTS AND DISCUSSION

Overall, 803 records of the salamander's occurrence were excerpted from 68 orographic units (Fig. 1). The highest number of sites was recorded in Spišsko-Gemerský kras mountains (88 records), but the occurrence was also considerable in the Malé Karpaty mountains and the Malá Fatra mountains. The observed altitudinal range of locations was 200 to 1000 m with a gradual reduction of occurrence above and below this range (Fig. 2). The majority of recorded sites were located in deciduous or mixed forests. Most of the locations (43 %) were situated in a cool climatic region, 38 % in a moderately warm and 19 % in a warm climatic region. The highest number of sites (326 data from a total number of 803) was observed in a cool climatic region, with a moderately cool, very damp character of the district which had the climatic characteristics in July with temperatures of 12 to 16 °C.

The total number of captured individuals during monitoring in FMU Železná Studienka were 388, from which 363 individuals were identified. We carried out 69 retraps with 52 individuals (19 % of the total number). The most preferred site for the occurrence of salamanders was the area between Bystrička tributary and the tributary flowing into the Vydrica near the hotel Veronika. This area was characterized by a continuous presence of approximately 50 years old beech stand with a minimal level of shrubs, a low steepness of slope and a high presence of underground shelters. The northern, southern and eastern foot of the mountain Hrubý Drieňovec and also surroundings of Snežienka, Klanec and Húštie appeared to be unsuitable for the salamanders. These sites were characterized by the fragmentation or absence of forest or the presence of a young forest with a highly developed level of shrubs. Localities without tributaries and those affected by human building activities were also unsuitable. The overall population density in the area of interest was 0.77 salamanders per hectare.

The distribution of individuals in different parts of the monitored site was not uniform. From the total number of individuals (363 animals), 76.7 % (280 animals) were located in an area the size of 14.4 hectares (3.05 % of the total monitored sites). The home range had been previously determined by Schulte *et al.* (6) in individuals which had been captured more than 2 times during the period of monitoring (5 females, 8 males). The mean home range was set at 372.8 m^2.

In our observations, the mean home range of males was 511.3 m^2 (3.32 m^2–3200 m^2) and of females 151.3 m^2 (23.4 m^2–443 m^2). The mean distance of females from the nearest water area was 39.7 m, and for males 49.4 m. The minimum and maximum values ranged from 0 m to 178 m, while 17.33 % of all findings were located directly in the forest stream. The majority of individuals were monitored inside the forest, or at the edge near the tributary. Individual salamanders were often observed freely in the terrain without nearby shelter (30 % of the total number), or directly in the forest stream (17 %), but the majority of them were found in close proximity to the shelter to which salamanders actively escaped. Salamanders were situated in the terrain of different slope. We found 24 % of all individuals on the plain, most frequently in the area of lateral tributaries near the entry to river Vydrica with 0° slope. Other categories showed significantly decreasing tendencies.

After complete analysis of the excerpted data, we found that the majority of recorded sites were located in forested areas at altitudes of 200 to 1000 m, with a moderately cool and very damp character. Gregor (1) pointed to a general trend of a decline in salamander abundance in all orographic units of Slovakia. Salamanders were captured only in some areas of our monitored sites despite the fact that most areas fulfilled habitat's assumptions of occurrence. It seems that a high fidelity to the site and small home range are characteristic of salamanders. We recorded the size of the home range to be 151 to 511 m^2, but these values varied considerably for each individual; therefore, based on a relatively small statistical sample, we cannot draw unambiguous conclusions.

The first record of a strong fidelity to the limited area was provided by Joly (2). He observed that adults remained in a small area (only 68 m^2) during 7 years. Strong fidelity to the site in the combination with small home range was also demonstrated in alpine salamanders, *Salamandra atra* and *Salamandra lanzai* (6). In the monitored sites, females showed almost 10 m lower mean distance to the nearest water area than the males. This closer relationship originated probably from the importance of water bodies for the females when laying larvae. Based on the type of habitat in which the individuals were found, we can conclude that salamanders spent most of their time in the immediate vicinity of the shelter.

Seven % of the individuals were observed near anthropogenic objects – walls of buildings and bunkers. In these areas, salamanders were seen especially in the autumn and winter because they were probably looking for a suitable place for wintering. This was confirmed also by some other authors, for example, Stojanová and Opatrný (7). In terms of the slope rating, we found a significant inverse relationship. With increasing slope the number of individuals significantly decreased.

In the future we plan to obtain additional data on the occurrence of fire salamander and use special geographic methods to obtain new knowledge about this species in our country.

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Selected papers from the Seminar of PhD. students, organized in September 2012 by the UVMP in Košice, devoted to the memory of outstanding veterinary physiologist Academician Boďa.

FOLIA VETERINARIA, 56, 4: 7–10, 2012

ANALYSIS OF SELECTED MARKERS OF ENERGY METABOLISM IN DAIRY COWS IN THE PERIPARTAL PERIOD

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ABSTRACT

This study evaluated, under practical conditions, the level of nutrition relative to rumen fermentation and its influence on the adaptation of energy metabolism of saccharides and fats in cattle. The degree of lipomobilization and metabolic transformation of non-esterified fatty acids (NEFA) in relation to the functional state of the liver was evaluated in two groups of dairy cows. The investigation was conducted during the peripartal period. The mean level of propionic acid in the rumen of cows of the Ist group (6 herds were fed a bulk type ration) was 16.44 ± 2.54 mmol.l^{-1} and the mean serum level of NEFA in this group reached 0.52 ± 0.18 mmol.l^{-1}. In the IInd group (6 herds were fed a total mixed ration of the concentrate type) the mean propionic acid level reached 21.32 ± 2.13 mmol.l^{-1} and the NEFA mean level was 0.24 ± 0,04 mmol.l^{-1}, which indicated a significantly lower level of lipomobilization.

Key words: dairy cows; energy metabolism; peripartal period

INTRODUCTION

The peripartal period is of critical importance for the health, production and profitability of dairy cows. It is associated with dramatic changes in the coordination of the metabolism that is required for covering the needs of the mammary gland on energy, glucose, amino acids (AA) and calcium during the early lactation period (4, 5). The nutrient requirements on day 4 of lactation compared to day 250 of gestation are: about 2-fold higher for AA (1), 4-fold for Ca (3), 5-fold for NEFA, and 3-fold higher for glucose (1). The serum glucose levels are important for: the synthesis of lactose; determination of the milk yield; and are the limiting factor for the degree of lipomobilization and supply of liver with NEFA. In ruminants, glucose is synthesized mostly from propionic acid which is absorbed through the rumen wall and carried *via* the portal vein into the liver. During the transition period, the contribution of propionic acid to gluconeogenesis remains approximately at the level of 50–60 %. Glucose is synthetized also from lactic acid (10–15 %) and glycerol (2–4 %). During this phase of negative energy balance, low voraciousness and deficiency of precursors of glucose synthesis, the amino acids play an important role. They enter the metabolism as a result of muscle proteolysis and, also from the total mixed rations (TMR) they may support the energy metabolism even at the 20–30 % level (6).

Increased energy intake through the TMR during the peripartal phase is associated with a decrease in the excessive lipomobilization and production of ketone substances. The key preventative measures are the support of gluconeogenesis and the physiological levels of blood glucose. The essential substrate for the production of glucose in the liver, is propionic acid which develops during fermentation of rapidly fermenting saccharides in the rumen. The content of nutrients (particularly of easily fermentable and fibrous saccharides) is an important criterion for optimisation and evaluation of the quality of rations during the preparation for parturition, with the aim to maximise production of propionic acid in the rumen and stabilise the metabolism of glucose.

MATERIAL AND METHODS

We evaluated the level of rumen and metabolic adaptations and the influence of the feed supplied in the peripartal period in 12 herds of dairy cows (all together 69 cows) with an annual yield of 8 000 litres of milk per head. The farms were located mainly in the lowland areas, where the bulk portion of rations consisted of maize and alfalfa silage. The herds were divided to two groups with 6 herds in each (34 cows in the Ist group and 35 in the IInd).The Ist group was fed bulk type rations and the IInd group was fed a total mixed rations with a higher proportion of concentrates. We focused on the composition of the rations (bulk and concentrate types) and their influence on the level of rumen fermentation evaluated by means of volatile fatty acids (VFA) and their influence on the level of NEFA and ketone substances. For all herds we summarised and evaluated the TMR and rumen profile and observed the impact on; energy metabolism, the level of lipomobilization, and the metabolic load on the liver.

RESULTS AND DISCUSSION

Evaluation of TMR during preparation for parturition

Table 1 summarizes the mean levels and ranges of nutrients in TMR in the peripartal period. In the Ist group, the mean level of neutral detergent fibre (NDF) in the TMR reached 394.1 g.kg^{-1} DM (Dry Matter) and the recommended upper limit of NDF was exceeded in 5 of 6 examined herds. The mean level of non-fibrous saccharides (NFS) in this group was 372 g.kg^{-1} DM and 3 herds exceeded the recommended upper limit of NFS. In the IInd group the mean level of NDF reached 382 g.kg^{-1} DM and in 2 herds the respective limit was exceeded.

The mean level of NFS in the TMR supplied to group I cows was 390.9 g.kg^{-1} DM and in 4 herds it exceeded the maximum limit.

Rumen profile level

The rumen profile level is shown in Table 2. The mean acidity of the rumen content in the Ist group (6.80 ± 0.15) exceeded the upper reference level and so did the acidity in 65 % of the cows. In the IInd group, the mean level of the pH in the rumen and rumen acidity in 71 % of cows were within the reference range. The mean level of the VFA in the Ist group (86.4 ± 12.83 mmol.l^{-1}) was close to the lower limit with 44 % of the cows below the lower reference limit. In the IInd group, the mean level of the VFA was in the middle (104.45 mmol.l^{-1}) of the reference range, and 88 % of the animals had the VFA in the reference range. The mean propionic acid level in the rumen in the Ist group was below 20 mmol.l^{-1}1 and in the IInd group, it was above 20 mmol.l^{-1}.

The mean propionic acid level in the Ist group reached 16.44 mmol.l^{-1}. In 61.8 % of the cows, the propionic acid in the rumen was below 20 mmol.l^{-1} and in 32 % of the animals even below 15.0 mmol.l^{-1}. In the IInd group, the mean level of the propionic acid was 21.32 ± 2.33 mmol.l^{-1}and 25.6 % of the cows had levels lower than 20 mmol.l^{-1}.

Energy metabolism and internal environment

Selected blood indicators of the internal environment of dairy cows related to energy metabolism and quantification of the degree of lipomobilization may be seen in Table 3. In

Table 1. Mean content of analysed nutrients in TMR in the peripartal period

Parameter	Recommended Range	Group I		Group II	
		x	Range	*x*	Range
Dry matter (DM) [g.kg^{-1}]	450–500	465.3	394–544	425.4	352–498
NS [g.kg^{-1}]	130–150	136.0	102–157	124.7	107–145
Fat [g.kg^{-1}]	–	38.1	31–47	35.8	26–55
NDF [g.kg^{-1}]	320–380	394.1	366–406	382.8	333–435
ADF [g.kg^{-1}]	230–250	237.3	179–258	222.5	188–263
NFS [g.kg^{-1}]	300–350	372.9	314–434	390.9	346–467
Starch [g.kg^{-1}]	–	245.6	182–299	269.6	222–337
NEL [MJ.kg^{-1}]	6.2–6.5	6.4	6.15–6.71	6.5	6.27–6.68

x – mean; NS – nitrogen substances; NDF – neutral detergent fibre; ADF – acid detergent fibre
NFS – non-fibre saccharides; NEL – net energy of lactation

Table 2. Mean peripartal levels of rumen metabolic parameters in the cows

Rumen content		Reference Values	Group I (Mean ± STD)	Group II (Mean ± STD)
pH	P < 0.001	6.4–6.6	6.80 ± 0.15	6.52 ± 0.14
NH_3 [mg.100ml^{-1}]		15–25	17.49 ± 3.05	16.46 ± 3.64
Sum of VFA [mmol.l^{-1}]	P < 0.001	80–130	86.37 ± 12.83	104.45 ± 10.20
Propionic acid [mmol.l^{-1}]	P < 0.001	> 20	16.44 ± 2.54	21.32 ± 2.13
Acetic acid [mmol.l^{-1}]			59.37 ± 9.60	69.76 ± 7.64
Acetic acid [%]	P < 0.01	60–65	68.71 ± 1.82	66.54 ± 1.79
Propionic acid [%]	P < 0.01	20–25	18.84 ± 1.30	20.55 ± 1.52
Butyric acid [%]	**NS**	10–20	12.45 ± 1.44	12.90 ± 0.98
C_3 : C_2	P < 0.01	1 : 2.5–3.0	1 : 3.75 ± 0.34	1 : 3.29 ± 0.34

NS – not significant

Table 3. Serum levels of energy metabolism parameters in dairy cows in the peripartal period

Blood serum parameters		Reference Values	Group I		Group 2	
			x	STD	x	STD
Glucose [mmol.l^{-1}]		2.2–4.1	3.00	0.38	3.32	0.32
Cholesterol [mmol.l^{-1}]		2.3–4.2	2.24	0.39	2.39	0.37
NEFA [mmol.l^{-1}]	P<0.001	> 0.35	0.52	0.18	0.24	0.04
AcAc [mmol.l^{-1}]	NS	> 0.5	0.30	0.08	0.28	0.09
BHBA [mmol.l^{-1}]	P<0.001	0.2–1.24	0.75	0.14	0.53	0.14
AST [μkat.l^{-1}]	P<0.001	0.76–1.38	0.80	0.12	1.35	0.24
Bilirubin [μmol.l^{-1}]	P<0.01	0.17–5.13	5.33	2.18	3.33	1.37

SD – standard deviation; NS – not significant; NEFA – non-esterified fatty acids
AcAc – acetoacetic acid; BHBA – beta-hydroxybutyric acid; AST – aspartate aminotransferase

this regard, the NEFA are a direct marker. In the Ist group of animals, the mean level of NEFA reached 0.52 ± 0.18 mmol.l^{-1} with increased levels observed in 70 % of the cows. Some of these cows also showed increased levels of AST (52.9 %) and bilirubin (61.7 %), indicating a metabolic load on the liver. In the IInd group of dairy cows, the mean NEFA was 0.24 ± 0.04 mmol.l^{-1} and increased levels of AST and bilirubin were found in 31.4 % and 34.3 % of the cows, respectively. A high level of lipomobilization was observed in the Ist group, with propionic acid level in the rumen below 20 mmol.l^{-1} in 61.8 % of cows and increased NEFA (> 0.35 mmol.l^{-1}) in 86 % of the cows. The load on the liver was indicated by an increase in the AST in 66 % of the animals and increased bilirubin in 48 % of the cows from this group.

The rapid fermentation of sugars, along with a sufficient and synchronised supply of degradable proteins in the rumen, support the synthesis of microbial proteins and leads to the absorption of propionic acid which stabilises the level of blood glucose in peripartal dairy cows. The increased levels of NFS in the TMR cover the increased requirements of dairy cows on nutrients, particularly glucose. The changing pattern of glucose metabolism is subject to considerable ho-

meorhetic influence which maximizes gluconeogenesis in the liver and minimizes the utilization of glucose by peripheral tissues (6). Glucose is purposefully diverted to the mammary gland to support the synthesis of lactose which eventually determines the milk yield. The principal substrate of gluconeogenesis in the liver is propionic acid that develops especially by fermentation of rapidly fermentable saccharides in the rumen. These above facts are related to the regulation of the synthesis of insulin, a hormone with significant antilipolytic effects, which can result in decreased lipomobilization and metabolic load on the liver (2).

The blood levels of glucose, insulin, and glucagon are the key factors in the management of metabolic adaptation and prevention of metabolic disorders. Our observations showed that in the Ist group of herds, fed TMR of the bulk type with a mean level of propionic acid in the rumen reaching 16.44 ± 2.54 mmol. l^{-1}, there was a considerably higher degree of lipomobilisation and metabolic load on the liver than in the IInd group of dairy cows with the production of propionic acid in the rumen exceeding the minimum recommended level of 20 mmol.l^{-1}.

ACKNOWLEDGEMENT

The study was supported by the grant VEGA 1/0970/11.

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FOLIA VETERINARIA, 56, 4: 11–13, 2012

CHARACTERIZATION OF DIFFERENT PHENOTYPES OF CELLS IN THE CENTRAL CANAL LINING OF RAT SPINAL CORD DURING EARLY POSTNATAL PERIOD

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ABSTRACT

Despite the fact that many studies have dealt with the development of the nervous system, the information about postnatal gliogenesis is still incomplete. The aim of this study was to analyse processes which take place in the lining of the spinal cord central canal during the early postnatal period. Using an endogenous marker of proliferation (Ki-67), we observed that the proliferation in the central canal lining of the rat spinal cord occurred throughout the monitored period, reaching its peak during the second postnatal week. Furthermore, the cells that formed the lining of the central canal rarely underwent apoptosis. The lining of the central canal consisted of various cell types which were immunoreactive to markers of radial glia, ependymal cells, neurons and astrocytes, as well as a small number of cells with the oligodendrocyte phenotype.

Key words: central canal; glial cells; proliferation; rat; spinal cord

INTRODUCTION

The development of the spinal cord during the embryonic period represents a comprehensive sequence of events involving the formation of the neural tube and neural crest which consist of neuroepithelial cells. The neuroepithelial cells extensively proliferate and differentiate into progenitor cells or radial glia (1). Bipolar radial glial cells represent a transient cell type that is present in the central canal lining during a restricted period. Radial glial cells gradually disappear during the late embryonic development as a consequence of their transformation into astrocytes (7), ependymal cells (8), oligodendrocytes (10) or even neurons (5). Several studies have described the lining of the central canal as an ependymal layer consisting of homogenous ependymal cells. Nowadays, it is widely accepted that the ependymal layer is a highly heterogeneous region of the spinal cord (3). The cells in the central canal lining are mitotically active during embryonic and postnatal periods and only a small number of these proliferating cells are ependymal cells (9).

Despite the numerous investigations in the area of neuronal development, there is still lack of information about postnatal gliogenesis in the lining of the central canal. Our study focused on the early postnatal development of the rat spinal cord with an emphasis on the processes taking place in this zone.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethical Committee of the Faculty of Science, Pavol Jozef Šafárik University in Košice, in accordance with requirements of the National Veterinary and Food Administration of Slovak Republic. Wistar albino rats of age 1, 8, 15, 22, 29 and 43 days (P1–P43) were used in this study. At each time point we examined groups of three animals. The rats were deeply anesthetized with sodium thiopental and intracardially perfused with saline followed by 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Isolated spinal cords were fixed overnight in 4 % paraformaldehyde and immersed in a cryoprotectant (30 % sucrose in PBS). The 40 µm thick tissue sections were produced with a cryostat and processed by conventional immunohistochemical and immunofluorescence techniques (1). The proliferative cells were visualised using an endogenous marker of proliferation Ki-67

(Abcam, 1 : 500). Diaminobenzidine (DAB) was used to visualize the Ki-67 immunoreactive cells. We examined 14 tissue sections per animal for all three animals in each group. The number of proliferative cells was evaluated as a number of Ki-67 positive cells per 1 mm^3 of the tissue. We utilized the method of *in situ* hybridization TUNEL (2) to determine the presence of apoptotic cells in the lining of the spinal cord central canal. The primary antibodies used for identification of cell phenotype are shown in Table 1. The secondary antibodies (Vector) were conjugated with biotin or with fluorophores (AlexaFluor 594 and 488, Jackson).

Tissue sections were examined under an Olympus BX41 light microscope using QuickPHOTO Micro 2.2 software and Ellipse 2 software. The sections for fluorescent microscopy were examined under a confocal microscope Leica TCS SP5X with LAS AF software.

