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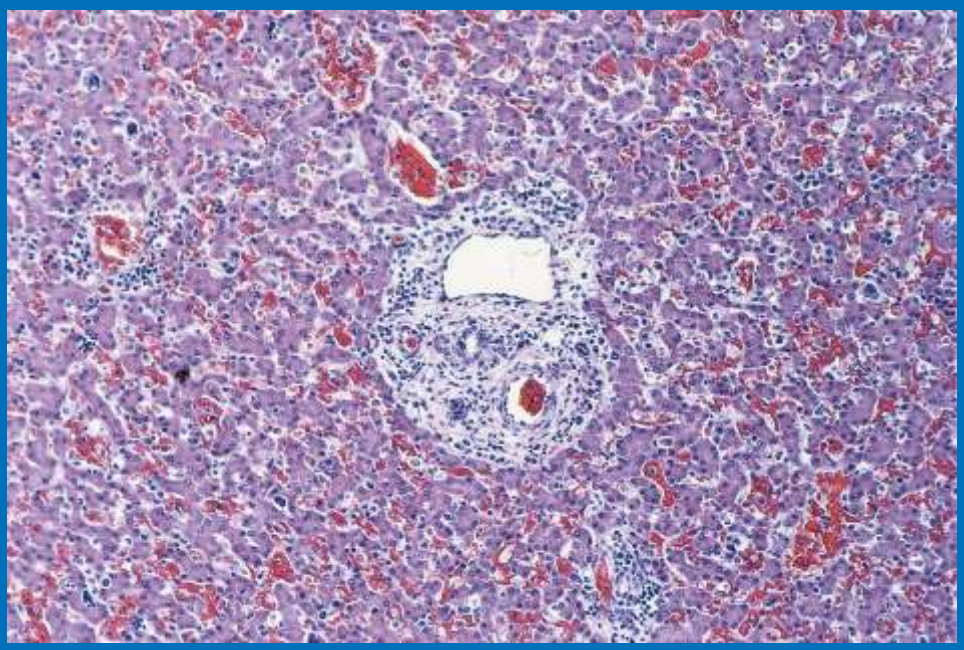

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Review paper

THE OESTRUS CYCLE IN THE BITCH: A REVIEW ARTICLE

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Summary: The regulation of the oestrous cycle in the bitch is quite unique among the various animal species commonly encountered in veterinary medicine. The cycle, which has an average length of about 3 months, consists of the pro-oestrus, oestrus and metoestrus phases and is followed by the anoestrus phase that varies in duration. The duration and regulation of the luteal phase is the same in both cyclic and pregnant bitches. In contrast to some other species, the uterus is not involved in the regulation of the cyclic corpus luteum. While the first stage of the luteal phase is completely autonomous, the second depends on pituitary factors, mainly prolactin. It is still unclear whether LH has luteotrophic properties in the bitch. Recent studies centred on the role that hormones play in the oestrous cycle have led to developments such as the FSH threshold concept, which could lead to the induction and manipulation of the oestrous cycle. They have also led to the use of progesterone-receptor antagonists to control some of the physiological consequences of the luteal phase, such as pseudopregnancy. This article presents a review of the new scientific insights concerning the oestrous cycle of the bitch, with the emphasis on the regulation and complications of the luteal phase, such as the cystic endometrial hyperplasia–pyometra syndrome, acromegaly, insulin resistance, diabetes mellitus and the incidence of mammary tumours.

Key words: oestrous cycle; luteal phase; bitch

Introduction

The oestrous cycle of the bitch consists of the pro-oestrus, oestrus, and metoestrus phases. After each oestrous cycle, which has a length of about 3 months, there is an anoestrus phase, which varies in duration. The mean interval from the onset of one oestrous cycle to the next is about 7 months, within a range of between 4 and 12 months. The inter-oestrous interval of individual bitches may be either regular or variable (1).

Pro-oestrus is defined as the period when the bitch has become sexually attractive but is rejecting the male's advances until the first signs of its willingness to accept the male. As early behavioural signs may be indistinct, the onset of a serosanguineous vaginal discharge and swelling of the vulva are used to mark the first day of pro-oestrus. The duration of pro-oestrus ranges from 3 to 17 days with an average of 9 days. Oestrus is the period when the female allows breeding and has an average duration of 9 days, within a range of 3 to 21 days. During oestrus the vulva begins to shrink and soften and the vaginal discharge generally diminishes. Metoestrus begins when the bitch will no longer accept the male and usually lasts about 70 days. The end of metoestrus can be defined in a number of ways such as when the progesterone secretions of the luteal phase subside, mammary development declines, progesterone secretions no longer effect the endometrium or when the plasma-progesterone concentration initially declines to a level of 1 μg/L or less (1, 2).