Table 1. Markers used for identification of cell phenotypes in central canal lining

Cell type	Marker	Producer and solution
Radial glia cells	Nestin	Santa Cruz (1:200)
	Vimentin	Abcam (1:200)
	GLAST	Millipore (1:1000)
Astrocytes	GFAP	Sigma-Aldrich (1:1000)
Oligodendrocytes	Olig2	Millipore (1:500)
	PDGFRα	Santa Cruz (1:50)
	Sox10	Santa Cruz (1:100)
Ependymal cells	S100β	Abcam (1:100)
	CD24	Santa Cruz (1:50)
Neurons	Doublecortin	Millipore (1:3000)

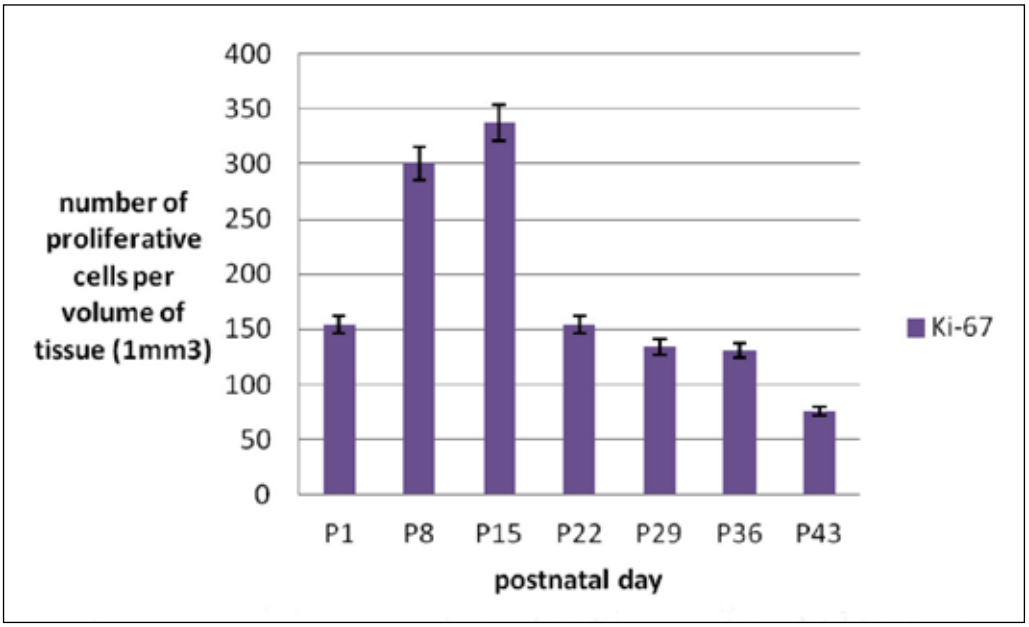

Fig. 1. The number of Ki-67 immunoreactive cells per 1mm^3 of the tissue

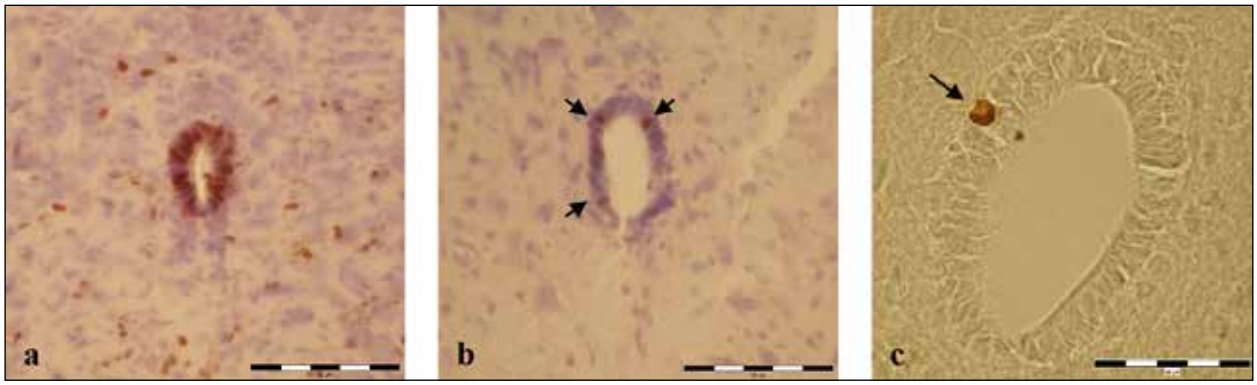

Fig. 2. Ki-67 positive cells in the lining of the central canal at P15 (a) and P43 (b). Tunel-positive cell in the spinal cord ependyma at P43 (c)

RESULTS AND DISCUSSION

Our study investigated the processes which take place in the lining of the central canal of the rat spinal cord during the early postnatal period. In our previous study, using exogenous proliferation marker BrdU (5-bromo-2-deoxyuridine), we discovered two waves of cell proliferation in the ependymal layer of the rat central canal during the late development of the spinal cord (9). The first wave occurs around embryonic day 18 and the second between postnatal days 8 and 15 (9). In our recent study, we used an endogenous marker Ki-67, which in contrast to BrdU, labels proliferating cells in every phase of the cell cycle (except G0). We found that the proliferation in the ventricular zone occurred throughout the studied period (P1–P43) (Fig. 1) with a maximum at the second postnatal week (Fig. 2a).

The next step was to detect the fate of the newly formed cells. The first possibility is that new cells are required for the elongation of the central canal on account of the growth of the spinal cord and they remain in the lining of the central canal. The second possibility was that part of these cells migrate to the distant regions of the spinal cord and become the source of the glial cells. The last assumption was that some of the cells undergo apoptosis. We have applied *in situ* hybridization method TUNEL for verification of the last hypothesis. TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. We detected that there is a very small number of apoptotic cells in the lining of the central canal (Fig. 2c). Our results indicate that apoptosis has only a negligible impact on the fate of cells generated in the ventricular zone of the central canal.

In the next step, we tried to identify the phenotype of postnatally generated cells in the lining of the central canal. We used immunohistochemical markers for radial glia, ependymal cells, astrocytes, oligodendrocytes and neurons at various stages of differentiation. Radial glia presents in the lining of the central canal during the first postnatal week and shows immunoreactivity for vimentin, nestin and GLAST. After the second postnatal week, radial glial cells slowly disappear. However, we could observe subtle vimentin positive fibres around the central canal even at the sixth postnatal week. Similar trends in the expression of glial cell markers were described by Barry and McDermott (1). The highest proliferative activity was observed in the first and second postnatal week. During this period, the cells lining the central canal showed activity mainly as ependymal cells ($S100\beta^+$) and neurons contacting the central canal (Doublecortin$^+$). In the near vicinity of the central canal we detected GFAP$^+$ astrocytes. We found the presence of Olig2$^+$, Sox10$^+$ and PDGFRα^+ cells in close contact with the lining of the central canal. These cells were most probably immature oligodendrocytes or precursors of oligodendrocytes. We observed Olig2 positive cells directly in the central canal lining. The presence of Olig2$^+$ cells in the ependymal layer could indicate that the postnatal oligodendrocytogenesis occurred in this region, as was already described in the ventricular zone of brain ventricles (4, 6) but not in the spinal cord of rodents until now.

ACKNOWLEDGEMENT

The study was supported by projects VEGA 1/0967/12 and VVGS-PF-2012-49.

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Selected papers from the Seminar of PhD. students, organized in September 2012 by the UVMP in Košice, devoted to the memory of outstanding veterinary physiologist Academician Boďa.

FOLIA VETERINARIA, 56, 4: 14–16, 2012

IN VIVO TESTING OF THE EFFICACY OF BEE PROBIOTIC LACTOBACILLI

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ABSTRACT

American foulbrood is amongst the most dangerous diseases of the bee-family affecting many honeybee colonies worldwide. In most of the countries, based on veterinary legislation, the bee colonies that test positive for American foulbrood are eradicated with resulting high economic losses. It is therefore necessary to find effective preventions of American foulbrood, especially by using natural methods available, such as probiotics. Probiotics constitute a potential alternative to antibiotic use in the prevention, as well as in the therapy of various diseases. However, the knowledge regarding their use in invertebrates is poor. In this study, we used lactobacilli isolated from the digestive tracts of adult healthy honey bees and selected the isolates based on their good probiotic properties and their ability to inhibit the growth of *Paenibacillus larvae*. These isolates were identified as *Lactobacillus brevis* and *Lactobacillus plantarum*. Night cultures of both strains were used for the preparation of the probiotic solutions and pollen was selected as an appropriate carrier for the application of the probiotic lactobacilli to the hives. Half a litre of a pollen solution was prepared for each hive. The solution for the experimental hives contained probiotic lactobacilli in the concentration of 10^7–10^8 CFU.ml^{-1}. Bees in control hives received pollen solution without added probiotic lactobacilli. The solutions were administered three times, once a week. Before and during administration of the probiotics, samples of honey bees from each hive were taken every week and the numbers of lactobacilli, *P. larvae*, enterobacteria and coliform bacteria were determined in their digestive tracts. Four weeks after the first application of the probiotic-pollen solution, the numbers of lactobacilli were increased approximately by 0.5 log. Before starting this experiment, *P. larvae* (approx. 10^7 CFU.ml^{-1}) were detected in the digestive tracts of the honey bees. In the experimental group after 3 weeks and in control group after 4 weeks no viable counts of *P. larvae* were found. The numbers of enterobacteria and coliform bacteria decreased in both groups. Based on these results, we assumed that the probiotic-pollen solution had a positive influence on the composition of microflora in bee digestive tracts and it can increase the resistance to *P. larvae*. Probiotic-free pollen solution showed similar but weaker effects.

Key words: administration of probiotics; microflora; *Paenibacillus larvae*

INTRODUCTION

American foulbrood is one of the most serious diseases of honey bees, resulting in high mortality rates. It is being spread worldwide and causing major economic losses. It has been classified as a disease subject to the reporting obligation in terms of the Office of International Epizootics (OIE) list of transmissible diseases. The disease is caused by the Gram-positive rod-shaped bacterium, *Paenibacillus larvae* (1). The administration of antibiotics has only minor effect, as the antibiotics only affect the vegetative forms of *P. larvae*, while the spores continue to spread. Moreover, residues of these drugs persist in bee products, primarily in honey, contributing to the increasing antibiotic resistance of pathogens. These are the reasons why the administration of antibiotics against American foulbrood has been prohibited by European Union legislation. Infection by *P. larvae* is dealt with in a radical way – by eradicating the hives and

by burning all the combustible materials. Various teams of scientists have been trying to find a worthy alternative to use of antibiotics. Excellent possibilities are offered by probiotics.

This study deals with the testing of the administration of probiotic lactobacilli in bee hives by monitoring their impact on the composition of the intestinal microflora of honey bees.

MATERIAL AND METHODS

Probiotic lactobacilli

Lactobacillus brevis and *Lactobacillus plantarum* used for this experiment were isolated from digestive tracts of adult healthy honey bees. Both strains were identified by rep-PCR and the MALDI TOF-MS method. These strains showed in previous studies, positive growth and probiotic properties and the ability to inhibit the growth of *P. larvae* (5). The night cultures of both strains were cultured in MRS broth at 37 °C under aerobic conditions. These cultures were washed 3 times in saline solutions followed by centrifugation at 600xg for 30 min. The sediment was resuspended in autoclaved tap water and the concentration of the lactobacilli was adjusted to 10^7–10^8 CFU.ml^{-1}.

Probiotic-pollen solution

Based on previous testing of the viability of *L. brevis* and *L. plantarum* in different media (4), pollen was selected as the best carrier for probiotic lactobacilli. For each administration and each experimental hive, we prepared half a litre of the probiotic-pollen solution composed of 50 g of pollen and probiotic lactobacilli suspension. For control hives we prepared half a litre of pollen solution composed of 50 g of pollen in autoclaved tap water without the added probiotic lactobacilli.

Experimental design

Six bee colonies (3 experimental and 3 control) were used in the experiment. Probiotic-pollen solution was administered to the experimental hives, while honey bees in the control hives received pollen solution without the probiotic lactobacilli. The solutions were supplied to honey once a week for three subsequential weeks.

Analysis of intestinal microflora of honey bees

Before and during administration of the solutions, approximately 100 bees (a handful) from each colony were taken every week and the honey bees were killed by ether vapours. The digestive tracts (intestine, stomach and rectal sack) were used for the evaluation of the microflora composition. Bacterial counts were determined by plating on selective media after preparing ten-fold dilutions.

Lactobacilli were cultured on MRS agar (Oxoid, Basingstoke, UK) for 48 h at 37 °C in 80 % CO_2 and 20 % N_2 atmosphere. *Paenibacillus larvae* were grown in MYPGP agar (yeast extract 15 g, K_2HPO_4 3 g, sodium pyruvate 1 g, D(+) glucose 2 g.l^{-1}, or agar 20 g.l^{-1}, pH 7.2) for 48 h at 37 °C. Endo agar (Oxoid, Basingstoke, UK) was used for the cultivation of *Enterobacteriaceae*, and Mc Conkey agar (BBL, Becton Dickinson, Cockeysville, USA) for cultivation of for *E. coli*. Both agars were incubated for 24 h at 37 °C.

The significance of differences between experimental and control bee hives was tested by *t*-test.

RESULTS

No problems with the intake of probiotic-pollen or pollen solutions by honey bees were observed during the study.

After administration of the probiotic-pollen solutions, the numbers of lactobacilli showed an increasing tendency throughout the experiment. In the last two samplings, the numbers of lactobacilli were significantly higher in the experimental colonies, in comparison with the control (Table 1).

Table 1. Plate counts of lactobacilli expressed as $\log_{10}$ CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen solution

	Sampling				
	1	2	3	4	5
Experimental hives	6.2 ± 0.4	6.3 ± 0.6	6.5 ± 0.4	6.2 ± 0.4	6.7 ± 0.1
Control hives	6.3 ± 0.4	6.3 ± 0.5	6.1 ± 0.6	5.3 ± 0.4	6.2 ± 0.5
***t*-test**	ns	ns	ns	P < 0.001	P < 0.01

ns – not significant; sampling 1 – before administration; samplings 2–4 – one week after each administration; sampling 5 – two weeks after the last administration

Before starting the experiment, *P. larvae* (in a concentration of approximately 10^7 CFU.ml^{-1}) was found in the digestive tracts of honey bees. After three applications of probiotic-pollen solution, *P. larvae* completely disappeared from the digestive tract. After three applications of pollen solution in the control colonies, the numbers of *P. larvae* were significantly reduced (P < 0.001) in comparison with the 3rd sampling and in the last sampling we detected no viable *P. larvae* (Table 2).

Table 2. The numbers of *Paenibacillus larvae* expressed as $\log_{10}$ CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen solution

	Sampling				
	1	2	3	4	5
Experimental hives	7.1 ± 0.3	7.0 ± 0.3	6.5 ± 0.1	0	0
Control hives	6.8 ± 0.4	6.3 ± 0.2	6.1 ± 0.4	1.9 ± 2.6	0
***t*-test**	ns	P < 0.1	ns	ns	ns

ns – not significant; sampling 1 – before administration; samplings 2–4 – one week after each administration; sampling 5 – two weeks after the last administration

During the experiment, the numbers of *Enterobacteriaceae* and coliform bacteria decreased after administration of probiotic-pollen and pollen solutions by 1–1.5 log, but the differences between the control and experimental groups were insignificant (Tables 3 and 4).

Table 3. The numbers of *Enterobacteriaceae* expressed as $\log_{10}$ CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen solution

	Sampling				
	1	2	3	4	5
Experimental hives	6.8 ± 0.6	6.5 ± 0.5	7.0 ± 0.6	6.4 ± 0.7	5.6 ± 0.9
Control hives	7.1 ± 0.7	7.0 ± 0.4	6.8 ± 0.6	6.5 ± 0.5	5.8 ± 0.6
***t*-test**	ns	ns	ns	ns	ns

ns – not significant; sampling 1 – before administration;
samplings 2–4 – one week after each administration;
sampling 5 – two weeks after the last administration

Table 4. The numbers of coliform bacteria expressed as $\log_{10}$ CFU.ml^{-1} ± SD (n = 9) in the digestive tracts of honey bees before and after administration of probiotic-pollen or pollen solution

	Sampling				
	1	2	3	4	5
Experimental hives	6.5 ± 0.7	6.7 ± 0.4	7.2 ± 0.6	6.4 ±0.5	4.8 ±1.8
Control hives	6.5 ± 0.8	6.9 ± 0.6	7.2 ± 0.5	6.8 ± 0.8	5.8 ± 0.7
***t*-test**	ns	ns	ns	ns	ns

ns – not significant; sampling 1 – before administration;
samplings 2–4 – one week after each administration;
sampling 5 – two weeks after the last administration

DISCUSSION

Probiotics represent one of the promising possibilities for replacement of antibiotics in prevention and therapy of many diseases in humans and other animals. Our results indicate the ability of *L. brevis* and *L. plantarum* to inhibit the growth of *P. larvae*. They stabilized microflora in the digestive tract of honey bees. Similar conclusions were presented in the study by Canganella and Balsamo (2), who demonstrated the inhibitory effects of lactobacilli against *P. larvae* and *P. alvei* (European foulbrood). Forsgren *et al.* (3) also observed the ability of lactobacilli and bifidobacteria isolated from the stomach of honey bees to inhibit the growth of *P. larvae*. They administered such lactobacilli to bee larvae through the mother bread and noted significantly lower numbers of infected larvae. Colonisation of the gut by beneficial microflora can prevent proliferation of spores of *P. larvae* and thus prevent infection. Application of pure pollen without addition of probiotic lactobacilli also showed positive effect on the composition of intestinal microflora of honey bees and the elimination of *P. larvae* from their digestive tract. Pollen can stimulate the immune response of honey bees, because it naturally contains lactobacilli, nutrients which increase the food quality, and some immunostimulating substances (e.g. vitamins, fatty acids. enzymes, etc.).

CONCLUSION

The following conclusions were drawn.

- Honey bees willingly drank probiotic-pollen and pollen solutions.
- *P. larvae* disappeared from the digestive tract of honey bees after administration of test solutions.
- Numbers of *Enterobacteriaceae* and coliform bacteria were reduced by 1–1.5 log.

ACKNOWLEDGEMENTS

The study was carried out within the Scientific Grant Agency project of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences No. 2/0016/12 New methods of biological protection against microbial diseases of honey bees.

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Selected papers from the Seminar of PhD. students, organized in September 2012 by the UVMP in Košice, devoted to the memory of outstanding veterinary physiologist Academician Boďa.

FOLIA VETERINARIA, 56, 4: 17–20, 2012

HYPERICIN DECREASES CISPLATIN CYTOTOXICITY IN HUMAN OVARIAN ADENOCARCINOMA CELL LINES

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ABSTRACT

Cisplatin is one of the most widely used chemotherapeutic agents. However, cisplatin resistance is a major problem for the successful treatment of cancer. It is known, that many natural compounds are able to modulate various resistance mechanisms. Since cancer treatment failure and herb-drug interactions are a topical and serious issue, we investigated the impact of hypericin, St John's wort (*Hypericum perforatum* L.) a secondary metabolite, on cisplatin action in human ovarian carcinoma cell lines (A2780, A2780cis). The results confirmed our assumption about hypericin-mediated resistance development and revealed that hypericin decreases cisplatin cytotoxicity in cisplatin sensitive cells A2780 as well as in cisplatin resistant cells A2780cis (MTT assay, cell viability analysis). These findings point out the risk of St John's wort extracts and chemotherapy during co-administration.

Key words: cisplatin; drug resistance; hypericin; St John's wort

INTRODUCTION

Despite the progress in anticancer therapy, chemotherapy still remains the first-line of treatment for many forms of advanced or metastatic tumors. Cisplatin, the effective cytostatic agent, primarily targets DNA and plays a crucial role in the treatment of many solid tumors, including ovarian cancer. However, the intrinsic or acquired cisplatin resistance is the major limitation to clinical treatment and may lead to disease relapse by way of the expansion of drug-resistant cancer clones. It is known, that many natural compounds are able to modulate various resistance mechanisms. Such inductive or inhibitory effects may result in the development of negative herb-drug interactions, which may even cause treatment failures. In recent years many experimental and clinical studies have revealed the negative impact of St John's wort (SJW, *Hypericum perforatum* L.) extracts on some co-administered drugs; particularly those which are substrates of cytochrome P450 enzymes (CYP450) and/or P-glycoprotein (P-gp), one of the ATP-binding cassette (ABC) transport proteins (2). Our results suggest that hypericin, SJW secondary metabolite, can induce expression and activity of some ABC transporters (4).

Since some of these efflux pumps may have a negative impact on cisplatin action (5, 6), we have investigated the possibility of cisplatin resistance developing through the potential hypericin-mediated ABC transporters induction. We examined the effect of hypericin pre-treatment on cisplatin action in cisplatin sensitive (A2780) and cisplatin resistant (A2780cis) human ovarian carcinoma cell lines.

MATERIAL AND METHODS

Cell lines and growth conditions

The human ovarian carcinoma cell line A2780 was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA) and its cisplatin resistant subline A2780cis was kindly provided by Prof. Alois Kozubík (Institute of Biophysics, Brno, Czech Republic). The cells were grown in complete RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10 %

fetal bovine serum, 7.5 % $NaHCO_3$ and antibiotics (penicillin 100 U.ml^{-1}, streptomycin 100 μg.ml^{-1} and amphotericin 25 μg.ml^{-1}; Invitrogen, Carlsbad, CA, USA) at 37 °C, 95 % humidity in 5 % CO_2 atmosphere. The cisplatin resistance of A2780cis cells was maintained using 1 μM cisplatin (EBEWE Pharma GmbH., Nfg. KG, Unterach, Austria) added to the culture every 2nd passage.

Experimental design

Cells were seeded in 96-well plates for MTT assay, 6-well plates for flow cytometry analyses or Petri dishes for cell number analyses and left to settle for 24 h in the stated conditions. Subsequently, cells were pre-treated with hypericin (4,5,7,4',5',7'-hexahydroxy-2,2'-dimethylnaphtodiantron, HPLC grade; AppliChem GmbH, Darmstadt, Germany) for another 24 h, prior to cisplatin addition. Depending on the type of analysis, the results were analyzed at 24 h and/or 48 h after treatment.

Metabolic activity of the cells analyzed by MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Aldrich) assay was performed to evaluate the metabolic activity of cells as was previously reported (3). MTT was added to the cells 24 h and 48 h after drug treatment (final concentration 0.5 mg.ml^{-1}). The results were evaluated as percentages of the absorbance of the untreated control. In the case of cisplatin, the estimated IC50 values were extrapolated from an exponential fit to the metabolic activity data using Origin Pro 8.5 (OriginLab, Northampton, USA). The hypericin effect on cisplatin action was evaluated by CalcuSyn software (BIOSOFT, Ferguson, MO, USA and Cambridge, UK) according to the method of Chou and Talalay (1).

Cell number analysis

The number of floating cells and total cell number were assessed separately using a Coulter Counter (Coulter Electronics Ltd, Luton, Beds, England) 24 h and 48 h after cisplatin treatment. The floating cells were analyzed directly in the medium removed from the individual experimental groups. The adherent cells were harvested by trypsinization and subsequently counted.

Phosphatidylserine externalization and cell viability analysis

For phosphatidylserine externalization and cell viability analysis, adherent cells were harvested by trypsinization and collected together with floating cells 24 h and 48 h after cisplatin treatment and an Annexin V-FITC/propidium iodide (PI) double staining kit (APOPTEST™-FITC; Dako, Glostrup, Denmark) was used according to the manufacturer's instructions, as was previously described (3). Analyses were performed on a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the results were evaluated using FlowJo software (Tree Star, Inc., Ashland, USA).

Statistical analysis

Data were analyzed using one-way ANOVA with Tukey's post test or *t*-test and were expressed as mean ± standard deviation (STD) of at least three independent experiments. The experimental groups given combined treatment were compared with the groups treated with cisplatin alone (* – $P<0.05$; ** – $P<0.01$; *** – $P<0.001$).

RESULTS AND DISCUSSION

SJW is one of the most popular herbal remedies for treating depression and mood disorders and a growing percentage of the population is using its various preparations for preventive and therapeutic purposes. Such antidepressive action is mediated by biologically active compounds synthesized by SJW (hyperforin, hypericin, flavonoids etc.). However, SJW metabolites contained in the extracts may be responsible for negative herb-drug interaction development. This drawback is caused by hyperforin, a potent inducer of some CYP450 enzymes and P-gp (8). Recent results revealed that hypericin may also induce some ABC transporters, mainly multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) (4). Because cisplatin represents a potential ABC transporters substrate (5, 6), we suspected a possible hypericin-mediated decrease in cisplatin action and cisplatin resistance development in human ovarian adenocarcinoma cells (A2780, A2780cis).

Table 1. Effect of hypericin ($H_{0.1}$, $H_{0.5}$), cisplatin (C_{IC50}) and hypericin pre-treatment in combined therapy with cisplatin ($H_{0.1}$ C_{IC50}, $H_{0.5}$ C_{IC50}) on cell metabolic activity

Cell line	Time	Cell metabolic activity [%]					
		Control	$H_{0.1}$	$H_{0.5}$	C_{IC50}	$H_{0.1}$ C_{IC50}	$H_{0.5}$ C_{IC50}
A2780	24 h	100.00 ± 4.72	106.75 ± 6.01	130.80 ± 4.95	72.28 ± 4.47	79.48 ± 2.82	98.51*** ± 4.58
	48 h	100.00 ± 3.54	97.22 ± 3.59	119.09 ± 4.52	70.17 ± 4.22	78.47 ± 3.51	95.88*** ± 3.44
A2780cis	24 h	100.00 ± 4.51	111.39 ± 5.93	141.61 ± 7.93	64.88 ± 6.31	68.75 ± 7.12	97.21*** ± 7.85
	48 h	100.00 ± 3.45	103.91 ± 4.80	131.12 ± 7.89	70.01 ± 5.38	83.80* ± 5.70	107.49*** ± 9.57

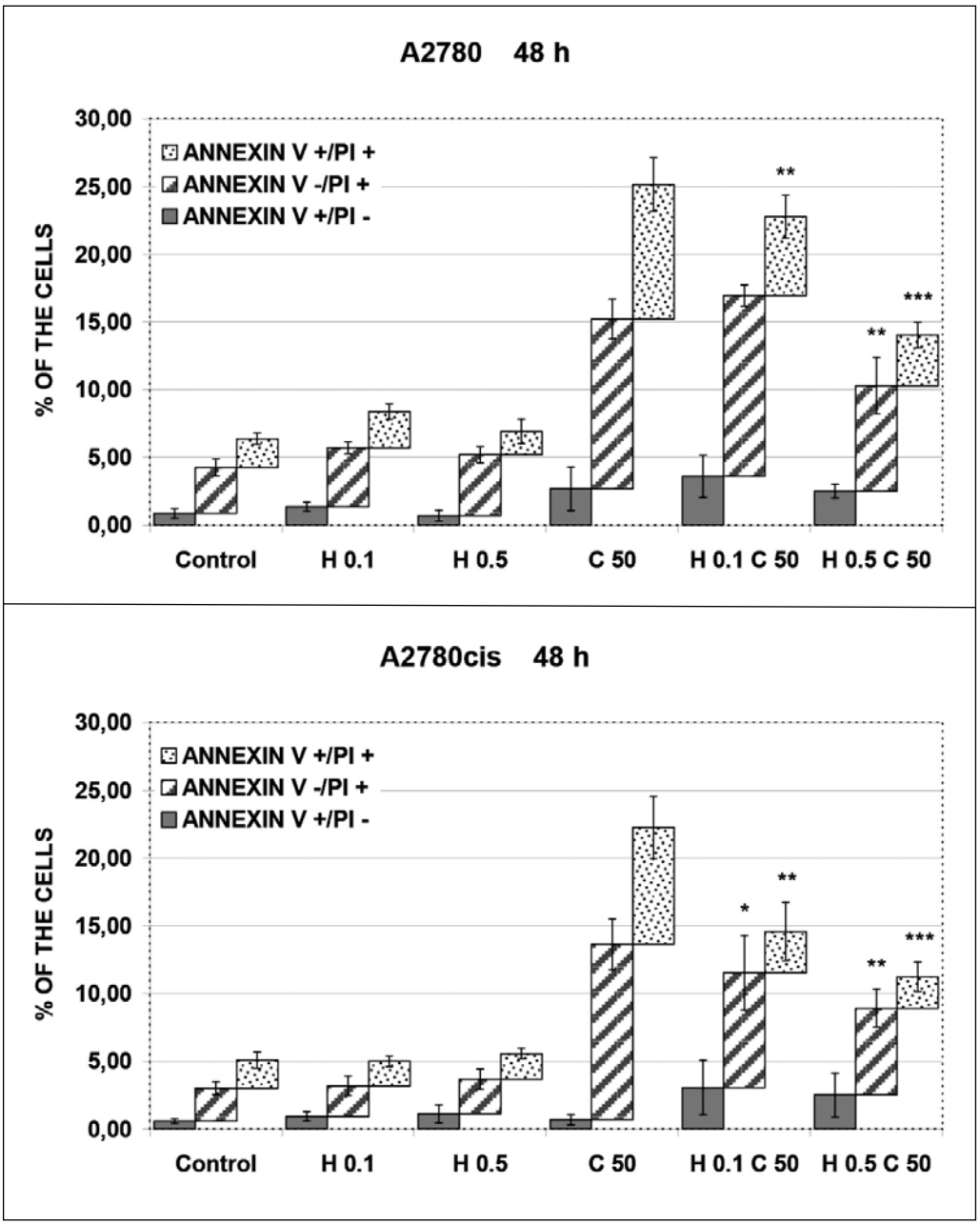

Fig. 1. Effect of hypericin (H 0.1, H 0.5), cisplatin (C 50) and hypericin pre-treatment in combined therapy with cisplatin (H 0.1 C 50, H 0.5 C 50) on cell viability

At first, cisplatin concentrations (IC50) were chosen based on MTT metabolic assay for each cell line alone (data not shown). Two low hypericin concentrations (0.1 μM and 0.5 μM) were chosen pursuant to previous results in our laboratory (4). We found, that hypericin is able to reduce cisplatin action not only in cisplatin sensitive A2780 cells, but also even in cisplatin resistant A2780cis cells. Hypericin pre-treatment in combined therapy with cisplatin caused significant time- and dose-dependent increase in cell metabolic activity in comparison to the groups treated with cisplatin alone (Table 1). According to these results, hypericin-cisplatin combination index values were evaluated using CalcuSyn software and revealed antagonistic effect of this phytochemical on cisplatin action (data not shown).

Although there were no significant alterations in the number of floating cells and total cell number (data not shown), hypericin affected cell viability and phosphatidylserine externalization, one of the programmed cell death markers. Compared with cisplatin alone, a significant decrease in the percentage of apoptotic and necrotic cells was evident after combined treatment (Fig. 1). These findings correlated with the effects on cell metabolic activity, since we determined diminished cisplatin action in both cell lines after hypericin pre-treatment.

In conclusion, the results of this study revealed the strong antagonistic effect of hypericin, SJW secondary metabolite, on cisplatin action, demonstrated by the reduced cisplatin

cytotoxicity. Since hypericin pre-treatment had a positive impact on the viability of both A2780 and A2780cis cells, we may suppose that this phytochemical could be another potential modulator of some resistance mechanisms and could thereby affect resistance development. Cisplatin resistance is a multifactorial phenomenon, which can occur by several mechanisms (reviewed in: 7), including increased drug efflux. Therefore, further studies will be necessary to elucidate, which of those mechanisms could be influenced by hypericin (ABC transporters, DNA repair, glutathione, etc.). This study contributes towards the discovery of negative herb-drug interactions and may possibly lead to cancer treatment complications. We would like to call attention to the chronic use of SJW extracts, especially during the period of cancer treatment.

ACKNOWLEDGEMENTS

This work was supported by NEXO (Network of Excellence in Oncology) under contract ITMS 26220120039.

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Selected papers from the Seminar of PhD. students, organized in September 2012 by the UVMP in Košice, devoted to the memory of outstanding veterinary physiologist Academician Boďa.

FOLIA VETERINARIA, 56, 4: 21–24, 2012

GDF-15 AS A FACTOR POTENTIALLY INFLUENCING EFFICACY OF HYPERCIN-BASED PHOTODYNAMIC THERAPY

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ABSTRACT

The aim of this study was to investigate the influence of a multifunctional cytokine, GDF-15, on the efficacy of photodynamic therapy with hypericin (HY-PDT). The initial experiments were carried out to evaluate the effects of GDF-15-rich conditioned media on the cell death of HCT116 p53$^{-/-}$ after HY-PDT. Subsequent experiments were aimed to confirm the involvement of GDF-15 in the effects of the conditioned media by evaluating the influence of GDF-15 on the metabolic activities of the cancer cell lines HCT116 p53$^{+/+}$, HCT116 p53$^{-/-}$ and A549 (MTT assay) and their proliferation and migration ability (wound-healing assay). The initial results indicated the presence of signals promoting programmed cell death; however, subsequent analyses did not support the hypothesis that this effect was caused by GDF-15.

Key words: GDF-15; hypericin-based photodynamic therapy

INTRODUCTION

Photodynamic therapy (PDT) is an effective therapeutic approach for the treatment of both malignant and benign tumors. During PDT, a photosensitizer is activated by the light of an appropriate wavelength. Subsequent photochemical and photobiological processes result in reactive oxygen species formation causing damage to the target cells or tissues and launch a programmed cell death cascade (1). Hypericin is a naturally occurring highly efficient photosensitizer with a strong affinity for cancer tissues and minimal dark toxicity (5).

GDF-15 (growth/differentiation factor 15) is a multifunctional cytokine of the TGF-β family. It plays a role in various processes including; prenatal development, cellular responses to stress signals, and immune system regulation (4). Its expression and secretion is rather low under most physiological conditions, however, it is considerably elevated in association with various pathological conditions including oncological disorders. Secreted GDF-15 may be implicated in both the inhibition and promotion of cancer progression by positively or negatively modifying cell proliferation, differentiation and motility, depending on the cell type, stage of disease and overall context (2, 3, 4). Increased GDF-15 serum level is also a promising marker of cancer progression, malignancy grade, lymph node infiltration and metastasis formation for various types of oncological disorders (4).

Little is known about the role of GDF-15 in cancer cell response to photodynamic therapy. The use of 5-aminolevulinic acid-based PDT caused an increase in *gdf-15* mRNA (6), however, there is a lack of information about the final protein production and secretion, and its subsequent effect on cancer cells. Our previous experiments revealed that hypericin induces an increase in GDF-15 secretion, whereas HY-PDT leads to GDF-15 intracellular accumulation. The aim of this work is now focused on the influence of pleiotropic cytokine GDF-15 on HY-PDT efficacy on various cancer cell lines.

MATERIAL AND METHODS

Cell lines, cell culture and experimental design

The p53 expressing HCT116 (HCT116 p53$^{+/+}$) and p53-null (HCT116 p53$^{-/-}$) colorectal adenocarcinoma cell lines were created

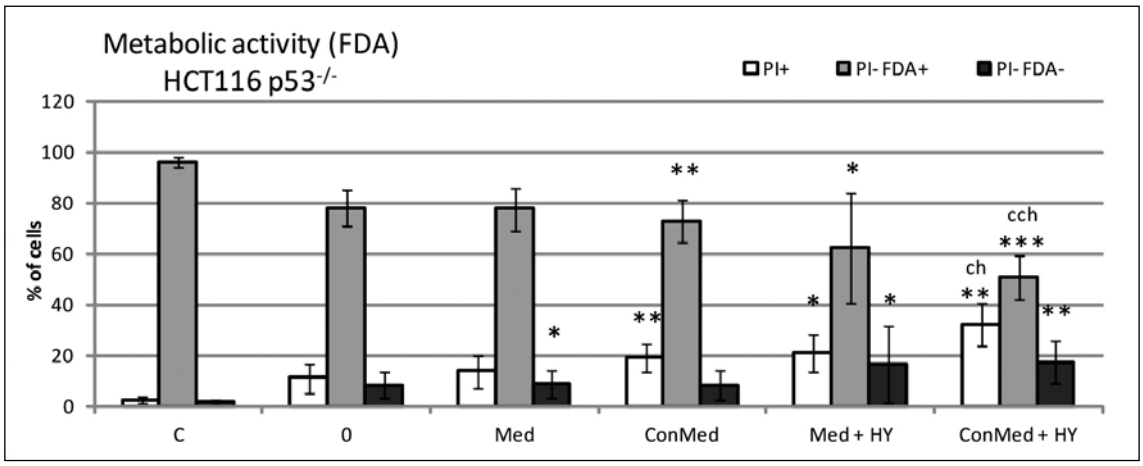

Fig. 1. Percentage of metabolically active cells in experimental groups

C – control; 0 – group without media changes; Med – medium; ConMed – conditioned medium; HY-hypericin; * – statistical significance in comparison to control; c – statistical significance in comparison to ConMed goup; h – statistical significance in comparison to Med+HY group; PI+ – dead cells; PI-FDA+ – metabolically active cells; PI-FDA- – cells with impaired metabolic activity

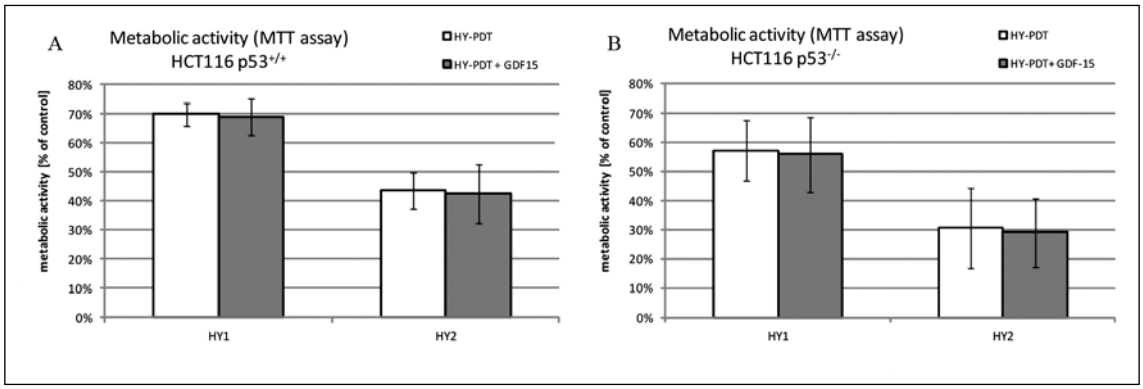

Fig. 2. Metabolic activity of HCT116 p53$^{+/+}$ (A) and p53$^{-/-}$ (B) cells evaluated by MTT test 48 hours after HY-PDT

HY1 – hypericin concentration 0.05 μmol.l^{-1}; HY2 – concentration 0.075 μmol.l^{-1}

by targeted homologous recombinations and was a gift from Professor Bert Vogelstein (7) (both kindly provided by Dr. Alois Kozubík, Institute of Biophysics, Brno, Czech Republic). A549 lung adenocarcinoma cell line was purchased from American Tissue Culture Collection (ATCC, Rockville, MD USA). HCT116 cells were grown in McCoy's 5A medium and A549 in Ham's F12K medium (both from Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, penicillin (100 U.ml^{-1}), streptomycin (100 mg.ml^{-1}), amphotericin (25 mg.ml^{-1}) (Gibco Invitrogen Corp., Carlsbad, CA, USA) and gentamycin (50 mg.ml^{-1}; Sigma-Aldrich). All cell lines were cultured at 37 °C, 95 % humidity in an atmosphere containing 5 % of CO_2.

For the experiments, cells were seeded into 6 wells, 96 well tissue culture plates or 60 mm Petri dish (TPP, Trasadingen, Switzerland) and cultivated 30 hours before hypericin addition. Hypericin, at different concentrations, was activated after 16 hours of incubation with cells in the dark, by light at a total dose of 3.6 J.cm^{-2}. In some experiments, the cells were pretreated with GDF-15 (1000 ng.ml^{-1}) for 24 hours before the PDT.

Reagents

GDF-15 – growth/differentiation factor 15 (PeproTech, London, UK) was prepared as a 100 μg.ml^{-1} stock solution in PBS containing 0.1 % of bovine serum albumin.

Hypericin (4,5,7,4´,5´,7´-hexahydroxy-2,2´-dimethylnaphtodiantron, HPLC grade, Applichem GmbH, Darmstadt, Germany) was dissolved in DMSO.

MTT assay

For MTT assay, cells were seeded to 96 well plates. MTT (3-[4.5-dimethylthiazolyl]-2,5diphenyl-tetrayolium bromide)(Sigma Chemicals Co., St. Louis, MO, USA) was added 24 or 48 h after PDT with 0.05 or 0.075 μmol.l^{-1} hypericin. In the case of the experimental groups treated with GDF-15 alone (concentrations 250, 500, 750 and 1000 ng.ml^{-1}), MTT was added 48 or 72 h post-GDF-15 and allowed to metabolize for 4 h. The reaction was stopped and crystals of formazan were dissolved by the addition of 10% SDS (Serva, Heidelberg, Germany). The absorbance of dissolved formazan was measured by FLUOStar Optima (BMG Labtechnologies GmbH, Offenburg, Germany) at 585nm.

Conditioned media and cell death markers (flow cytometric analysis)

The conditioned media collected from the HCT116 $p53^{+/+}$ cells were added to HCT116 $p53^{-/-}$ 0, 8 and 24 h after HY-PDT. The adherent and floating cells were harvested together 24 or 48 h after PDT, washed with PBS and stained either with fluoresceindiacetate (FDA) (100 ng.ml^{-1} /20 min/ 37 °C) and propidium iodide PI (25 µg.ml^{-1}/5 min/37 °C) for metabolic activity and viability evaluation, or with Annexin V-FITC (DAKO, Medesa s.r.o., Polička, Czech Republic) for phosphatidylserine externalisation detection. Flourescent substrate for caspase 3 was used to analyze caspase 3 activation. For the measurement of all parameters a BD FACSCalibur (Becton Dickinson, San Jose, CA, USA) flow cytometer was used.

Wound-healing assay

For the wound healing assay, cells were seeded into silicon adapters in a 60 mm Petri dish. GDF-15 (1000 ng.ml^{-1}) was added either as a pretreatment, 24 hours before HY-PDT, or immediately after HY-PDT as a post-treatment. Hypericin: 0.05 µmol.l^{-1} for HCT-116 and 0.1 or 0.125 µmol.l^{-1} for A549 cells were the concentrations used. The width of the interspace between the two confluent cell layers was monitored 0, 6, 12, 24, 32, 48 and 72 h after HY-PDT by microscopy (Leica DMI6000, Leica Microsystems GmbH, Wetzlar, Germany) and subsequently analyzed by LAS AF Lite 2.6.0 Software.

Statistical analysis

The results were analyzed using a *t*-test comparison and the differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In our experiments, we used GDF-15-rich conditioned media from HCT116 $p53^{+/+}$ cells with normal GDF-15 expression. The conditioned media were added to HCT116 $p53^{-/-}$ cells with impaired GDF-15 expression due to lack of p53 protein, 0, 8 and 24 h after HY-PDT treatment. Cell death parameters of p53-null HCT116 cells were analyzed by flow cytometry. The experimental group treated with the conditioned medium from cells incubated with hypericin, where the highest concentration of GDF-15 in the medium was assumed, showed the highest amount of cells with externalized phosphatidylserine. Also, the percentage of cells with impaired metabolic activity was significantly increased in this group compared to controls and groups treated with conditioned medium and with medium containing hypericin only (Fig. 1). These effects were caused partially by the conditioned medium and partially by hypericin. These factors also caused an increase in cells dying by caspase independent cell death 48 h after HY-PDT.

The MTT test was used as a tool to confirm the role of GDF-15 in the changes of the metabolic activity of cells caused by GDF-15-rich conditioned medium. The results showed a significant decrease of metabolic activity of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells treated with GDF-15 only; these cell lines differed however, in sensitivity to concentration of GDF-15 and the time of treatment. No significant changes of HCT116 cells metabolic activity 24 and 48 h after HY-PDT treatment, between GDF-15 supplemented experimental groups and experimental groups without GDF-15 addition were observed (Fig. 2). A549 cells did not respond to GDF-15 treatment by changes in metabolic activity. However, GDF-15 supplemented cells demonstrated an increased resistance to PDT 24 h after HY-PDT treatment. These cells also show a decreased ability of metabolic activity regeneration 48 h after HY-PDT, compared to GDF-15 untreated group.

The proliferation and migration potential of the cells was observed using the wound-healing assay. Briefly, neither HCT116 nor A549 cells exhibit any significant changes in their ability to enclose the gap between two confluent cell layers treated with GDF-15 only or with HY-PDT combined with GDF-15 pretreatment or co-treatment independently on hypericin concentrations.

Even though the preliminary experiments with conditioned media indicated the presence of signals promoting programmed cell death after HY-PDT, subsequent series of experiments did not support the hypothesis that these effects are caused by the activity of GDF-15. This cytokine inhibits the metabolic activity of HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells and in combination with HY-PDT influences the metabolic activity of A549 cells. The wound-healing assay did not confirm a role for GDF-15 in cell migration and proliferation, whether used alone or in combination with HY-PDT treatment. In these experiments, we did not manage to confirm the hypothesis of GDF-15 influence on PDT efficacy by direct action on the cancer cells. However, the effects of GDF-15 described in various studies can be caused by the cytokine action, not only to cancer cells, but also, the cells of the immune system that are involved in anticancer immune response.