In addition to this behaviour-oriented classification of the oestrous cycle, a new and far more appropriate classification system has been introduced. It is based on the ovarian function and divides the oestrous cycle into four phases: the

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follicular, the pre-ovulatory luteinization and ovulation, the luteal and the anoestrus phases (3, 4).

Follicular phase

Tertiary follicles developing in the ovaries produce oestradiol. The increased concentration of oestradiol is responsible for external signs of prooestrus, such as hyperaemia and oedema of the vulva, the bloody vaginal discharge and for behavioural changes. It also causes a lengthening and hyperaemia of the uterine horns, an enlargement of the cervix and a thickening of the vaginal wall (1, 5). Plasma-oestradiol levels increase constantly throughout the follicular phase and reach peak plasma values 1 to 2 days before the pre-ovulatory LH surge (6, 7, 8). The oestrogen levels decline rapidly thereafter, while the level of plasma progesterone starts to increase as a result of the partial luteinization of the follicles (7). Both LH and FSH plasma concentrations are relatively low during the follicular phase (9, 10).

Pre-ovulatory luteinization and ovulation

The pre-ovulatory LH surge starts 1 to 2 days after the oestradiol peak and coincides with the declining oestradiol and rising plasma-progesterone concentrations (6, 7). It has been suggested that the pre-ovulatory LH surge is triggered by a decline in the plasma oestrogen: progesterone ratio in the latter stages of the follicular phase (7, 11). The pre-ovulatory LH surge, which lasts from 24 to 72 hours and produces a rapid and final enlargement and luteinization of the mature follicles, causes ovulation and in the process transforms oestrogen-secreting follicles into progesterone-secreting corpora lutea (7). Thus the LH surge represents the transition from the follicular phase to the luteal phase. Ovulation appears to occur synchronously about 36 – 48 hours after the LH peak (7, 12). Most ova in the bitch are ovulated in an immature state as primary oocytes (13) and cannot be fertilized until they undergo the first meiotic division to become secondary oocytes, which usually occurs about 60 hours after ovulation (12, 14). By this time the ova have descended through two thirds of the oviduct. Plasma-progesterone concentrations are between 2 and 4 µg/L at the LH peak and by the time ovulation occurs, usually 36 to 48 hours later, they rise from 5 to 8 µg/L (15). Concurrent with the LH peak, there is also a pre-ovulatory surge of FSH that reaches its peak concentration 1 to 2 days later (9).

Luteal phase

The concentration of progesterone, which originates from the corpora lutea, increases in the peripheral blood during the latter stage of oestrus and the onset of metoestrus and reaches its maximum level 10 to 30 days after the LH peak. Thereafter, in non-pregnant bitches, the progesterone secretion slowly declines and reaches a basal level of 1 µg/L for the first time about 75 days after the start of the luteal phase (15). The transition from oestrus to metoestrus takes place during the initial stage of the luteal phase.

Regulation of the luteal phase

In many species, the regulation of the cyclic corpus luteum is influenced by both luteotrophic and luteolytic factors. Prostaglandin F2? originating from the endometrium, which is the causative factor for luteolysis in the cow and sheep, is not present during the luteal phase of the cyclic dog (15). This is demonstrated by the fact that a hysterectomy does not influence the length of the luteal phase. Therefore the uterus is not involved in the regulation of the cyclic corpus luteum (15). Moreover, in the initial stage of the luteal phase the canine corpus luteum functions completely autonomously. Studies of dogs that had undergone hypophysectomy demonstrated that the canine corpus luteum functions independently of pituitary support for 24 to 28 days from the onset of the luteal phase (16).