ACKNOWLEDGEMENTS

The study was supported by NEXO (Network of Excellence in Oncology) under contract ITMS 26220120039.

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Selected papers from the Seminar of PhD. students, organized in September 2012 by the UVMP in Košice, devoted to the memory of outstanding veterinary physiologist Academician Boďa.

FOLIA VETERINARIA, 56, 4: 25–30, 2012

FEEDING A FOOD ADDITIVE CONTAINING BETA-GLUCAN AND ITS EFFECTS ON SELECTED IMMUNE AND ENERGY INDICES IN SOWS AND PIGLETS IN A FIELD EXPERIMENT

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ABSTRACT

The aim of this study was to investigate the feeding of a food additive based on beta-glucan (IMUNOL P, Polychem spol. s r. o., Slovakia) to pregnant sows and their suckings. The experimental group received the additive, while the control group did not. The parameters of comparison involved the animal's active non-specific immuno-modulation and various other chosen indices of immunological and energy profiles.

The sows showed no essential differences between the experimental and control groups, except for the immunological profile. However, in the experimental group of sucklings (A) significant changes were found for various indices compared with the control sucklings (B).

Key words: beta-glucan; immunity; swine

INTRODUCTION

Neonatal diseases with high morbidity and mortality are serious problems on animal farms throughout the world. Neonatal, suckling and newly weaned piglets face various challenges including, diarrhoea, low feed intake, and body weight loss. These adverse factors can cause damages to the intestinal health and function; which can influence their whole immune system (11). During the neonatal and suckling periods, the piglets can be passively protected by antibodies, which are present in the milk, but disappear at weaning. Weaned piglets need an active immunity in order to be properly protected against infectious agents. Piglets may gain protection by oral immunisation or vaccination, but at times, these can be insufficient. The use of dietary antibiotic supplementation to improve the growth and feed efficiency of swine may result in bacterial resistance to the antibiotics and their residues may be hazardous to human health. Their use in Europe have been prohibited since 2006 (2, 13).

Therefore, at present, as an alternative to antibiotics, it appears to be possible to use various natural-based supplements with beneficial effects on the immune system to increase the resistance to various infections. It is well known, that beta-glucan, as a natural substance, have potential immune-modulating effects (1). Besides the reported higher weight gain, there are also reports of their stimulating effects on an increased resistance of sucklings against infectious agents. In addition, beta-glucan have beneficial influences on: phagocyte activity of cells; production of reactive forms of oxygen; proliferative activity of lymphocytes; and the expression of specific receptors (2, 3).

To examine some of these factors, we performed an experiment on weaned piglets with the immunomodulation of sows. We anticipated higher trans-colostral transfer of immunoglobulins and enhancement of certain chosen indices in the weaned piglets.

MATERIALS AND METHODS

The experiment conducted on a middle-sized pig farm in the south of Slovakia.

Animals

Twenty sows (crossbreeds of Large White and Landrace) and their piglets (n = 40) were included in the experiment. The sows were homogenously divided into two groups, depending on their intake of beta-glucan – experimental sows (ES), (n = 10), and control sows (CS), (n = 10). The sucklings (two biggest piglets from every litter in both groups of sows) were divided to two groups (A: n = 20; B: n = 20) depending on their intake of beta-glucan by their mothers (Table 1.).

Table 1. Dividing of experimental pigs

Group	Feeding	Sucklings
Experimental sows (ES) n = 10	Feedstuff + Imunol P	(A) n = 20
Control sows (CS) n = 10	Feedstuff	(B) n = 20

The experimental sows were fed feedstuff with an admixture containing beta-glucan (IMUNOL P) in a 5 % concentration (5 kg in 100 kg of feedstuff) from day 14 before parturition, until the weaning of the piglets (day 28). The control sows were fed only standard foodstuff. The composition of Imunol P is presented in Table 2.

Table 2. Composition of the dietary additive IMUNOL P

Compound	Content [% weight]
Beta-1,3/1,6-D-glucan	0.8
Zeolite	38.8
Glycerol	34.0
Silicaoxide	16.7
Water	9.6
$NaCl_2$	9.0
Cholinchloride	$4.5.10^{-3}$
DL-methionine	$0.9.10^{-3}$
$ZnCl_2$	$0.8.10^{-3}$
E-vitamin (alpha tocopherol 100 % weight)	$8.9.10^{-5}$
Beta carotene	$3.8.10^{-5}$
$CoCl_2$. $6H_2O$	$1.8.10^{-5}$
B_3-vitamin (niacin 100 %)	$1.6.10^{-5}$
KI	$0.6.10^{-5}$
Na_2SeO_3. $5H_2O$	$0.4.10^{-5}$

The collections of biological materials from the sows were done three times: 14 days before parturition (sampling 0), during parturition (1st sampling) and during the weaning of the piglets (2nd sampling). Blood was taken by venipuncture from the vena cava cranialis. In sucklings, the samples were collected five times: immediately after birth (sampling 0), 14 days (1st sampling), 28 days (2nd sampling), 35 days (3rd sampling), and 60 days (4th sampling) after birth. Blood was taken by venipuncture from sinusopthalmicus. The blood samples were analysed for the following profiles: immunological (total immunoglobulins – TIg; phagocyte activity – PhA; index of metabolic activity of phagocytes – IMA; sub-populations of lymphocytes: CD4, CD8, CD4CD8, CD3, CD 21, CD4:CD8), and energy (total cholesterol – TCH; and total lipids – TL).

Analysis of samples

The indices of the energy profile from the blood serum were determined by an automatic analyser ALIZE (Lisabio, France) using commercial Randox kits. Phagocyte activity was analysed by a commercial set Phagotest® (ORPEGEN Pharma, Germany) on a flow cytometer BD FACSCanto™ (Becton Dickinson Biosciences, USA) using BD FACS Diva™ software. The tests were conducted according to the manufacturer's instructions, and 10 000 leukocytes were counted for interpretation. The stimulation index of leukocytes (SI) was assessed by a commercial test (Cell Proliferation ELISA Kit, BrdU-colorimetric, Roche) and measured by an ELISA reader (8). The blood serum total immunoglobulins (TIg) were analysed spectrophotometrically by the turbidimetric method with the zinc-sulphate test (12). Identification of sub-populations of lymphocytes in the peripheral blood was performed by flow cytometry. We used a flow cytometer BD FACSCanto™ (Becton Dickinson Biosciences, USA) equipped with BD FACS Diva™ software. Fluorochromes were excited by a blue 488 nm laser. For fluorescence measurement, FITC filter (530/30 nm) and a RPE filter (585/42 nm) were used. The position of lymphocytes was labelled by a dot plot FSC against SSC and consequently their representations evaluated on dot plots FITC against RPE. For testing of the oxidative burst of phagocytes, we used the iodine-nitro-tetrazolium test according to Lokaj and Oburkova (5).

Statistical evaluation

The results of the biochemical and immunologic analyses were evaluated in groups on the basis of their average values (x) and standard deviations (SD). The statistical significance was evaluated by statistic software GraphPad Prism 5, using the non-paired *t*-test and one-way analysis of variance ANOVA (Tukey's multiple comparison test). Statistical significance within groups is marked with letters: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$; and between groups: d = $P < 0.05$; e = $P < 0.01$; f = $P < 0.001$.

RESULTS

Except for the immunological profile, the dynamics of other indices of the examined profiles in the sows showed no significant changes related to immune-modulation. In the experimental group of sucklings (A), significant changes were recorded for various indices compared with the control sucklings (B).

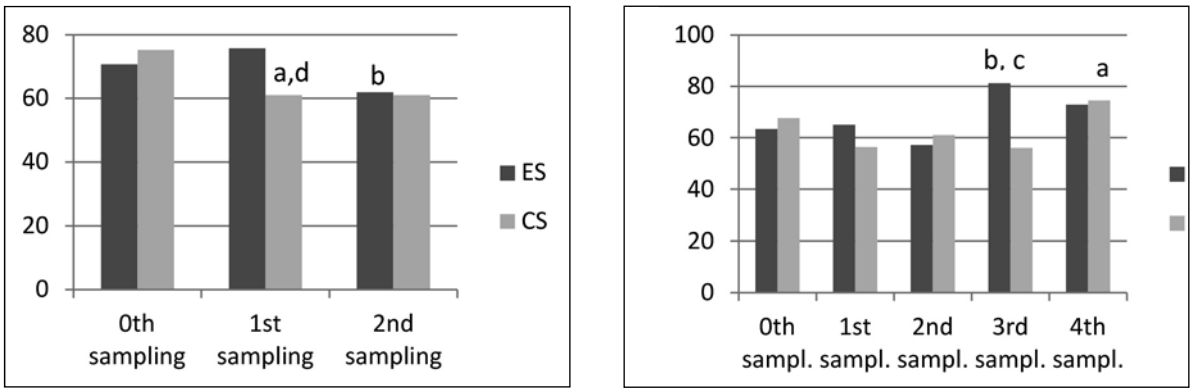

Fig. 1. Phagocyte activity in peripheral blood of sows and piglets

ES – experimental sows; CS – control sows; A: G+; B: G–
a: P < 0.05; b: P < 0.01; c: P < 0.001 – statistical significance within group
d: P < 0.05; e: P < 0.01; f P < 0.001 – statistical significance between groups

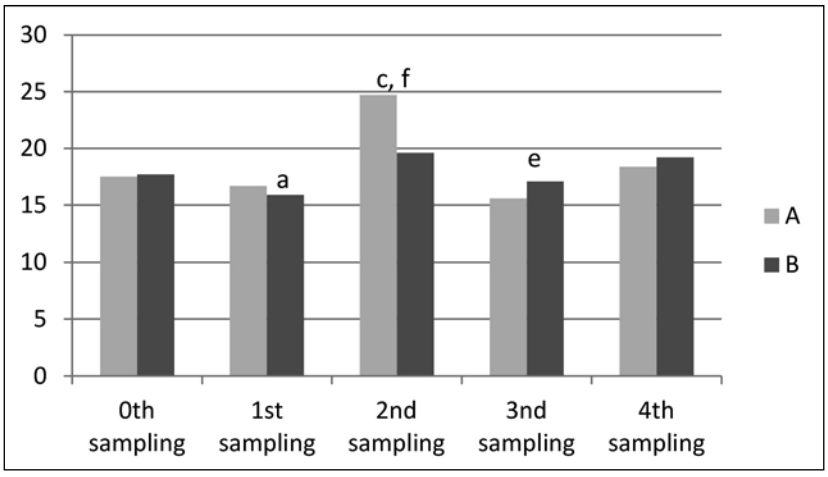

Fig. 2. Dynamics of changes in TIg in piglets

A: G+; B: G–
a: P < 0.05; b: P < 0.01; c: P < 0.001 – statistical significance within group
d: P < 0.05; e: P < 0.01; f: P < 0.001 – statistical significance between groups

An immunomodulating influence of the food additive containing beta-glucan (IMUNOL P) was demonstrated in sows only partially in the dynamics of immunologic profile indices, particularly during the parturition period, when significantly higher ($P < 0.05$) values of phagocyte activity were recorded in the experimental group compared to the controls. This indicated a higher responsiveness and resistance to birth stress. In the experimental group of piglets (A) we recorded significantly higher values of: TIg, FA and IMA at weaning ($P < 0.01$; $P < 0.001$, resp.) in comparison with the control group of piglets (B).

In the experimental sows, we recorded significantly higher (d: $P < 0.05$) phagocytic activity (FA) at the first sampling (Fig. 1.). During the experiment, there was a significant decrease in FA in the control sows at the first (a: $P < 0.05$) and in the experimental sows at the second sampling (b: $P < 0.01$).

Statistically significant differences within tracking of time-dependence in piglets were recorded in groups A (b: $P < 0.01$) and B (a: $P < 0.05$). In group A, there was a significant difference between the zero and third sampling (b: $P < 0.01$) and between the second and third sampling (c: $P < 0.001$), and in group B between the first and fourth sampling (a: $P < 0.05$) and the third and fourth sampling (a: $P < 0.05$).

Throughout the experiment, the levels of TIg in piglets were below the lower limit of the reference range (20–35 UZST); only in group A, at the second sampling were they within the physiological range (Fig. 2.). Significant differences (f: $P < 0.001$; e: $P < 0.01$) were observed between groups A and B at the second and third samplings. The levels

Table 3. Index of metabolic activity of phagocytes in the peripheral blood of piglets expressed as arithmetic mean ± standard deviation

Piglets	Sampling 0	Sampling 1	Sampling 2	Sampling 3	Sampling 4	ANOVA
A	1.28 ± 0.19	1.14 ± 0.06	1.57 ± 0.14	1.61 ± 0.08	1.62 ± 0.15	c : P < 0.001
B	1.14 ± 0.08	1.10 ± 0.07	1.31 ± 0.16	1.32 ± 0.11	1.48 ± 0.07	b: P < 0.01

A: G+; B: G–
a: P < 0.05; b: P < 0.01; c: P < 0.001 – statistical significance within group
d: P < 0.05; e: P < 0.01; f: P < 0.001 – statistical significance between groups

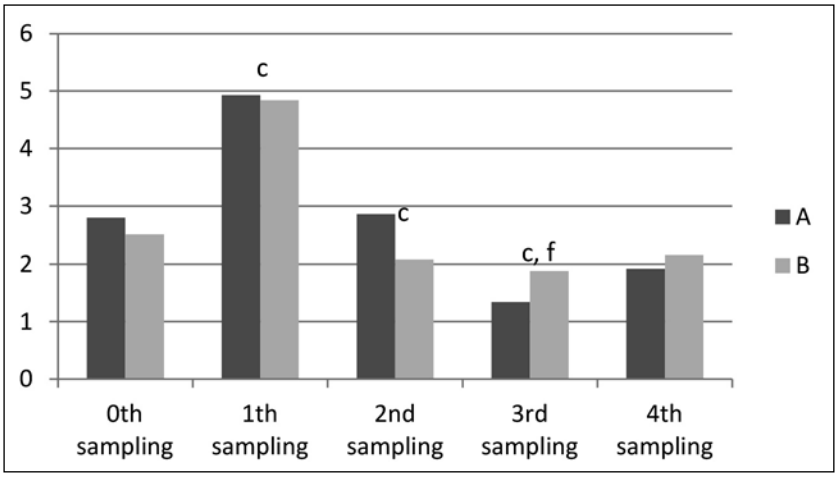

Fig. 3. Dynamics of changes of TCH in piglets

A: G+; B: G–
a: P < 0.05; b: P < 0.01; c: P < 0.001 – statistical significance within group
d: P < 0.05; e: P < 0.01; f: P < 0.001 – statistical significance between groups

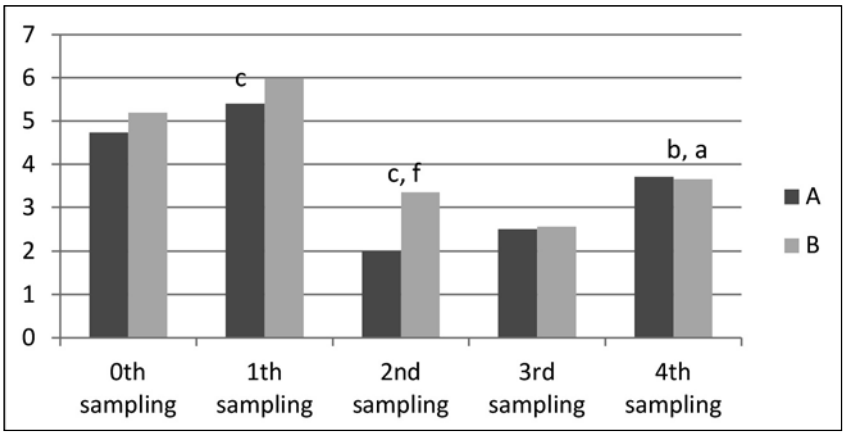

Fig. 4. Dynamics of changes in TL in piglets A: G+; B: G–

a: P < 0.05; b: P < 0.01; c: P < 0.001 – statistical significance within group
d: P < 0.05; e: P < 0.01; f: P < 0.001 – statistical significance between groups

of TIg in the group A were significantly higher (c: $P < 0.001$) at the second compared to other samplings, and in the group B significantly lower (a: $P < 0.05$) at the first, compared to the second sampling.

During the experiment we noticed, in both groups of piglets, a decrease in IMA at the first sampling and a subsequent increase, when significant changes were observed in the group A (c: $P < 0.001$) and B (a: $P < 0.05$) (Table 3.).

The comparison of the results of specific sub-populations of lymphocytes, showed statistically significant differences between the groups only in case of double-positive CD4CD8 lymphocytes. In piglets, we noticed the same favourable trend of changes in the observed sub-populations of lymphocytes in the peripheral blood compared with the control group. Swine possess no IgD and in the differentiation of lymphocytes gene conversions, it was not applied. In the peripheral lymphocyte population, there was a notable number of γδ cells and double-positive $CD4^+CD8^+$ lymphocytes (14), which was also demonstrated in our study.

In both groups of piglets we documented an initial increase in the level of total cholesterol (above reference range 2.6–3.9 $mmol.l^{-1}$) and subsequent decrease (below reference range), when the level of TCH persisted until the end of the experiment (Fig. 3.). A significant difference (f: $P < 0.001$) was found between the group A and control group B at the third sampling. The mentioned increase achieved the same statistic significance in both groups of piglets at the first sampling and a significant decrease at the second sampling (c: $P < 0.001$). A significant decrease in TCH was shown in the group of piglets from immunostimulated sows (A) at the third sampling (c: $P < 0.001$) which indicated anti-lipolytic or a cholesterol-decreasing effect.

An anti-lipolytic effect was also indicated by the level of total lipids (Fig. 4.). At the second sampling, the levels in group A were, by contrast, significantly lower than those in the group B (f: $P < 0.001$). In general, we could observe a continuing significant increase at the first sampling in group A (c: $P < 0.001$), a significant decrease at the second sampling in both groups (c: $P < 0.001$), and a subsequent, significant increase (A – b: $P < 0.01$; B – a: $P < 0.05$) at the fourth sampling, when the TL levels in both groups again reached the lower limit of the reference range (3.5–5.5 $g.l^{-1}$).

DISCUSSION

This study investigated the immunomodulating effects of beta-glucan on a large-scale pig farm, with the aim of achieving non-specific immune stimulation of sows and improving the immune responses of piglets.

Fungi are highly appreciated and consumed for their nutritional and dietetic properties (low caloric value, low fat intake, high level of proteins, minerals, and polysaccharides). Many species of them are used as food additives, especially thanks to the content of polymers in their cell walls (beta-glucan, chitin...), which show beneficial effects in the treatment of various diseases (7, 9). Early studies on the use of beta-glucan in swine were oriented on production indices in an effort to use them as growth-stimulants to replace antibiotic-medicated foodstuffs. Many authors (2, 6, 15) reported relatively positive results, even if some of them provided conflicting data. In addition to higher weight gain, there are also reports of their stimulating effect on: the resistance of sucklings against infectious agents; increased level of serum cholesterol and hepatic lipoprotein with low density; influence on phagocyte activity of cells; production of reactive forms of oxygen; proliferation activity of lymphocytes; and expression of specific receptors (2, 3,7).

Chau *et al.* (4) studied the potential use of beta-glucan as an oral adjuvant in weanlings vaccinated against Haemophilus pleuropneumoniae. The authors suggested the beneficial effects of orally administered beta-glucan, which may improve the piglets' response to vaccination. Oral administration of beta-glucan improved the activity of NKcells and peritoneal macrophages. Animals fed diets with beta-glucan showed an increase in pig lymphocyte subpopulations MHC II, CD4, and CD8 compared with animals fed another diet.

The effects on the lymphocyte subpopulations of the beta-glucan fed to the experimental sows was observed also in our experiment, when the experimental piglets showed significantly higher ($P < 0.01$) proportion of CD4 lymphocyte at the 2nd and 3rd samplings. On the contrary, the percentage of CD8 lymphocytes significantly increased between the 1st and 2nd samplings (both $P < 0.001$) followed by a slight decrease after weaning (3rd and 4th sampling), with a subsequent increase to values comparable with adult animals (30–40%). Shan *et al.* (10) investigated the effects of dietary lactoferrin on immune functions and blood serum concentrations of iron in suckling piglets. The authors concluded that lactoferrin may improve lymphocyte proliferation in the peripheral blood and spleen, increase serum IgG, IgA, IgM, IL-2, and iron concentrations with regulation of immune functions, which may lead to improved growth of weanlings and protection against infections and post-weaning stress.

In conclusion, by comparing the experimental and control groups, our study of selected indices and the effects of beta-glucan feeding to sows confirmed the immunomodulating effects of long-term beta-glucan administration.

ACKNOWLEDGEMENTS

This work was supported by Grant Agency for Science, VEGA of the Slovak Republic, Grant No. 1/0727/09.

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Received September 4, 2012

FOLIA VETERINARIA, 56, 4: 31–35, 2012

INTRA-PARTURIENT UTERINE TORSION IN A BOERBOEL BITCH (A Case Report)

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ABSTRACT

This paper reports a case of intra-parturient uterine torsion (270°) in a 2 year old boerboel bitch (44 kg) that has had two successful whelpings previously. The bitch had whelped two puppies 48 hours prior to presentation, but could not whelp more puppies despite obvious abdominal enlargement, vulva discharges and discomfort. The bitch's physiological parameters were slightly high, presumably due to labor stress, while physical and sonographic examinations revealed a gravid uterus with severely distressed fetuses. Following caesarian section, the right uterine horn was empty and involuting while the left horn was markedly enlarged, congested/discolored, and had twisted counterclockwise by 270 degrees at the base. The torsion was corrected, and 4 dead fetuses were evacuated from the horn. The bitch's recovery was uneventful. The rarity of the condition and the associated aetio-pathogenesis form the objective of this report.

Key words: bitch; rare; torsion; uterine

INTRODUCTION

Uterine torsion occurs when one or both uterine horns twist along the long axis or around the opposite horn (17, 1). It may also occur following the rotation of the entire uterine body (17). It is most frequently associated with late pregnancy and is an uncommon cause of canine dystocia (1, 3, 6, 21). A clockwise or counter clockwise twist of the gravid or non- gravid uterus from 90 to more than 200 degrees has been reported (1, 23, 26). The aetio-pathogenesis of the condition is still poorly understood (15, 25). In the bitch, the occurrence of the disorder is higher in the gravid than non-gravid uterus (12); with unilateral torsion more likely to occur than bilateral (21). Torsion of the uterus is rare in the bitch and queen but common in cows and mares (3, 17, 25). The wide disparity in the occurrence among animal species has been associated with differences in the suspension of the tubular genital tract which affects the "stability" of the gravid uterus (15). Jumping and running in late pregnancy, active fetal movement, premature uterine contraction, partial abortion, variation in the length and mobility of the proper ovarian ligament, and abnormality of the uterus have been linked with the aetio-pathogenesis (12, 17, 23). Uterine torsion is associated with high mortality due to complications such as peritonitis, septicaemia, endotoxaemia and disseminated intravascular coagulation (11, 14, 16, 19). In this paper a report of uterine torsion in a boerboel bitch which occurred during parturition following whelping of two live puppies is reported. The rarity of the condition in bitches and the paucity of information on the aetio-pathogenesis of this case form our objectives for this report.

CASE REPORT

A two and half year old boerboel bitch weighing 44 kg was presented at the Veterinary Teaching Hospital, University of Ibadan, Ibadan, Nigeria (on December 2, 2011) together with two puppies she had whelped 48 hours unaided before the presentation (Fig. 1). The bitch had two successful whelping previously. The history consisted of the inability of the bitch to whelp more puppies despite obvious abdominal enlargement, vulva discharges, and oxytocin medication which was given by the owner. The bitch was bred to a sire of the same breed and of relatively equal size. She started

Fig. 1. Bitch with her puppies before surgery

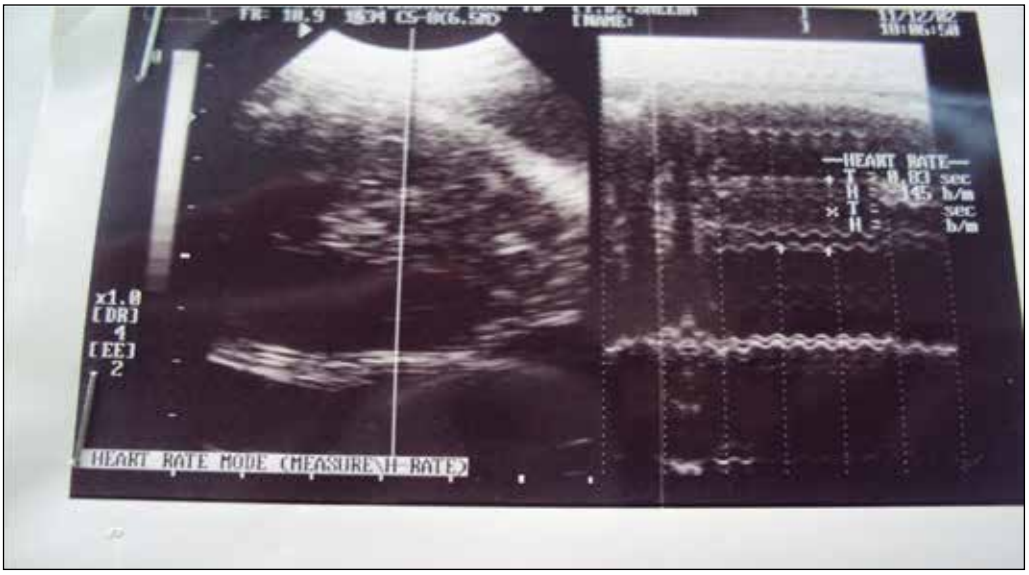

Fig. 2. Ultrasound image of bitch uterus showing fetal heart rate measurement prior to surgery

whelping on the 63rd day of gestation counting from the last day of mating.

Clinical examination

The bitch's temperature was 39.9 °C, heart rate, 147 beats.min^{-1} and respiratory rate was 61 breaths.min^{-1} and she displayed slightly pale mucous membranes. The bitch had a swollen vulva with chocolate brown discharges. She appeared weak, although she could ambulate, and elicited no straining, nor did she strain upon the manipulation of her vagina. The cervix was patent with no fetus presented in the birth canal. Ultrasonography revealed the presence of stressed foetuses in-utero (Fig. 2). The bitch's heamatological parameters (with normal values in brackets) were as follows: packed cell volume (PCV) 20 % (37–55 %); haemoglobin (Hb) 6.6 g.dl^{-1} (12–18 g.dl^{-1}); red blood cells (RBC) 3.31×10^6/µl ($5.5–8.5 \times 10^6$/µl); mean corpuscular volume (MCV) 60 fl (60–77 fl); mean corpuscular haemoglobin concentration (MCHC) 33 g.dl^{-1} (32–36 g.dl^{-1}); platelets 1.27×10^5 µl ($2–9 \times 10^5$ µl); total white blood cells (WBC) 8.1×10^3 µl ($6–17 \times 10^3$ µl); differentials (segmental neutrophils 76 % (60–70 %); band neutrophils 7 % (0–3 %; lymphocytes 9 % (12–30 %); monocytes 5 % (3–10 %); eo-

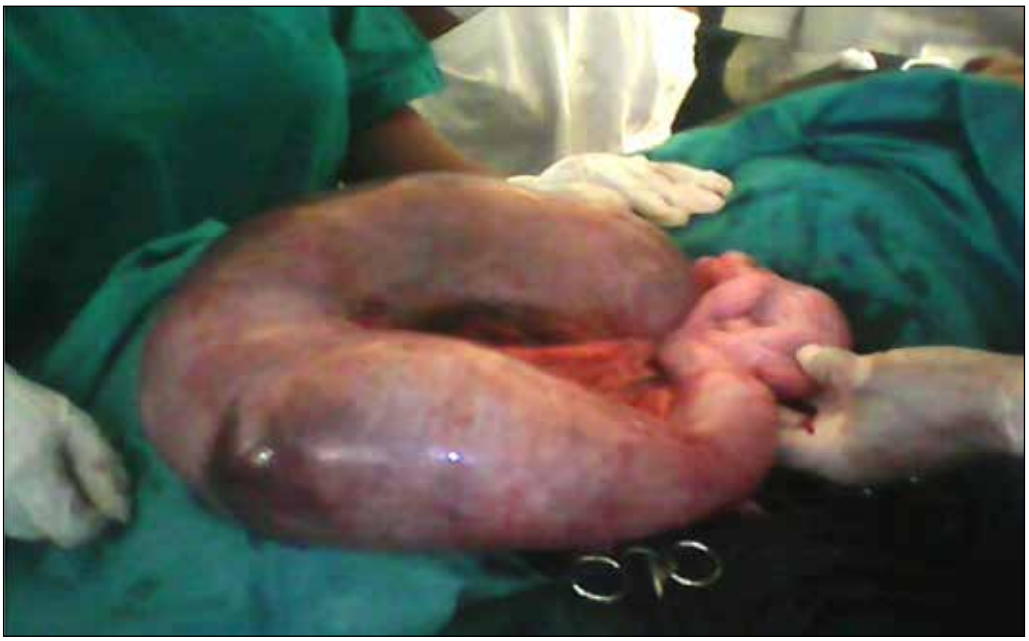

Fig. 3. Torsioned uterine horn

Fig. 4. One of the dead foetuses exteriorized from the uterus following hysterotomy

sinophils 3 % (2–20 %); basophils 0 (rare). No parasite was observed in the blood sample. Based on the result of the clinical examination, a diagnosis of dystocia was given, and an emergency caesarean section was planned.

Surgical procedure

Following premedication with xylazine (0.5 mg.kg^{-1}), and epidural anaesthesia (regional nerve block) with bupivacaine–lignocaine (1 : 1) (Bupivacaine, Alcaine®, Alpha pharmacy, Nigeria; Lignocaine, Glocain, Vital Health Care Limited, India) at a dose rate of 6 ml.kg^{-1}, the ventral abdomen was prepared for aseptic surgery. A ventral midline abdominal incision was made from the umbilicus to the pelvic brim to approach the uterus. Upon exposure of the abdomen, the right uterine horn was markedly enlarged and congested. On exteriorization, the gravid horn was observed to have twisted counterclockwise (270°) at its base (Fig. 3). The horn was untwisted, and four dead fully formed fetuses were evacu-

ated (Fig. 4) *via* the hysterotomy incision as earlier described (7). The laparotomy incision was closed routinely (7). Five hundred mg Ciproflxacin (Ciprotab 500® Medibos Laboratories Limited. J/76 Tarapur, Thane-401506, India) antibiotics was administered *per os* with twice-daily regimen (b.i.d.) for 7 days. Fluid therapy was continued until the bitch could drink and eat. The bitch had an uneventful recovery and stitches were removed ten days post surgery. Follow-up observation showed that the bitch had shown signs of estrus twice since the surgery: at 5 months post surgery and later at 9 months and the owner confirmed that the bitch had been bred and showing possible signs of pregnancy.

DISCUSSION

Uterine torsion is an uncommon cause of dystocia and a life threatening condition in dogs (12, 25). The condition has been reported more frequently in gravid bitches (12) than in non-gravid bitches (13, 14, 18). In non-gravid bitches, it has been reported in association with uterine carcinoma (11), focal adenomyosis of the horn (24), inflammatory endometrial polyp (2) and sometimes without obvious underlying disease (4, 14). In gravid bitches, however, causes are yet to be determined (22). The condition is regarded as a complication of the first or second stage of labour (3, 12, 15).

The bitch under discussion has had two previous successful whelpings without difficulties, and had commenced labour at 63 day of gestation counting from the last mating. It was therefore a complication of labour as earlier reported (3, 12, 15). The condition has been linked with jumping or running in late pregnancy, active foetal movement, premature uterine contraction, and partial abortion (12). Variations in the length and mobility of the proper ovarian ligament and abnormalities of the uterus, lack of foetal fluid and violence such as sudden falls or rolling in late pregnancy has also been associated with the aetiology (12, 17). This case however, lacks evidence of the occurrence of the above mentioned inciting factors. It may be hypothesized that the two whelped puppies were in the empty uterine horn and when they evacuated, they created a gravitational derangement, leading to the torsion of the second horn. Or, that the smaller horn contained only a single pup, and following evacuation of that pup, and one pup from the alternate horn, the torsion occurred at the base of the horn causing the obstruction. In one study where fetuses were marked in-utero, the birth of a pup from one horn was alternated by a next pup from the contra-lateral horn in 78.2% of the births studied (28). This case may have fallen under the 21.8% of the cases, where at least two puppies were evacuated from one horn before alternation, and may be responsible for the gravitational derangement that led to the uterine torsion. However, active fetal movement as well as premature uterine contractions (17) may also be contributory to the aetiology of this case.

The bitch had whelped two puppies before the torsion occurred. The occurrence of uterine torsion as a sequel to the whelping of live puppies has been reported earlier. Geigeenmuller (6) reported a case of a dog that had whelped 4 puppies prior to uterine torsion. The twisted horn retained 2 dead fetuses. Ritt and Fossum (19) also reported a case of torsion in a young dog which contained two retained dead fetuses. In this case, the fetuses may have died from hypoxia following the twisted horn and an occluded blood supply. The placenta of fetuses was friable and weakly attached to the maternal endometrium and the fetuses were bathed in a large volume of endometrial fluid. Sonographic Doppler flowmeter measurement of the fetal heart rate had shown evidence of severely distressed fetuses by a heart rate of 145 beats.min^{-1} before intervention (Fig. 2). Fetuses are considered to be: normal when their heart rate, determined by a Doppler flowmeter, is > 220 beats.min^{-1}; suffering from slight fetal distress when their heart rate is between 180 and 220 beats.min^{-1}; and suffering from severe fetal distress when heart rate was < 180 beats.min^{-1} (29). The 270 degree unilateral cornual torsion results in complete obstruction with severe consequences on the fetuses and the bitch (21). Varying degrees of uterine torsion have been reported in literature. Bilateral uterine torsion with one horn wrapped around the other 180 degrees and the other twisted 180 degrees was reported by Shull *et al.* (21), a case of 180 degree torsion of the left horn and 135 degree torsion of the left ovary has also been reported (9). Three hundred and sixty degree torsion with concurrent pyometra was reported by Misumi *et al.*, (14). Apart from fetal death, heamatometra, endotoxaemia, and peritonitis have been reported as a sequel to uterine torsion especially following a delay in bitch presentation (27). The relatively high neutrophilia shows evidence of bacteraemia and toxaemia. However, the low PCV of the bitch may be associated with pregnancy associated haemodilution, while other physiological data were reflective of a stressed bitch under difficult parturition (5).

Although, oxytocin was administered, uterine rupture did not occur. Oxytocin induced contraction against obstructed uterus have resulted in rupture in cases of torsion (10, 17, 18, 20). Oxytocin therapy is contraindicated in cases of obstructive dystocia (27). The diagnosis of uterine torsion has been reported to be difficult in the bitch, even with good quality imaging techniques, except at laparotomy (15) which may reveal the diagnosis at surgery.

Ovariohysterectomy has been suggested for bitches with uterine torsion (25). Kumru *et al.* (12) reported a case of unilateral en bloc ovario-cornectomy as a treatment for uterine torsion in a bitch with consequent reduced litter sizes. In this case, reperfusion of the affected uterine horn was rapid following correction of the torsion, and precluded the need for ovariohysterectomy (25). Prolong uterine congestion due to torsion could result in development of venous infarction (6, 25), hence a need for quick intervention. The owner reports that the bitch has come into heat twice following the surgery, at the time of this follow up report.

Dystocia in animals is a clinical emergency and requires immediate attention (15, 25), more so when the cause of the dystocia cannot be accurately determined as in the case of uterine torsion. Intra-parturient uterine torsion could occur in the bitch. Survival of dam and intrauterine fetuses, and prevention of attendant complications depends on the time of presentation and intervention by the surgeons.

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Received November 5, 2012

FOLIA VETERINARIA, 56, 4: 36–38, 2012

THE CHANGES IN LACTATE DEHYDROGENASE ACTIVITY DURING RIPENING PROCESS OF KILLED AND SHOT WILD RABBIT'S MEAT

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ABSTRACT

In this study we examined the changes in the total activity of lactate dehydrogenase (LDH) during the ripening process in rabbit's meat. The experiment was carried out on 36 wild rabbits (*Cuniculus oryctolagus*) divided into 2 groups: farmed wild rabbits killed by bleeding (n = 18) and wild rabbits shot during hunting (n = 18). The samples of thigh muscles were analysed spectrophotometrically by measuring their absorbance at the wavelength of 505 nm on days 1, 7 and 14 of storage at 4 °C. The results of our study showed a significant decrease in LDH activity on day 7 ($P \leq 0.05$) and 14 ($P \leq 0.001$) of ripening in shot, as well as killed rabbits. This decrease between days 1 and 14 was more pronounced in the shot (1.25 μkat.l^{-1}) than in the killed rabbits (0.80 μkat.l^{-1}). Our results indicate that changes in the activity of the LDH in rabbit's meat were influenced by both the way of killing and the storage conditions.

Key words: LDH total activity; meat; ripening; wild rabbit

INTRODUCTION

The metabolism of glycogen plays a key role during the muscle-to-meat transition. For this reason it is interesting to know how specific enzymes of the glycolytic pathway change during this process, especially in relation to the *ante mortem* situation (transport of animals, way of killing) as well as *post mortem* situation (time and temperature of meat storage). Lactate dehydrogenase (LDH) is found in all tissues of organisms and consists of a system of 5 tetrameric isoenzymes. The total LDH activity is composed of the activity of all five isoenzymes. The isoenzyme 5 is located in skeletal muscles (9). LDH isoenzymes in tissues are found in different quantities and their occurrence is species specific (10). After the death of animals, glycogen is metabolized by anaerobic glycolysis to lactate which is accompanied with a reduction in the pH value (1). The pH of the meat decreases due to lactate accumulation within the tissues (13). The LDH enzyme participates in the metabolism of glycids by catalysing the reversible conversion of pyruvic acid to lactic acid in the presence of NADH/NAD$^+$ as a coenzyme system (2). For this reason, the cooling of rabbit's meat after slaughter should be fast and long enough for the muscle to be depleted of energy (7). Storage of meat at low temperature, during the ripening process, is important for the development of the major consumption qualities, including flavour and tenderness (11).

The aim of this study was to determine the activity of LDH during the ripening process of farm killed and hunted wild rabbit's meat at 4 °C.

MATERIAL AND METHODS

Experimental design

The experiments were carried out on 36 wild rabbits (*Oryctolagus cuniculus*), divided into 2 groups: the first group consisted of 18 farmed wild rabbits, bred in cages, transported by car from the farm (7 hours) to the place of the experiments at the University of Veterinary Medicine and Pharmacy (UVMP, Košice) and killed by bleeding. The welfare conditions of the animals during transport were regularly checked and appropriately maintained. The second group was composed of 18 wild rabbits, shot during hunting. Un-

skinned carcasses of all slaughtered rabbits were kept at +4 °C in a refrigerator until analysis. Thigh muscles of the rabbits (killed by bleeding (n = 6) and by shot (n = 6)) were taken subsequently on days 1, 7 and 14 after killing by bleeding or hunting and analysed for LDH total activity.

Preparation of tissue homogenates

A 5 g thigh muscle sample in 10 ml Tris-HCl buffer system was homogenized for 5 min and centrifuged (3000 r. p. m.; 10 min). The supernatant was decanted and used for analysis.

Total activity of LDH

The principle of the determination of LDH activity is based on the catalysis of the reversible conversion of pyruvic acid into lactic acid. Pyruvic acid is condensed with 2,4-dinitrophenylhydrazine to form a reddish-brown pyruvate hydrazone, which is then analysed.

The calibration curve was prepared using individual volumes. The standard solution consisted of buffer (50 mmol.l^{-1} Tris-HCl, pH = 8.5), sodium pyruvate (2 mmol.l^{-1}) and 0.5 ml DNPH (2, 4-dinitrophenylhydrazine, 1 mmol.l^{-1} in 1 mol.l^{-1} HCl) and was allowed to stand for 20 minutes at laboratory temperature. Subsequently, NaOH (0.4 mol.l^{-1} NaOH) was added and after 10 minutes the absorbance of the calibration solutions were determined spectrophotometrically (Spekol 11, Carl Zeiss Jena 11, Germany) at a wavelength of 505 nm against a blank.

The total activity of LDH in the meat homogenates was determined as follows: calcium L-lactate hydrate and NAD^+ were added to the samples as well as to the control and the mixtures were heated on a water bath at 37 °C for 5 minutes. The samples were added to the homogenate and incubated for 15 min. Then DNPH was added and the contents were mixed. The homogenate was added to the control sample after 20 min incubation at laboratory temperature. Then 5 ml of NaOH was added to the samples and the control and the contents were mixed. The absorbance of the meat samples were measured spectrophotometrically after 10 minutes waiting time against the control (blank sample). The absorbance of the samples were compared with the calibration curves of LDH standards and evaluated in μkat.l^{-1} (3).

Statistical analysis

The results obtained were analysed statistically using the Student's ***t***-test and correlation coefficients (Microsoft Office Excel 7.0), setting the significance levels at P ≤ 0.001, P ≤ 0.01 and P ≤ 0.05.

RESULTS AND DISCUSSION

The total activity of LDH depends on the collection time of the thigh muscle samples *post mortem*. In the first group of wild rabbits hunted by shooting, the total activity of LDH decreased significantly (P ≤ 0.05; r = 0.427) on day 7 of storage in a refrigerator (Table 1). The lowest total activity of LDH (P ≤ 0.001; r = 0.747) was detected on day 14 in comparison with day 1 of the experiment.

Our results showed that the highest total activities of LDH were recorded 24 hours *post mortem* in both groups of rabbits. During *post mortem*, the glycogen within the muscle tissue is initially metabolized by the aerobic metabolism which is followed by hypoxia and a shift to anaerobic glycolysis. It could be suggested, that the delayed increase in the LDH activity is due to the initial aerobic glycolysis (1). Increased activities of LDH were also observed in other animals (pigs) compared to pre-slaughter time immediately (40 min) after slaughter (5). Similarly, an increase in lactate concentration after slaughter was observed (12).

Table 1. Total activity of LDH in muscles of shot rabbits during ripening process

Day *post mortem*	Total activity of LDH [μkat.l^{-1}]			
	Number	Mean	SD	X_{Max}
1	6	2.50	0.14	2.75
7	6	2.26*	0.13	2.50
14	6	1.25***	0.50	1.80

Significant at * – P ≤ 0.05; *** – P ≤ 0.001
X_{Max} – mean of the maximum values

In the second group of wild rabbits (Table 2) killed by bleeding, the total activity of LDH decreased significantly (P ≤ 0.05; r = 1.95) on day 7 of the experiment. A statistically significant decrease (P ≤ 0.001; r = 0.413) was observed in the total activity of LDH on day 14 compared to the first day of the experiment.