Administering aglepristone in the early part of the luteal phase does not effect its duration (17). During the second half of the luteal phase, pituitary luteotrophic factors – prolactin and possibly LH – are necessary to sustain the luteal function (16, 18, 19, 20). Whether LH has luteotrophic properties in the bitch is still unclear. Concannon et al. (1987) reported that passive immunization against LH caused a decline in the progesterone concentration. However, the luteotrophic role of LH has been brought into question by studies in which LH-inhibition had no effect on the plasma-progesterone concentration, whereas prolactin-inhibition caused it to fall abruptly, indicating that only pro-

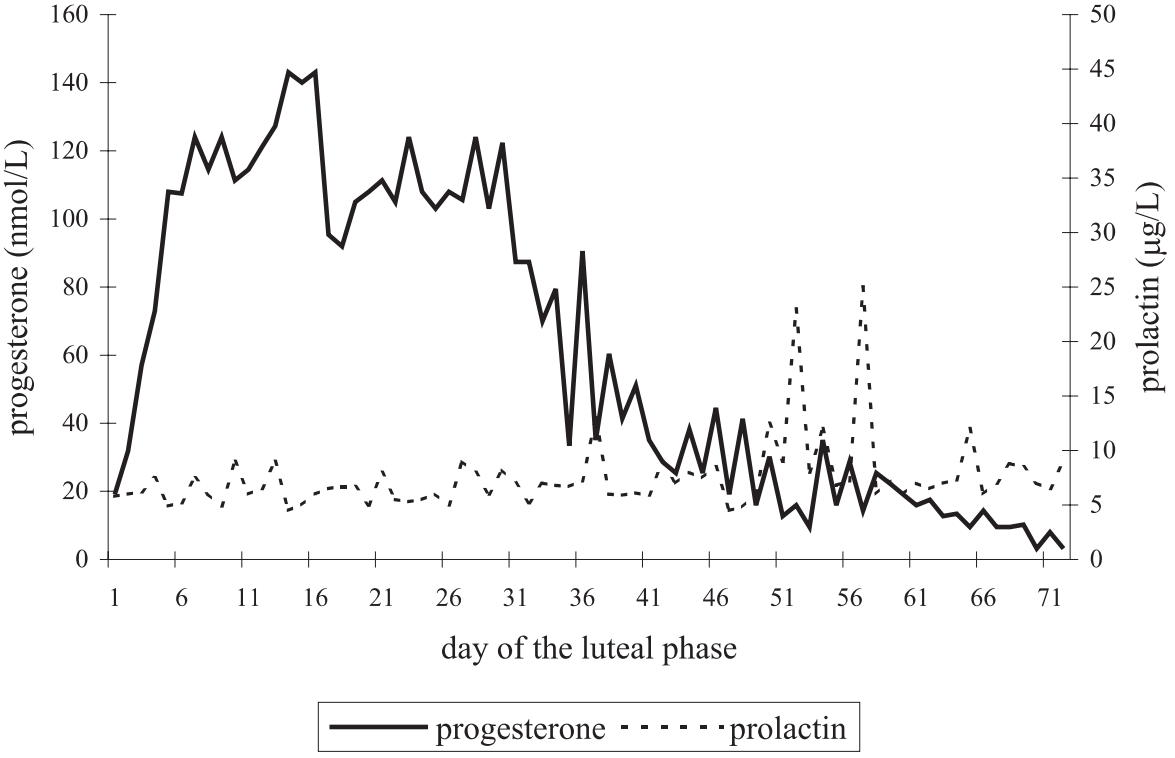

Figure 1: Mean plasma concentrations of progesterone and prolactin in 3 healthy beagle bitches, starting from the day of ovulation (Day 1) to the end of the luteal phase. (From Galac S. The effect of aglepristone, the progesterone receptor antagonist, on the hypothalamic-pituitary-ovarian axis, pregnancy and luteal phase in bitches. In: doctoral thesis. Ljubljana, 2001. Reproduced with the author's permission)

lactin is luteotrophic in cyclic dogs (19). It is possible that LH has an indirect luteotrophic role that is mediated by the secretion of prolactin (21).

Anoestrus

The transition from the luteal phase to anoestrus is gradual and varies considerably among bitches. The onset of anoestrus depends on which criteria are being used to define the end of the luteal phase. It can be defined as the period when mammary development subsides, which is usually after 2 to 3 months, or when the plasma-progesterone concentration reaches a level below 1 µg/L for the first time or as the moment that the influence of progesterone on the endometrium is no longer evident (1, 22). In anoestrus, the normal bitch is neither attractive nor receptive to the male, the mucoid vaginal discharge is minimal and the vulva is small (5).