Table 2. Total activity of LDH in muscles of killed rabbits during ripening process

Day *post mortem*	Total activity of LDH [μkat.l^{-1}]			
	Number	Mean	SD	X_{Max}
1	6	2.26	0.10	2.40
7	6	1.91*	0.23	2.10
14	6	1.46***	0.21	1.80

Significant at * – P ≤ 0.05; *** – P ≤ 0.001
X_{Max} – mean of the maximum values

In our study, a decrease in the total LDH activity was observed on day 7 of the experiment in both groups of wild rabbits. *Post mortem* hypoxia would explain the decrease in the complex activity of LDH. The significant decrease in lactic acid, as a product of LDH activity, was observed in the experiment with shot European brown hares (Lepus Europaeus Pallas). The authors observed a significant decrease in lactic acid concentration on days 9 and 16 compared to day 3 of the experiment (4).

The comparison of the two ways of killing of rabbits in our study revealed a significant decrease in the total LDH activity between days 1 and 14 of the ripening process in shot rabbits compared to the killed by bleeding rabbits (difference 1.25; 0.80 μkat.l^{-1}). Significantly higher activities of LDH were observed in shot rabbits on day 1 of the experiment ($P \leq 0.01$; $r = -0.159$) as well as on day 7 of the experiment ($P \leq 0.05$; $r = -0.421$) compared to the killed by bleeding rabbits. The difference on day 14 of the experiment between the two groups was insignificant.

The transport time of the rabbits to the slaughterhouse where the rabbits are killed is a factor which can influence the animal's welfare during road transport and subsequent affect the quality of the meat. Mazzone *et al.* (8) found that the LDH activity in the blood of hybrid commercial rabbits was not influenced by transport and the loading method did not affect the meat quality. In an experiment with wild rabbits (*Oryctolagus cuniculus*), higher levels of lactic acid were observed during ripening in the muscles of hunted wild rabbits compared to transported rabbits (6).

CONCLUSION

The results of our study indicate that the changes in the total activity of LDH in the rabbit's meat were influenced by the way of killing and by chilling conditions during storage. The results showed the importance of the observation of dynamic changes in LDH enzyme activity on the muscle-to-meat transition during the ripening process from the point of view of meat quality.

ACKNOWLEDGEMENT

This study was supported by grant VEGA SR No. 1/0403/08.

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Received December 12, 2012

FOLIA VETERINARIA, 56, 4: 39–47, 2012

USE OF TOTAL INTRAVENOUS ANAESTHESIA (TIVA) IN DOGS (A Review)

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ABSTRACT

There are several different ways to induce general anaesthesia in dogs. One of the modern, but in practice less used, ways is the total intravenous anaesthesia (TIVA). This article is a review of the techniques and the most commonly used drugs along with their effects on the animals. We focused mainly on anaesthetics available in the Slovak Republic and the conventional methods of their administration. The articles reviewed were concerned with the use of intravenously administered anaesthetics in dogs and were searched in ordinary scientific databases and book publications. Depending on the anaesthetic used, this method is effective and safe in terms of cardiovascular stability. Moreover, as opposed to the use of inhalation anaesthesia, it does not result in the contamination of the environment. The equipment needed to perform TIVA is relatively inexpensive and is easy to use. Therefore, we consider this article on TIVA to be of benefit regarding the use of anaesthetics available in our country.

Key words: continuous rate infusion; dog; propofol; total intravenous anaesthesia

TIVA

Total intravenous anaesthesia (TIVA) is a technique of general anaesthesia, in which all of the anaesthetic agents used for the induction and maintenance of anaesthesia are administered only intravenously. This represents an alternative to the use of inhalation anaesthesia (10, 33, 52, 57). In the past, intravenous or injectable anaesthetics were mainly used for the induction of anaesthesia for short durations and interventions requiring longer anaesthesia were maintained by inhalation anaesthetics (17). In recent years, new intravenous anaesthetics and analgesics (e.g. propofol, alfaxalon, fentanyl, remifentanyl) with a rapid onset of action, redistribution and exclusion are becoming available, which help maintain the anaesthesia (5, 57, 64). The ideal anaesthetic must induce a loss of consciousness, amnesia, analgesia and muscle relaxation. Since all these effects can not be induced by a single administration of one anaesthetic, it is necessary to use a combination of anaesthetics. Analgesic and anaesthetic sparing effect of the combined drugs used, reduces the rate of the infusion of the intravenous general anaesthetics (64). This method is called balanced anaesthesia (56, 64). The principle consists of the intravenous administration of drugs with hypnotic, analgesic, and neuromuscular effects in a combination to reach the so called balanced anaesthesia and through the influence of various receptor systems to achieve the surgical stage of general anaesthesia with minimal depression of other organ systems (25). Balanced intravenous anaesthesia can be achieved by the administration of sedatives and analgesics during premedication, as well as using a variety of sedatives, analgesics and anaesthetics during general anaesthesia (57, 64).

The instrumentation for intravenous anaesthesia is less expensive and easily handled (25). The use of intravenous anaesthetics for induction and maintenance of anaesthesia does not require endotracheal intubation, supplemental oxygen or mechanical ventilation. This fact, together with the dangers of exposure to inhalation anaesthetics, explains the increased interest of veterinarians in the total intravenous anaesthesia (33, 64). Intravenous anaesthesia is easier to balance and can provide a good cardiovascular stability. In addition, the selective receptor effect helps to minimizing organ toxicity

(25). When using injectable anaesthesia, its depth of inducement significantly depends on the intensity of surgical stimulation when compared with inhalation anaesthesia. Undisturbed animals can breathe calmly, the chewing and abdominal muscles can be relaxed, which gives an appearance of a deep general anaesthesia. However, surgical stimulation can accelerate and deepen the breathing, causing loss of muscle relaxation and also, reflex movement of limbs may occur, and it may result in a rapid rise in blood pressure and heart rate. These are some of the risks of intravenous anaesthesia. If to deepen the anaesthesia, larger volumes of anaesthetic are administered, then this can cause significant respiratory depression and may lead to a longer period of awakening from anaesthesia (17).

METHODS OF ADMINISTRATION

The easiest way to maintain intravenous anaesthesia is the intermittent manual bolus administration. In this technique, the plasma concentration of an anaesthetic oscillates between peaks (increased incidence of side effects such as hypotension or apnoea) and periods of inadequate anaesthesia. The unstable plasma concentrations of anaesthetics lead to unstable anaesthesia and prolonged recovery times. To maintain such anaesthesia, large amounts of anaesthetics are needed and the risk of side effects is thus increased (8, 51, 67). Intermittent bolus administration is also a time consuming technique and so the anaesthetist has less time for patient monitoring (42).

Another method of maintaining the general anaesthesia is the administration of anaesthetics using a continuous infusion. In this technique, there are no major oscillations in plasma concentrations of the anaesthetic (8, 48). The cheapest, but least accurate method, to ensure such continuous infusion anaesthesia, is the use of the drop infusion set and infusion bottle containing anaesthetics. In this technique, the rate of administration depends on: gravity, diameter and length of the infusion set, the volume of drops, diameter of the intravenous cannula, the viscosity of the infusion, and the height at which the infusion is placed in the context of the patient's venous pressure (8).

For the precise automated intravenous administration of anaesthetics, there are two basic groups of devices. Infusion pumps are used for the continuous administration of larger volumes. Injectomats (syringe drivers) are devices which are filled syringes and are suitable for administration of small volumes (8, 25, 30).

By the form of dosing, we can distinguish two infusion systems. They are pharmacokinetically independent (dose of administered anaesthetic or infusion rate in these systems are controlled manually by the anaesthetist) and pharmacokinetically dependent infusion systems, where a microprocessor controls the anaesthetic dosage based on pharmacokinetic and pharmacodynamic studies (8).

The pharmacokinetically independent infusion systems are called constant rate infusion (CRI) and rate-controlled infusion (RCI) (8). The aim of CRI is to achieve a stable plasma concentration of anaesthetic after the concentration reaches a steady state (11, 25, 44). CRI has several advantages. It allows for the titration of the dose of anaesthetic to the effect, which reduces the amount of anaesthetic used, thus minimizing side effects and also reducing the cost (17, 30, 44). The CRI disadvantage is a slow elevation to the therapeutic plasma concentration level, which can cause the lack of deep anaesthesia (8, 30). However, the bolus administration at the beginning of anaesthesia can provide a rapid initial increase and sufficient plasma concentration of the anaesthetic at the start of the procedure (10, 25). With long-term use, this method increases the plasma concentration of the anaesthetic and side effects may occur associated with the accumulation of the anaesthetic. The accumulation and risk of side effects can be reduced by changing the dose rate according to the needs of the patient. This dosing system is then called RCI (8).

The pharmacokinetically dependent infusion systems are referred to as stepped infusion systems (SIS) and target-controlled infusion systems (TCI). The stepped infusion system is actually a series of several different CRI with well-defined speed and order. The rate of administration is determined based on a software simulation utilizing pharmacokinetic data specific for the anaesthetic (8). The TCI systems are actually computer- algorithm- controlled injectomats able to calculate and change the rate of administration and to maintain a target plasma concentration of the anaesthetic (8, 7, 19, 40, 41). These systems, based on information from studies of population pharmacokinetics and pharmacodynamics, are able to calculate and change the rate of administration according to individual patient's needs after entering the patient's age and weight and the desired plasma concentration of the anaesthetic (6). These systems are also referred to as open loop control systems (the infusion rate is governed by the model and is independent of the actual depth of anaesthesia (15). The accuracy of TCI systems is dependent on the pharmacokinetic variables used in their programming. As a result, they must be validated before clinical use (8). If the infusion rate (input) depends on the measured output (e.g., evoked potentials, bispectral index, blood pressure, heart rate), such systems are referred to as closed loop systems (15).

The use of intravenous anaesthesia in horses (9, 31, 32, 37, 53, 61, 62), cats (23, 24, 34, 45) and other species has been reported.

Drugs used for TIVA

Numerous clinical studies and experiments have been carried out on dogs that focused on the investigation and use of intravenously administered sedatives, analgesics, and anaesthetics. In these studies, anaesthesia was induced without premedication (6, 33, 35, 36, 46, 65, 66), or with different premedications. As premedication, acepromazine (0.01–0.05 mg.kg^{-1} i.m., i.v.) was the most commonly used in combination with various opioids (3, 4, 5, 7, 22, 29, 40, 43, 51, 52, 54, 58, 59,). Other commonly used drugs include -2 agonists (xylazine, medetomidine) (13, 16, 20, 21, 49, 50, 62), and opiates (morphine, fentanyl, remifentanil, pethidine, a.o.). Anticholinergics (atropine) were most frequently used in combination with -2 agonists or opioids.

The most widely used for induction and maintenance of anaesthesia is propofol alone (3, 16, 35, 46, 47, 50) or in combination with ketamine (16, 50), respectively μ-opioid agonists (fentanyl, remifentanyl) (5, 12, 20, 27) or alfaxalon (3, 4, 52). The doses used for premedication, induction and maintenance, are shown in Table 1.

Weaver and Raptopoulos (66) conducted a clinical study to evaluate the induction of anaesthesia with propofol in dogs and cats with different premedication. The incidence of side effects was minimal in this study, the induction was rapid and smooth, and with minimal side effects (tremor and swimming movements of

the limbs). Similar results have been seen by Watkins *et al.* (65), Cullen and Reynoldson (13), Hellebrekers and Sap (20), Huges and Nolan (22), Lerche *et al.* (29) and Aguiar *et al.* (2).

In order to evaluate the cardiovascular effects and quality of the induction after rapid administration of propofol or alfaxalon, Amengual *et al.* (4) performed a prospective randomized blind study of 60 dogs. As a premedication, acepromazine at a dose 0.03 mg.kg^{-1} i.m., and pethidine 3 mg.kg^{-1} i.m. were used. Anaesthesia was induced 30 minutes after premedication with a quick (about 5 seconds) administration of propofol (3 mg.kg^{-1} i.v.) or alfaxalon (1.5 mg.kg^{-1} i.v.) and measurements were performed. Overall, the induction was smooth in most patients. Intubation was easy to perform in both groups. After administration of propofol and alfaxalon, there was a slight decrease in HR (heart rate) and BP (blood pressure). The most common side effect was the post-inductional temporary apnoea (propofol 57 %, alfaxalon 48 %). A high incidence of apnoea (100 %) after induction with propofol (8 mg.kg^{-1} i.v.) and a slight decrease in BP and HR were also recorded by Mohamadnia *et al.* (35) in their randomized cross-over experiment. Apnoea of different durations was the most common side effect also seen by: Morgan and Legge (36), Robertson *et al.* (47), Cullen and Reynoldson (13), Keegan and Greene (26), Reid and Nolan (46), Aguiar *et al.* (2), Murison (38), Adetunji *et al.* (1), Kuusela *et al.* (28), Tusell *et al.* (60), Covey *et al.* (12) and Tsai *et al.* (59). In contrast, Watkins *et al.* (65), Weaver and Raptopoulos (66), Lerche *et al.* (29), Murrel *et al.* (39) did not observed so high an incidence of apnoea, and Thurmon *et al.* (55) observed no apnoea at all. This was probably due to the fact that the induction of propofol had been made slowly and administrated to effect, or a lower dose of propofol may have been used for premedication. This argument also supports one study made by Musk *et al.* (40), in which the incidence of apnoea was directly proportional to the administered dose and rate of administration. Also, in a study carried out by Morgan and Legge (36), the initial occurrence of apnoea in the non-premedicated group of dogs and cats was reduced when the induction was performed by slow administration until it was possible to intubate. There was a low incidence of transient myoclonus, twitching or paddling and pain of injection, after induction with propofol (4, 5, 35, 40, 54, 59, 66). Tsai *et al.* (59) reported a 20 % incidence of side effects after induction of propofol at the dose 1 to 2.5 mg.kg^{-1} administered within 3 seconds and the subsequent slow administration of propofol until intubation was allowed. Neurological symptoms occurred in 14 %, cyanosis in 5 %, and other side effects (vomiting, salivation) in 1 % of the dogs.

Most authors used propofol alone or in combination with other anaesthetics, for example, those with analgesic activity (ketamine, μ-opioid agonist) applied in different ways for maintaining the TIVA in dogs.

Cullen and Reynoldson (13) evaluated the duration of action and cardiopulmonary effects of once administered: propofol (6.55 mg.kg^{-1} i.v.), xylazine (0.8 mg.kg^{-1} i.m.), medetomidine (0.03 mg.kg^{-1} i.m.), xylazine plus propofol (3 mg.kg^{-1}) or medetomidine plus propofol (3 mg.kg^{-1}) in their cross-over experiment in 5 dogs. The results revealed that xylazine and medetomidine premedication prolonged propofol anaesthesia and propofol alone reduces BP, transiently (short-term) increases HR, causing apnoea and hypoxemia, which was most pronounced with the combination of medetomidine with propofol. Time to get to the sternal position after a single administration of propofol in the premedicated group was 22.3 ± 3.35 min, in the group pre-treated with xylazine and medetomidine 54.0 ± 7.23 min or 68.4 ± 6.97 min.

For the comparison of anaesthesia maintained with intermittent bolus every 10–17 minutes (2.5 mg.kg^{-1}), and CRI (0.17 mg.kg^{-1} per min) of propofol in dogs premedicated with xylazine (2 mg.kg^{-1}) and atropine (0.04 mg.kg^{-1}) Adetunji *et al.* (1) compiled a study. In both groups an increase in heart rate occurred during the first 30 minutes of anaesthesia. The respiratory rate and rectal temperature were decreasing progressively during anaesthesia. The time of recovery in the group maintained by intermittent bolus and CRI was 18.6 min or 17.0 min. The incidence of side effects was less common in dogs maintained with CRI.

The technique of intermittent boluses of propofol or ketamine was used for the maintenance of anaesthesia in a clinical trial performed by Hellebrekers *et al.* (21) in 84 dogs. The dogs were categorized as ASA 1-2 (American Society of Anesthesiologist physical classification system) and anaesthesia was carried out due to elective diagnostics and surgical procedures. As the premedication, medetomidine (1 000 μg.m^{-2}, approximately 40 mg.kg^{-1} i.m.) was used. The induction of anaesthesia was performed 15 minutes after premedication with propofol (1–3 mg.kg^{-1} i.v.) or ketamine (2–5 mg.kg^{-1} i.v.) given into effect. Incremental doses of the drugs were administered as required to maintain an adequate depth of anaesthesia. For longer lasting interventions, anaesthetics had to be given more often. The HR was significantly higher ($P < 0.05$) in dogs anaesthetized using ketamine. Other significant physiological differences were not confirmed. The time, from the end of anaesthesia to recovery of the ability to walk, was on average 91 minutes. During maintaining the anaesthesia, there was pronounced higher tonus of the jaw muscles in dogs with ketamine (5 %) compared with propofol (1 %). The recovery was smooth in 89 % of dogs with propofol and 63 % of dogs with ketamine; therefore the incidence of the side effects (catalepsy, convulsion, muscle tremors, vomiting) was more frequent in dogs with ketamine.

Seliškar *et al.* (50) also came to similar conclusions in terms of side effects and the increase in heart rate in dogs anesthetized with propofol and ketamine. In his study, dogs premedicated with medetomidine (40 mg.kg^{-1} i.m. and additional 20 mg.kg^{-1} one hour later), the anaesthesia was induced with propofol (1 mg.kg^{-1}) or propofol (0.5 mg.kg^{-1}) and ketamine (1 mg.kg^{-1}). Anaesthesia was maintained with propofol CRI (0.15 mg.kg^{-1} per min, 70 min) or propofol (0.075 mg.kg^{-1} per min, 70 min) and ketamine (2 mg.kg^{-1} per h, 60 min). Eighty minutes after the induction of anaesthesia, all animals were given atipamezole (66 mg.kg^{-1}). During the maintenance of anaesthesia, the breathing was spontaneous and dogs were given 100 % oxygen. If the value of $EtCO_2$ (end tidal CO_2) exceeded 50 mm Hg for more than 90 sec, intermittent positive pressure ventilation was performed. Both groups developed progressive hypercapnia leading to respiratory acidosis, which was more pronounced in dogs that were anaesthetized with a combination of propofol and ketamine. Males who received propofol or propofol and ketamine were able to walk independently 42.2 min, and 39.2 minutes (respectively) after stopping the infusion of the anaesthetic. The faster recovery in dogs that were given propofol and ketamine to maintain the anaesthesia, may be due to the shorter duration of the infusion

of ketamine (60 min), and also that in this group a lower dose of propofol for the induction and maintenance of anaesthesia was used.

Kussela *et al.* (28) compared the anaesthesia induced with propofol (0.9 to 2.9 mg.kg^{-1}) and maintained for 60 minutes with propofol (0.2 mg.kg^{-1} per min) or propofol with isoflurane (MAC 1% – minimal alveolar concentration), in dexmedetomidine premedicated dogs. During the maintenance of anaesthesia, the heart rate was lower in the group which was administered propofol, than in the group anesthetized with isoflurane. Compared with the preinductional values, the heart rate increased in both groups. $PaCO_2$ (concentration of CO_2 in the blood) and mean arterial pressure (MAP) were higher in the group maintained only with propofol. The CRI of propofol alone in dogs premedicated with dexmedetomidine caused greater respiratory depression and a prolonged recovery. Many other authors used in their studies propofol CRI administration to dogs with different premedication (2, 3, 5, 16, 22, 26, 27, 33, 35, 43, 51, 52, 58, 59, 63). In these studies, anaesthesia was characterized by a good cardiovascular stability or moderate cardiovascular depression. If general anaesthesia was induced and maintained by propofol alone without premedication, the heart rates and blood pressures were declining in 15 to 25 minutes after induction. After this period, the heart rates and blood pressures were stabilized (13, 33). Tsai *et al.* (59) observed decreased heart rates and blood pressures in animals premedicated with acepromazine (0.05 mg.kg^{-1}) or diazepam (0.3 mg.kg^{-1}) after induction and maintenance of anaesthesia with propofol. After 15 minutes, the heart rate stabilized and the blood pressure began to rise slowly. Cullen and Reynoldson (13) studied, after the induction of anaesthesia with propofol (without premedication), the progressively lowering of MAP; the pressure was lowest 20–30 minutes after the induction. A slight temporary increase in the heart rate was seen in the postinductional period, if the animals were premedicated with xylazine or medetomidine and anaesthesia was induced by propofol. After this period, the heart rate slowed and progressively declined (13, 20), or did not change significantly (18, 50). If xylazine or medetomidine were used for premedication, there occurred a temporary slight increase in blood pressure after induction, then the MAP decreased (13). The same changes in blood pressure after premedication with medetomidine and induction and maintenance of anaesthesia with propofol were also seen by Vanio (63). If, after the pre-treatment of medetomidine (0.06 mg.kg^{-1}), the anaesthesia was induced using ketamine (3 mg.kg^{-1}), the heart rate increased for 10 minutes. After 10 minutes, the heart rate was stabilized and blood pressure was decreasing. After induction with fentanyl (2 mg.kg^{-1}), on the contrary, a reduction in the heart rate and blood pressure was reported (20). In dogs premedicated with medetomidine, after induction of anaesthesia combining propofol (0.5 mg.kg^{-1}) and ketamine (1.0 mg.kg^{-1}) and the maintenance using CRI with propofol (0.075 mg.kg^{-1} per min) and ketamine (1 mg.kg^{-1} per h), the heart rate after the start of infusion slowly rose to its zenith. The blood pressure (MAP) rose 5 min after induction, and then did not change significantly (50).

After induction of anaesthesia with propofol without premedication, the respiratory rate decreased 22.8% compared with pre-inductional values. If they were given xylazine or medetomidine before induction, the respiratory rate then decreased by 47.0%, and 35.5% respectively. In dogs with propofol induction, hypoxemia was more pronounced in the group pre-treated with medetomidine than with xylazine (13). The minute tidal volume during propofol anaesthesia decreased, and this decrease was more pronounced when it was administered with ketamine (50). If propofol with ketamine was used for induction, the incidence and duration of apnoea would increase (29). Anaesthesia maintained with propofol or propofol and ketamine leads to respiratory depression, with characteristic changes in the blood gases, but the values of pH, HCO_3 and base excess (BE) remain in the normal range (49, 50). If fentanyl is used alone for the induction and maintenance of anaesthesia, there is a significant risk of respiratory acidosis (20). Respiratory depression occurring during the maintenance of anaesthesia with propofol administered CRI, is directly proportional to the dose and rate of the administration of the anaesthetic (2, 40). Oxygen supported ventilation should be carried out, if the anaesthesia is maintained by the use of CRI with propofol and ketamine, as a result of the respiratory depression occurring during the induction and maintenance of such anaesthesia, (2, 16, 26, 28, 33, 52, 63).

Recovery from anaesthesia was with minimal side effects in most studies (tremor, spasm and swimming limb movements, hypersalivation, vomiting) (2, 47, 2). In a study carried out by Aguiar *et al.* (2), after 60 minutes of infusion with propofol at a dose 0.2, 0.3 and 0.4 mg.kg^{-1} per min, there were observed adverse effects in 10, 20 and 40% of the dogs. At a dose of propofol of 0.4 mg.kg^{-1} per min, a similar incidence of muscle tremor (50 %) was reported also by Robertson *et al.* (47). If the infusion rate of propofol (0.1–0.6 mg.kg^{-1} per min) was adjusted according to responses to surgical stimulations, the incidence of neurological symptoms was 10.3% (58). During the maintenance of anaesthesia with propofol infusion reported by Tsai *et al*. (59), generalized seizure-like muscle spasms in 4.5% was reported in acepromazine or diazepam premedicated dogs. These effects persisted even after increasing the infusion rate of propofol, but disappeared when isoflurane was used for the next maintenance of anaesthesia.

After premedication of acepromazine (0.02 to 0.04 mg.kg^{-1}) there is a possibility to reduce the dose of anaesthetic required for induction by approximately 30% (36, 65, 66). When using xylazine (0.8 mg.kg^{-1}) or medetomidine (0.03 mg.kg^{-1}), the dose of propofol necessary for induction was 50% less (13). The dose of anaesthetic agent needed to maintain the anaesthesia, was reduced by 50% after premedication (36, 65).

The recovery from anaesthesia after premedication with acepromazine was slightly extended. This difference was not significant (65). A slight extension of recovery time was observed by Morgan and Legge (36) and Weaver and Raptopoulos (66) after acepromazine premedication, and was reported also after premedication with tiletamine and zolazepam by Cullen and Reynoldson (14). An extended recovery time (by 58%) after premedication using xylazine or medetomidine was observed by Cullen and Reynoldson (13). Seliškar *et al.* (50) compared the recovery time from anaesthesia maintained with propofol (0.15 mg.kg^{-1} per min) or propofol (0.075 mg.kg^{-1} per min) and ketamine (1.0 mg.kg^{-1} per h) in dogs premedicated with medetomidine. The recovery was slower in the group maintained using propofol alone. However, this could be due to the fact that the dogs in the group which were administered propofol and ketamine generally got about only half the dose of propofol.

Keegan and Greene (26) compared the recovery from anaesthesia maintained with 120 minutes CRI of propofol (0.4 mg.kg^{-1} per min) and inhalation anaesthesia maintained with isoflurane.

Table 1. Overview of medications used in various experimental and clinical studies, and their dosages

Author	Premedication	Induction	Maintenance
Watkins *et al.*, 1987	ACE 0.02–0.04 mg.kg^{-1}, ATR 0.02 mg.kg^{-1}, without	PRO prem. 2.84 mg.kg^{-1}, without 5.18 mg.kg^{-1}	PRObol prem. 0.4 mg.kg.min^{-1}, without 0.8 mg.kg^{-1}
Morgan and Legge, 1989	ACE, ATR, DIA, XYL, without	PRO prem. 4.5 mg.kg^{-1}, without 6.5 mg.kg^{-1}	bol where appropriate, prem. 1.29 mg.kg^{-1}, without 1.53 mg.kg^{-1}
Weaver and Raptopoulos, 1990	ACE, ATR, DIA, PAP, PET, SCO, without	PRO prem. 3.6 mg.kg^{-1}, without 5.2 mg.kg^{-1}	INH
Vainio, 1991	MED 0.04 mg.kg^{-1}	PRO 4 mg.kg^{-1}	PROcri 0.15 mg.kg.min^{-1}
Cullen and Reynoldson, 1993	XYL 0.8 mg.kg^{-1}, MED 0.03 mg.kg^{-1} i.m.	PRO prem. 3.0 mg.kg^{-1}, without 6.5 mg.kg^{-1}	without
Nolan and Reid, 1993	ACE + PAP	PRO 4.0 mg.kg^{-1}	PROcri 0.4–0.6 mg.kg.min^{-1} + N_2O 67 %
Sap and Hellebrekers, 1993	MED 0.03–0.05 mg.kg^{-1} i.m.	PRO 1-2 mg.kg^{-1}	bol where appropriate
Reid and Nolan, 1996	without	PRO 5.0 mg.kg^{-1}	INH
Cullen and Reynoldson, 1997	TIL + ZOL 3 mg.kg^{-1} i.m.	PRO 3.0 mg.kg^{-1}	Without
Hellebrekers *et al.*, 1998	MED 0.04 mg.kg^{-1} i.m.	PRO 1-3 mg.kg^{-1}; KET 2–5 mg.kg^{-1}	bol where appropriate
Huges and Nolan, 1999	ACE 0.05 mg.kg^{-1} i.m. + ATR 0.04 mg.kg^{-1} i.m.	PRO 4.4 mg.kg^{-1} + FEN 2 μg.kg^{-1}	PROcri 0.2–0.4 mg.kg.min^{-1}, FENcri – 0.1–0.5 μg.kg.min^{-1}
Lerche *et al.*, 2000	ACE 0.05 mg.kg^{-1} + PET 3 mg.kg^{-1} i.m.	PRO 4 mg.kg^{-1}; PRO 2 mg.kg^{-1} + KET 2 mg.kg^{-1}	INH
Aguiar *et al.*, 2001	METH 1 mg.kg^{-1} i.v.	PRO 4.5 mg.kg^{-1}	PROcri 0.2–0.4 mg.kg.min^{-1}
Beths *et al.*, 2001	ACE 0.03–0.05 mg.kg^{-1} + MET 0.1 mg.kg^{-1} i.m., or PET 2 mg.kg^{-1} i.m.	PROtci 3.0 μg.ml^{-1}	PROtci 2.5–4.7 μg.ml^{-1}
Murison P.J., 2001	ACE 0.05 mg.kg^{-1} + MOR 0.25 mg.kg^{-1} i.m.	PRO 4.0 mg.kg^{-1}	HAL + N_2O
Adetunji *et al.*, 2002	YL 2.0 mg.kg^{-1} + ATR 0.04 mg.kg^{-1} i.m.	PRO prem 4,0 mg.kg^{-1}, without 6.0 mg.kg^{-1}	PROcri 0.17 mg.kg.min^{-1}
Kuusela *et al.*, 2003	DEX 10 mg.kg^{-1} i.m.	PRO 0.9–2.9 mg.kg^{-1}	PROcri
Musk *et al.*, 2005	ACE 0.03–0.05 mg.kg^{-1} + MOR 0.2 mg.kg^{-1} i.m.	PROtci 2.0–4.0 μg.ml^{-1}	without
Musk and Flaherty, 2007	MOR 0.5 mg.kg^{-1} i.m. + REM 0.2 μg.kg/min^{-1} + PAN 0.05 mg.kg^{-1}	PROtci 3.5 μg.ml^{-1}	PROtci 3.0–3.5 μg.ml^{-1}, REMvri 0.02–0.06 μg/kg/min^{-1}
Tsai *et al.*, 2007	ACE 0.05 mg.kg^{-1} i.m., DIA 0.3 mg.kg^{-1} i.v.	PRO 5.2 mg.kg^{-1}	PROcri 0.1–0.6 mg.kg.min^{-1}; I SO 1.5–3%

Table 1 Continued

Author	Premedication	Induction	Maintenance
Seliškar *et al.*, 2007	MED 0.04 mg.kg^{-1} i.m. + MED 0.02 mg.kg^{-1} 60 min later	PRO 1.0 mg.kg^{-1}; PRO 0.5 mg.kg^{-1} + KET 1.0 mg.kg^{-1}	PROcri 0.15 mg.kg.min^{-1}; PROcri 0.075 mg.kg.min^{-1} + KET 0.03 mg.kg.min^{-1}
Ambros *et al.*, 2008	ACE 0,02 mg.kg^{-1} iv+ HYD 0,05 mg.kg^{-1} i.v.	PRO 4.0 mg.kg^{-1}; ALF 2 mg.kg^{-1}	PROcri 0.25 mg.kg.min^{-1}; ALFcri 0.07 mg.kg.min^{-1}
Mohamadnia et al., 2008	without	PRO 8.0 mg.kg^{-1}	PROcri 0.3 mg.kg.min^{-1}
Tsai *et al.*, 2008	ACE 0.05 mg.kg^{-1} iv max 3 mg, DIA 0.3 mg.kg^{-1} iv	PRO 1.5-2.0 mg.kg^{-1}	PROcri where appropriate
Andreoni and Huges, 2009	ACE 0.03–0.05 mg.kg^{-1} i.m. + ATR 004 mg.kg^{-1} + CAR 4 mg.kg^{-1} s.c.	PRO 4.0 mg.kg^{-1} + FEN 2 μg.kg^{-1}	PROcri 0.3–0.4 mg.kg·min^{-1} + FEN 0.5 μg.kg.min^{-1}
Beier *et al.*, 2009	without	PROtci 5.9 μg.ml^{-1}; PROtci 6.0 μg.ml^{-1} + REMcri 0.3 μg.kg.min^{-1}	PROtci 2.0 μg.ml^{-1}; PRO 0.9 μg.ml^{-1} + REMcri 0.3 μg.kg.min^{-1}
Gasparini *et al.*, 2009	XYL 1 mg.kg^{-1} i.m. + ATR 0.05 mg.kg^{-1} i.m.	PRO 5.0 mg.kg^{-1}; PRO 3.5 mg.kg^{-1} + KET 1 mg.kg^{-1}	PROcri 0.4 mg.kg.min^{-1}; PROcri 0.28 mg.kg.min^{-1} + KETcri 0.06 mg.kg.min^{-1}
Silva *et al.*, 2011	ACE 0.03 mg.kg^{-1} i.m.+ ATRA 0.1 mg.kg^{-1} i.v.	PRO 6 mg.kg^{-1}	PROcri 0.3–1.5 mg.kg.min^{-1}
Amengual *et al.*, 2012	ACE 0.03 mg.kg^{-1} i.m.+ PET 3.0 mg.kg^{-1} i.m.	PRO 3.0 mg.kg^{-1}; ALF 1.5 mg.kg^{-1}	ISO
Mannarino *et al.*, 2012	without	PRO 6.0 mg.kg^{-1}; PRO 6.0 mg.kg^{-1} + LID 1.5 mg.kg^{-1}; PRO 4.0 mg.kg^{-1} + LID 1.5 mg.kg^{-1} + KET 1.0 mg.kg^{-1}	PROcri 0.5 mg.kg.min^{-1}; PROcri 0.41mg.kg.min^{-1} + LIDcri 0.25 mg.kg.min^{-1}; PROcri 0.31 mg.kg.min^{-1} + LIDcri 0.25 mg.kg.min^{-1} + KETcri 0.1 mg.kg.min^{-1}
Suarez *et al.*, 2012	ACE 0.01 mg.kg^{-1} + MOR 0.4 mg.kg^{-1}	PRO 6.0 mg.kg^{-1}; ALF 2.0 mg.kg^{-1}	PRO 0.3-0.5 mg.kg.min^{-1}; ALF 0.1–0.12 mg.kg.min^{-1}

ACE – acepromazine; ALF – alfaxalone; ATRA – atracurium; ATR – atropine; CAR – carprofenum; DIA – diazepam; FEN – fentanyl; HAL – halotan; HYD – hydromorphon; INH – inhalational anaesthesia; ISO – isofluran; KET – ketamin; LID –lidocaine; MED – medetomidine; MET – methadone; METH – methotrimeprezine; MOR – morphine; PAN – pancuronium; PAP – papavertum; PET – ptehidine; PRO – propofol; REM – remifentanil; SEV – sevoflurane; XYL – xylazine; bol – bolus; cri – constant rate infusion; prem. – premedication; tci – target controlled infusion; vri – variable rate infusion

The time since the stop of the supply of anaesthetic to extubation was shorter in the group maintained with isoflurane. Similar result has been reported by Kussela *et al. (*28) and Tsai *et al.* (59). Andreoni and Huges (5), in their study, found that the time to extubation significantly correlated with the total amount of administered propofol, and the time to get to the sternal position, strongly depended upon the duration of anaesthesia, and infusion. A similar effect of anaesthesia, concerning the total dose of propofol on recovery time was also observed by Weaver and Raptopoulos (66).

Dogs without premedication achieved light surgical anaesthesia after induction (5 mg.kg^{-1}) and maintenance of anaesthesia with propofol (0.44 mg.kg^{-1} per min), appropriate for diagnostics and minor surgical procedures (26). According to the results obtained by Mohamadia *et al.* (35), the anaesthesia induced (8 mg.kg^{-1}) and maintained with propofol (0.3 mg.kg^{-1} per min), without premedication, is sufficient to suppress the palpebral reflex and response of the limb to haemostat compression. After premedication with acepromazine and papaverine the anaesthesia was induced with propofol by Nolan and Raid (43). Subsequently, anaesthesia was maintained using CRI of propofol (0.4 mg.kg^{-1}) and inhalation of nitrous oxide. In all cases, this induced and maintained anaesthesia required an increased maintenance dose of propofol (0.6 mg.kg^{-1} per min) in order to achieve surgical anaesthesia. After acepromazine or diazepam premedication and the induction and maintenance of anaesthesia with propofol, this anaesthesia was insufficient to perform diagnostic flushing of the nasal cavity and rhinoscopy. The suc-

cessful performance of this procedure needed to increase the depth of anaesthesia (59). Suarez *et al.* (52) induced and maintained anaesthesia with propofol (induction 5.8 mg.kg^{-1}, maintenance of 0.37 mg.kg^{-1} per min) after premedication with acepromazine (0.01 mg.kg^{-1}) and morphine (0.4 mg.kg^{-1}). This level of anaesthesia was sufficient to perform ovario-hysterectomies. If the premedication with methadone (0.5 mg.kg^{-1}) and atropine (0.05 mg.kg^{-1}) was used, and anaesthesia was induced and maintained with propofol infusion (0.33 mg.kg^{-1} per min) and remifentanil (0.6 μg.kg^{-1} per min), it was sufficient to perform ovario-hysterectomyies (39). After medetomidine premedication (0.04 mg.kg^{-1}) and induction (4.0 mg.kg^{-1}) and maintenance of anaesthesia with propofol (0.15 mg.kg^{-1} per min) the anaesthesia was characterized as surgical anaesthesia, the palpebral reflex was absent and response to finger compression using haemostat was suppressed (63). Thurmon *et al.* (55) also observed the disappearance of the palpebral reflex and depressed response to direct nerve stimulation in medetomidine premedicated dogs during anaesthesia induced and maintained with propofol, and this anaesthesia was characterized as sufficient for surgery. Seliškar *et al.* (50) observed suppression of the palpebral reflex and disappearance of the response to the painful stimuli after the induction and maintenance of anaesthesia with propofol, in medetomidine premedicated dogs. However, the presence of mild palpebral and corneal reflexes caused the expression of doubt about sufficient anaesthesia for major surgical interventions.

CONCLUSION

The main benefit of TIVA is that the instrumentation is cheaper, easily handled and less space occupying when compared with inhalation anaesthesia and there is no contamination of the environment with inhalation anaesthetics. TIVA is characterized by good cardiovascular stability and the depth of anaesthesia can be changed easily as needed. One of the risks of TIVA is respiratory depression, which increases with the increased dose of the administered anaesthetics. Therefore, it is suitable for patients during TIVA to be served oxygen and ensure adequate ventilation. Using the appropriate premedication, the dose of anaesthetics required for the induction and maintenance of anaesthesia can be reduced. If propofol is used for the induction and maintenance of anaesthesia during the entire anaesthesia, there is a minimum of side effects. The risk of side effects increases with the administration rate and decreases if satisfactory sedation is provided before induction. Using ketamine or ketamine with propofol, the incidence of side effects compared with propofol alone is higher. TIVA using fentanyl alone provides adequate analgesia during surgery but the patient may respond to sound stimuli from the environment. Depending on the premedication, the recovery time can be minimal (acepromazine), or significantly prolonged (medetomidine). The repeated administration of a bolus for prolongation of anaesthesia does not provide balanced depth of anaesthesia and leads to delayed recovery. The recovery time is prolonged by the duration of anaesthesia, and is correlated with the dose of the administered anaesthetic. Depending upon the medication used as premedication, the induction and maintenance of anaesthesia using TIVA is suitable for non-invasive diagnostics and minor surgical procedures. It is appropriate, for premedication or during maintenance of anaesthesia, when there is a need to perform an extensive surgery (ovario-hysterectomy, orthopaedic surgery), to administer α-2 agonists (xylazine, medetomidine), opiates or opioids (morphine, fentanyl, remifentanyl), or local anaesthetics (lidocaine, bupivacaine).

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Received December 14, 2012

FOLIA VETERINARIA, 56, 4: 48–54, 2012

ANTIOXIDATIVE ACTIVITY IN LACTATING DAIRY COWS BEFORE AND AFTER THE SUPPLEMENTATION WITH FEED ADDITIVE AV3

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ABSTRACT

In this field study, 12 pluriparous Holstein dairy cows were fed with a commercial feed additive (AV3; Manghebati, France) in the first half of lactation. The product is declared to have, among other things, a positive antioxidative effect on animals. The study consisted of three phases. The first phase (Control 1) took two weeks and included the selection of the study animals and stabilisation of their diet. The second phase (Supplementation) took 58 days and in the course of it all, 12 dairy cows received 6.3–7.0 g of AV3 in their daily total mixed ration. The third phase (Control 2) took 28 days and included the same feed ration but without AV3. At the end of each phase, the mean total antioxidant capacity in the blood of the dairy cows under study was found to be 0.64, 0.68 and 0.63 mmol.l^{-1}, respectively. At the end of the supplementation period, the value was significantly ($P < 0.05$) higher than four weeks after the supplementation period. The blood bilirubin levels dropped significantly during the feeding with AV3 to within the reference range and then (at the end of the phase three) increased significantly (5.3, or 3.2 and 9.3 µmol.l^{-1}; respectively, all values $P < 0.001$). At the end of the supplementation period the animals showed insignificantly ($P > 0.1$) higher mean activity of glutathione peroxidase, which exceeded the minimum reference value (562, or 622 and 552 µkat.l^{-1}, respectively). Feeding with AV3 might also have contributed to a significant ($P < 0.01$) increase in the initially low test milk protein content in milk (30.8 or 32.7 and 33.3 g.l^{-1}, respectively).

Key words: blood chemistry; glutathione peroxidase; Holstein cattle; milk protein; total antioxidant status; total bilirubin

INTRODUCTION

Oxidative stress is a result of the imbalance between the formation of reactive oxygen species (ROS) and the ability of the body to metabolise reactive intermediate products. In ruminants, ROS are responsible for the development of nutritional muscle dystrophy, and their contribution to other diseases is assumed. A number of literature references reported the reduced incidence of mastitis, metritis, retained placenta or abomasal displacement following an increased supply of antioxidants (5, 15). Antioxidants are able to bind free oxygen radicals, thereby preventing oxidative damage to the target cell, or even eliminate free radicals and reduce the risk of oxidative stress. In ruminants, the most important exogenous sources of antioxidants such as vitamins, carotinoids, tannins, poly-phenols and compounds of some trace elements include good quality plant origin feeds, especially young alfalfa and whole crop cereals.

In high-producing dairy herds, high milk production is often accompanied with increased metabolic stress in lactating cows (18). This is caused by stressors of different intensities and lengths such as; poor housing, heat stress, poor feed quality and diet formulation, development of metabolic disorders, etc. The cows are exposed to the greatest stress during the transition period, when the effects of a number of stressors accumulate around calving time and may result in oxidative stress (1).

Lactating cows have their own antioxidative system, which can eliminate oxidative stress to a certain extent. The system consists of antioxidative enzymes (such as superoxide dismutase, catalase, peroxidases) and antioxidant substrates, either of lipophilic (such as vitamins A, E) or hydrophilic (such as vitamin C, glutathione, thiols, flavonoids, uric acid) nature.

In periods of increased stress, the feed ration should include components with sufficient contents of substances with antioxidative effects. These components include a wide range of natural substances (vitamins A, C, E, ß-carotene, lycopene, coenzyme Q10, rutine, resveratrol, selenium, zinc, manganese and copper compounds, etc.), which in addition to common high-quality feeds, are contained in large proportions, for example, in wild thyme (Thymus serpyllum), common thyme (Thymus vulgaris), mint, marjoram, garlic, rosemary and other herbs. Extracts from these plants can be applied in the original state but mostly they are used in combination with a suitable liquid, powder or gel carrier.

We investigated the effects of a feed additive. The manufacturer of this product (Manghebati, France) declares: positive effect on the immune system; improvement of reproduction parameters; increased dry matter intake with increase of daily milk yield in dairy cows; and reduction of oxidative stress.

The indicators of antioxidative capacity in the body include the activity of glutathione peroxidase (GSH-Px) and the total antioxidant status (TAS).

The aim of our trial was to verify the effects of the feed additive AV3 on antioxidant indicators and other blood chemistry parameters in Holstein dairy cows under field conditions in the Czech Republic.

MATERIALS AND METHODS

Animals

The trial was performed in a herd of 110 Holstein dairy cows with a mean milk yield of 8 700 kg during the standardised lactation (305 days). The cows were housed in a free stall barn, with straw bedding. The test population included 12 clinically healthy pluriparous dairy cows on their second to fifth lactation, with the mean parity 3.25. The selected animals were at least one week after the calving and at the same time with the lowest possible number of days in milk. At the beginning of the trial, the cows were 55 days in milk (DIM) on average (from seven to 92 DIM). The health status of the herd was stabilised, with the common incidences of metabolic and infectious diseases.

Trial progress

The tested feed additive AV3 was manufactured by Manghebati (France). It contained, among other things, extract from milk thistle (Silybum marianum) and Ginkgo biloba, with a silica powder carrier and no selenium content.

The experiment was realised using the single case reversal (ABA) design allowing the usage of small number of subjects without control group for the evaluation of the treatment response (8, 20). The model is commonly used for research in the fields of pharmacotherapy, psychology and behaviour of people and animals.

The trial started in the spring of 2011, took 100 days and was divided into three phases. In phase one, called Control 1 (C1; March) the selected dairy cows were stabilised for two weeks by feeding a fixed total mixed ration with the composition shown in Tables 1 and 2. Immediately afterwards the second phase followed, called Supplementation (SUPL; April and May) and lasting 58 days. In the course of this phase, the trial animals were fed with AV3 included in the standard diet given to the cows. The daily dose of AV3 per cow was 6.3–7.0 $g.day^{-1}$ on average (depending on the assumed ration intake affected by the milk yield and DIM). This was the minimum dose according to the instructions by the manufacturer. The third phase, called Control 2 (C2; June) and lasting 28 days, focused on the assessment of the condition of the trial animals after removal of AV3 from their diet. On the last day of each phase non-heparinised and heparinised blood was taken by puncture of vena coccygea and within the regular monthly milk testing test-day, milk production and milk fat and protein percentages were measured.

Dairy cow nutrition and feeding

The diet given as total mixed ration (TMR) was optimised for a standard dairy cow (600 kg) with a mean daily milk yield of 35 litres (fat = 4.0 %, protein = 3.4 %) and was fixed during the trial. The cows were presented feed twice daily and the feed was pushed up four times a day. The animals had *ad libitum* access to feed and drinking water all day long. The assumed intake of fresh matter of TMR components and their proportions (on dry matter basis) in the course of the trial are shown in Table 1. The composition of the complementary feed is given in Table 2. The experiment was assessed from the viewpoint of the need and intake of net energy of lactation (NEL) and metabolised protein (MP) for each trial phase separately according to the National research council (7).

Laboratory examination

The non-heparinised blood was used to obtain serum samples. Samples of serum were used to examine TAS and concentrations of the total protein, albumin, urea, total bilirubin and activities of aspartate aminotransferase (AST) and gama-glutamyl transferase (GGT). In the whole heparinised blood, the activity of the glutathione peroxidase (GSH-Px) was determined.

Laboratory analyses of all the samples were performed in the clinical biochemical laboratories at the Faculty of Veterinary Medicine of the Veterinary and Pharmaceutical University in Brno. The biochemical parameters were measured by the automatic analysers LIASYS (Analyser Medical systems S. r. l., Italy) and Konelab 20i (Thermo Scientific, Finland): TAS (Total Antioxidant Status, Cat. No. NX 2 332) – Randox Laboratories Ltd. (United Kingdom); total protein (^{L}Protein total, Cat. No. 12 751), albumin (L albumin colorimetric, Cat. No. 10 001), AST (^{L}AST, Cat. No. 10 351), urea (UREA L 250, Cat. No. 10 003 308) – BioVendor, Laboratorní medicína, a. s. (Czech Republic); total bilirubin (BIL 100, Cat. No. 10 003 138), GMT (GMT KIN 100, Cat. No. 10 003 208) – Erba Lachema s. r. o. (Czech Republic).

The TAS is specified by comparison of the antioxidant capacity of the sample with a standard in the form of an analogue of vitamin E dissolved in water (Trolox) and expressed by the corresponding quantity of the substance of the analogue in volumetric units ($mmol.l^{-1}$). The principle of the method of TAS determination is based on the inhibition of the artificially produced blue-green cation $ABTS^{+}$ by the antioxidants of the measured sample. The antioxidant concentration is then directly proportional to the change of the colour of the sample.

The GSH-Px was determined from full blood by Paglia and Valentine method (9) with use of the Ransel-Randox set (Ransel glutathione peroxidase, Cat. No. RS 505) – Randox Laboratories Ltd. (United Kingdom) on an automatic biochemical analyzer LIASYS (Analyser Medical systems S. r. l., Italy).

Table 1. Composition of total mixed rations (TMR) for lactating dairy cows (mean daily intake of 48.2 kg of original mass with 43.7 % of dry matter per cow)

Components of TMR	% of original mass	% of dry matter
Corn silage	33.6	24.3
Grass silage	18.7	16.2
Sugar pulp	18.7	6.7
Meadow hay	1.9	3.4
Corn grain	7.5	13.6
Beet molasses	1.3	2.1
DOVP BK[1]	16.4	30.5
Meadow hay (outside TMR)	1.9	3.2

[1] – DOVP BK – complementary feed for lactating dairy cows, for composition see Table 2

Table 2. Composition of complementary feed (DOVP BK) for lactating dairy cows (mean daily intake of 7.1 kg of original mass with 90.2 % of dry matter per cow)

Component	% of original mass	% of dry matter
Wheat	17.2	17.03
Triticale	10.0	9.66
Wheat bran	7.0	6.98
Flax seed	2.0	2.06
Rapeseed meal	20.0	19.65
Soybean meal	20.0	19.82
DDGS	16.0	16.02
Limestone	1.1	1.21
Salt	0.7	0.75
Soda	2.0	2.29
Magnesium oxide	0.4	0.46
Calprosan[1]	3.6	4.06
AV3[2]	0.094	0.095

[1] – mineral and vitamin supplement
[2] – commercial feed additive included in the supplementation phase (April and May 2011)

Table 3. Predicted dry matter intake, calculated requirements and intakes of net energy of lactation (NEL) and metabolizable protein (MP) in 12 dairy cows in the control 1 (C1), supplementation (SUPL) and control 2 (C2) phases (values in brackets = requirement fulfilment in %)

Indicator	Phase C1	Phase SUPL	Phase C2
Days in milk [day]	68.8	126.8	154.8
Dry matter intake [kg.day^{-1}]	21.8	21.7	19.7
Requirement of NEL [MJ.day^{-1}]	149.0	134.7	118.0
Intake of NEL [MJ.day^{-1}]	143.5 (96 %)	143.1 (106 %)	131.8 (112 %)
Requirement of MP [g.day^{-1}]	2 406	2 232	1 939
Intake of MP [g.day^{-1}]	2 311 (96 %)	2 301 (103 %)	2 077 (107 %)

Table 4. Daily milk yield, milk fat and protein levels for the control 1 (C1), supplementation (SUPL) and control 2 (C2) phases (means ± standard deviations)

Parameter	C1 (n = 12)	SUPL (n = 12)	C2 (n = 12)	P-value		
				C1 *vs.* SUPL	SUPL *vs.* C2	C1 *vs.* C2
Days in milk [days]	68.8 ± 11.5	126.8 ± 11.5	155.8 ± 11.5	–	–	–
Daily milk yield [kg.day^{-1}]	37.9 ± 2.6^{c}	31.9 ± 2.5^{b}	26.4 ± 2.0^{a}	**0.001**	**0.002**	**< 0.001**
Fat [g.kg^{-1}]	33.4 ± 2.2	34.5 ± 1.4	33.5 ± 1.4	0.538	0.161	0.970
Protein [g.kg^{-1}]	30.8 ± 0.7^{a}	32.7 ± 0.5^{b}	33.3 ± 0.5^{b}	**0.006**	0.149	**0.001**

^{a,b,c} – Values in the lines marked with different superscripts differ significantly ($P < 0.05$)

Statistical methods

The means and standard deviations were calculated from the raw data. Means of the recorded parameters between individual periods (C1, SUPL, C2) were compared using the GLM repeated measures procedure and Fisher's least significant difference (LSD) post hoc test of Systat 11.00 statistical software package.

RESULTS AND DISCUSSION

The predicted dry matter intake with the corresponding intake of NEL and MP at the end of the individual trial phases is shown in Table 3. In phases C1 and SUPL the NEL and MP needs were covered sufficiently regarding the actual daily milk yield found (37.9; 31.9 kg), because the TMR composition was optimised for comparable milk yields (35 kg). The National research council system bases dry matter intake prediction on days in milk (7), which approximates the predicted values to the actually lower intakes found at the beginning of lactation. As a consequence of the lower milk yield at the end of phase 3 (26.4 kg) NEL and MP intake with the same TMR composition was slightly over the framework of need.

The mean daily milk yield and protein and fat content measured at the end of each phase are shown in Table 4. The significant drop of milk yield in the individual trial phases ($P < 0.01$) corresponded to the lactation curve. At the end of the supplementation phase the low baseline concentration of milk protein increased significantly from 30.8 to 32.7 g.l^{-1} and the increased levels of milk protein (33.3 g.l^{-1}) were also measured in phase C2. The increased milk protein level might have been connected with the increasing saturation of the dietary energy requirements as documented by other trials (22). This value, in relation to the decreasing milk yield, increased moderately and slightly exceeded the requirements both in the supplementation and C2 phases (Table 3). Regarding the very low baseline milk protein level (30.8 g.l^{-1}) in comparison to the desirable minimum of 32 g.l^{-1}, it is rather necessary to consider the existence of an imbalanced fermentation processes in the rumen at the beginning of the trial (probably related to insufficient synthesis of microbial protein, or lack of readily available energy in the ration). The improvement of the microbial digestion may have been caused, among other things, by the effects of the trial product, which, according to its manufacturer, is designed to optimise the fermentation processes in the rumen. No significant

Table 5. Blood chemistry parameters of dairy cows in the control 1 (C1), supplementation (SUPL) and control 2 (C2) phases (means ± standard deviations)

Parameter	Reference range[1]	C1 (n = 12)	SUPL (n = 12)	C2 (n = 12)	P value		
					C1 vs. SUPL	SUPL vs. C2	C1 vs. C2
GSH-Px [μkat.l^{-1}]	over 600[2]	562 ± 20	622 ± 22	552 ± 22	0.126	0.104	0.609
TAS [mmol.l^{-1}]	–	0.64 ± 0.02	0.68 ± 0.02^{b}	0.63 ± 0.02^{a}	0.155	**0.045**	0.721
Total bilirubin [μmol.l^{-1}]	0.17–5.13	5.3 ± 0.4^{b}	3.2 ± 0.3^{a}	9.3 ± 0.3^{c}	**< 0.001**	< 0.001	< 0.001
AST [μkat.l^{-1}]	0.72–1.41	1.81 ± 0.17	1.79 ± 0.20	1.82 ± 0.13	0.932	0.827	0.961
GGT [μkat.l^{-1}]	0.14–0.55	0.54± 0.04	0.57 ± 0.04	0.61 ± 0.04	0.523	0.138	0.216
Urea [mmol.l^{-1}]	3.0–5.0	4.7 ± 0.2^{a}	5.5 ± 0.2^{b}	6.2 ± 0.2^{b}	**0.013**	0.069	**< 0.001**
Total protein [g.l^{-1}]	60.0–80.0[3]	85.9 ± 2.0^{b}	78.7 ± 2.4^{a}	80.0 ± 2.4^{a}	**0.001**	0.565	**0.013**
Albumin [g.l^{-1}]	30.0–42.0	35.1 ± 0.8^{b}	30.5 ± 0.6^{a}	29.6 ± 0.8^{a}	**<0.001**	0.298	**<0.001**

GSH-Px – glutathione peroxidise; TAS – total antioxidant status; AST – aspartate aminotransferase; GGT – gamaglutamyl transferase;
[1] – according to Pechová and Pavlata (12) except; [2] – according to Pavlata *et al.* (11); [3] – Stöber and Gründer (17);
[a,b,c] – Values in the lines marked with different superscripts differ significantly (P < 0.05)

changes of fat levels in the milk were observed in the course of the trial. The overall fat levels were relatively low, not reaching the breed standard of 38 g. l^{-1}. In the case of Holstein dairy cows, low fat content in milk in peak lactation may have been caused by the changed proportions of volatile fatty acids in the rumen due to the relatively higher proportion of concentrate and genetic dispositions.

The results of blood chemistry analyses are shown in Table 5. The antioxidant capacity of the dairy cow organism was monitored through activity of the glutathione peroxidase and total antioxidant status. The other parameters were selected mainly to assess the inner environment of the cow, metabolic activity and condition of the liver parenchyma regardless of their relationship to antioxidant capacity.

GSH-Px belongs to a wide spectre of intracellular enzymatic antioxidants. In the second stage of the response to the activity of the reactive oxygen species, GSH-Px catalyses the reduction of hydrogen peroxide produced in the first stage by the superoxide dismutase activity. The active centre of GSH-Px, includes the aminoacid selenocysteine and therefore it is also an indicator of selenium status of the animal. As in a number of recent publications, GSH-Px activity is expressed in the old enzymatic units (U.mg^{-1}), related to the weight unit of haemoglobin or total protein; it is difficult to compare the absolute values in μkat.l^{-1} measured by ourselves to other published results.

The mean values of GSH-Px activity (Table 5) measured at the end of each trial phase (562, 622 and 552 μkat.l^{-1}, respectively) fall within the wide range of values (23–1557 μkat.l^{-1}) obtained in the evaluation of selenium levels in dairy cows in the Czech Republic (10). The results correlate with the results (560–613 μkat.l^{-1}) published by Pechová *et al.* (13) and are relatively lower in comparison to the values (656–721 μkat.l^{-1}) published by Šustala *et al.* (19), both representing field experiments in dairy cows in the Czech Republic. The activities of GSH-Px observed in this experiment and recalculated for the purpose of comparison to the original units, amount to 0.39–0.48 U.mg^{-1} TP and fall within the interval 0.34–0.59 U.mg^{-1} TP published by Vázquez-Anón *et al.* (21). The activity of GSH-Px in phase C1 was below the limit of 600 μkat.l^{-1}, which was specified under our conditions as minimal with regard to the evaluation of the selenium status of the animals (11). Such a low activity level may, in relation to the assumed sufficient selenium supply, reveal increased oxidative stress probably related to metabolic stress occurring in the transition and fresh periods (1, 2). At the end of the SUPL phase, unlike the first control phase C1, the mean activity of GSH-Px already was above the reference, or the recommended value; although, this increase (from 562 to 622 μkat.l^{-1}) was not statistically significant. In the control phase C2 (after discontinuation of the trial product) there was a certain trend towards a decrease in GSH-Px

activity (from 622 to 552 μkat.l^{-1}; P = 0.1039). The numerically confirmed increased level of GSH-Px activity at the end of the supplementation phase might have been related to the positive effects of the tested product (AV3). As AV3 does not contain selenium and therefore the increased GSH-Px activity could not be caused by its increased synthesis, it may be assumed that the external supply of antioxidants by AV3, inducing the relatively good total antioxidant status at the end of the SUPL phase (see the TAS parameter discussed below), might have led to a reduced consumption of GSH-Px.

There is a wide range of antioxidants acting against oxidative stress in the body with complex mutual interactions between them and with frequent synergistic effects. The direct measurement of antioxidant levels to define the antioxidant capacity of the body is demanding and does not necessarily yield objective results. For these reasons, there is a method of specification of the cumulative effect of all these antioxidants and its expression with a single parameter called total antioxidant status – TAS (3).

The mean values of TAS (0.63–0.68 mmol.l^{-1}) observed in this field trial (Table 5) partly approximate the values of 0.84–1.13 and 0.82–1.02 mmol.l^{-1}published by Mandebvu *et al.* (6) and Pechová *et al.* (13), respectively. They are less than half of the TAS values 1.62–1.64 and 1.89–1.92 mmol.l^{-1} found in dairy cows by Souza *et al.* (16) and Gobert *et al.* (4), respectively. These absolute differences in the measured TAS concentrations between our results and the results obtained by the quoted authors are probably due to a different biological medium used for the analysis (serum versus plasma), and further, possibly also due to a different composition of TMR which contained components with higher natural levels of antioxidants [Souza *et al.* (16) included citrus pulp in TMR]. The TAS values in dairy cows lower than ours (0.07–0.42 mmol.l^{-1}) were published by Vázquez-Anón *et al.* (21). These low levels may be justified by the number of lactation days of the trial animals (2–6 weeks) because, at the beginning of lactation the oxidative or metabolic stress was demonstrated (1, 2, 14). Even though the mean TAS value at the end of the supplementation period was numerically higher (0.68 mmol.l^{-1}) than at the end of the preparatory phase (0.64 mmol.l^{-1}), the increase was not statistically significant. At the end of phase C2, i.e. four weeks after discontinuation of the trial product, a statistically significant (P < 0.05) decrease of TAS values in comparison to the end of the supplementation phase (from 0.68 to 0.63 mmol.l^{-1}) was found. The TAS decrease in C2 might have been connected with the reduced supply of antioxidants by TMR due to the discontinuation of AV3. As in the phase C2, the actual (increased) activity of substances with negative effects on the liver parenchyma, probably manifested itself, as suggested by the analysed total bilirubin levels. The TAS decrease may also have been caused by the increased consumption of antioxidants. Therefore, it cannot be concluded that the supply of AV3 significantly affected the total antioxidant capacity. A statistically significantly positive effect of addition of another antioxidant in the ration on the TAS parameter was proved by the results of an experiment in dairy cows published by Vázquez-Anón *et al.* (21). Pechová *et al.* (13) reported a statistically significant effect of the bypass form of fat and overall higher fat content in the diet of dairy cows (accompanied with higher levels of vitamin E in the blood) on TAS increase at the end of the second and the third month of lactation.

A statistically significant difference in the level of total bilirubin was also found at the end of each trial phase. Although this indicator also represents metabolites with antioxidant effect, it mainly reflects the hepatic condition and function. As shown in Table 5, at the end of the supplementation phase, the total bilirubin concentrations were highly significantly reduced (P < 0.001) from 5.3 to 3.2 μmol.l^{-1} in comparison to the first control phase C1. It may therefore be assumed that the trial product favourably affected liver function due to the hepatoprotective effects of silymarin contained in milk thistle, whose extract is included in the tested product. Another positive aspect of inclusion of AV3 in the diet of dairy cows is the reduced concentration of bilirubin fully falling within the reference range of 0.17–5.13 μmol.l^{-1} (12). In the second control stage C2 (after the discontinuation of trial product), the bilirubin concentration increased (P < 0.001), with its level (9.3 μmol.l^{-1}) being higher in this phase (P < 0.001) than the value of the initial control phase C1. Thus, the high concentration of total bilirubin is far above the upper reference value and probably reflects hepatic insufficiency caused by the actual effects of substances damaging the liver parenchyma.

The level of response of the other biochemical parameters shown in Table 5 on supplementation of the ration with AV3 cannot be specified unambiguously. Blood urea levels at the end of SUPL and C2 phases (5.5 and 6.2 mmol.l^{-1}, respectively), were significantly higher than at the end of phase C1 (4.7 mmol.l^{-1}). The values exceed the reference range (3.0–5.0 mmol.l^{-1}) according to Pechová and Pavlata (12), which usually reflects the relative or absolute excess of degradable crude protein in the diet. This was actually achieved, as shown in Table 3. Especially in the phase C2 (regarding the changes TAS and total bilirubin parameters) acute increase of stress can, in addition, be considered, accompanied by the possible activity of the stress hormone cortisol in the trial animals, which stimulates urea synthesis.

AST activity (1.79–1.82 μkat.l^{-1}) did not significantly fluctuate during the trial and was constantly above the reference values specified for the national conditions, i.e. 0.72–1.41 μkat.l^{-1} (12). The increased AST activity may be related to long-term stress, or moderate damage of hepatocytes, which is confirmed in the SUPL as well as C2 phases by the concurrent activity of GGT (0.57, or 0.61 μkat.l^{-1}), also slightly exceeding the upper reference limit of 0.55 μkat.l^{-1} (12).

The total protein and albumin concentrations showed significantly higher levels at the end of phase C1, where the total protein even exceeded the upper reference limit of 80 g.l^{-1} (17). Regarding the physiological concentrations of albumin in this sample, the exceeded limit of the total protein concentrations can be explained by the increased proportion of the globulin fraction (probably caused by past inflammations still manifested in some of the trial animals). The total protein concentrations were corrected in the course of the AV3 trial and after, with the exception of albumin at the end

of phase C2, while these two parameters fell within the reference range at the end of phases 2 and 3. The drop or the below-limit value of albumin at the end of phase C2, may reflect increased hepatic stress.

CONCLUSIONS

In the field trial with 12 Holstein dairy cows, blood chemistry parameters were used to investigate the effects of 58 daily supplementation of the commercial feed additive AV3 with declared antioxidative action. A numerical increase in the activity of glutathione peroxidase was observed. The activity reached the recommended values at the end of the supplementation period. A significantly better total antioxidant status was found at the end of the supplementation period in comparison to the values measured four weeks later. On the basis of these results, the potential positive effect of AV3 on the antioxidant capacity of the animal body may only be assumed, but not confirmed. Another positive finding at the end of the supplementation period, was a significant reduction of the total bilirubin levels which fully fell within the reference range. Bilirubin, even though it belongs to a wider range of endogenous antioxidants, is above all, a significant indicator of the liver parenchyma condition. In addition, a significant increase in low baseline milk protein level was observed. Both of these are favourable results and may be related to AV3 supplementation. The suggested positive results must be subject to additional testing of the trial product in more demanding studies.

ACKNOWLEDGEMENT

The study was performed with generous support of project MŠMT 2629608001 and the NAZV grant No. QI91A238.

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Received January 15, 2013