Although anoestrus seems an inactive part of the oestrous cycle in the bitch, neither the ovaries nor pituitary are quiescent (9). From early to late anoestrus, the hypothalamus releases increasing amounts of GnRH (23), which the pituitary becomes increasingly sensitive to (24). Additionally, increases in ovarian responsiveness to gonadotrophins (25) and the level of basal LH-concentrations towards the end of the anoestrus (9), as well as a brief period of increased LH pulsatility (26) have been reported as important determinants of the initiation of a new follicular phase. It has even been suggested that changes in the LH secretion may be more important than changes in the FSH secretion in the initiation of the follicular phase leading to ovulation (11). In line with this, the administration of pharmacological doses of LH can terminate anoestrus in bitches by inducing the follicular phase (27). However, the progression from early to late anoestrus is associated with an increase in the basal plasma-FSH concentration, suggesting that in the bitch an increase in circulating FSH levels is a critical event in the initiation of ovarian folliculogenesis (10). In this respect, there are similarities with the situation in primates. Observations during gonadotrophin-indu-

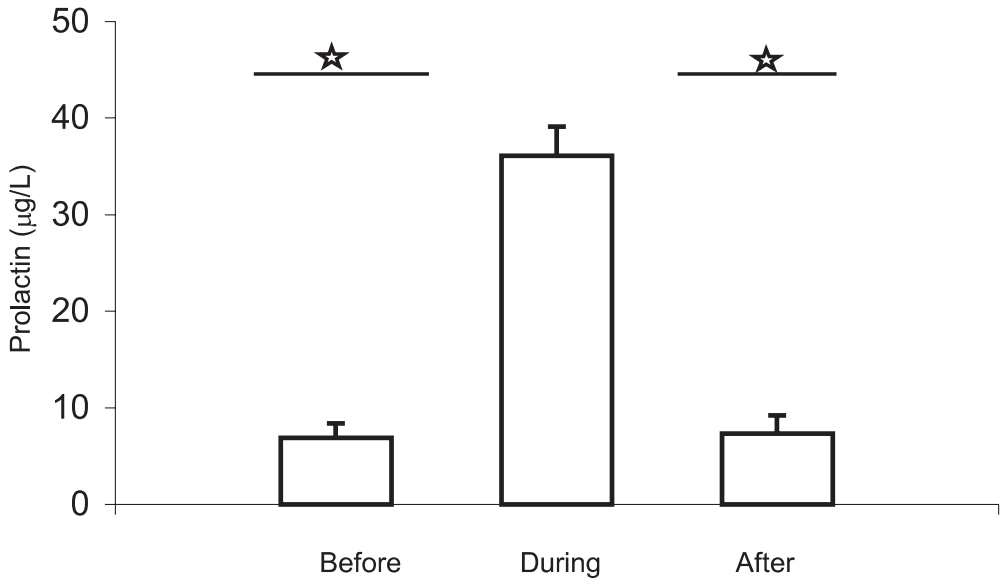

Figure 2: The average (± SEM) of the mean plasma prolactin concentrations in 6 beagle bitches before, during and after treatment with aglépristone. Asterisks indicate significant difference. (From Galac S. The effect of aglépristone, the progesterone receptor antagonist, on the hypothalamic-pituitary-ovarian axis, pregnancy and luteal phase in bitches. In: doctoral thesis. Ljubljana, 2001. Reproduced with the author's permission)

ced ovulation in women have emphasized that plasma FSH must exceed a certain concentration before preantral follicles reaching the FSH-dependent stage can progress to maturation (28). This has been labelled the FSH threshold concept. An increase of only 10 to 30 % above the threshold concentration of plasma FSH is sufficient to stimulate normal follicular development in women (29). The threshold for FSH may vary among individuals, and each follicle also has its own sensitivity to FSH (28). The study of Kooistra et al. (1999) indicate that the FSH threshold concept, as anticipated for women, could hold true for the dog as well.

The oestrous cycle can begin at any time throughout the year and there appears to be little, if any, seasonal influence. Breed differences and strains within breeds can form the basis of variation in mean inter-oestrous intervals. Environmental factors can also affect the inter-oestrous interval: the onset of pro-oestrus in an anoestrus bitch can be brought forward by several weeks by placing her in close proximity to a bitch in oestrus. Furthermore, bitches housed together often have synchronous oestrous cycles (11, 30).

Consequences of the oestrus cycle

The relatively long exposure to high levels of progesterone circulating during each oestrous cycle may result in disorders such as cystic endometrial hyperplasia-pyometra syndrome (31), acromegaly, insulin resistance, diabetes mellitus, (32) and an increased incidence of mammary tumours.

Cystic endometrial hyperplasia involves proliferation of the glandular epithelium and cystic dilatation of the endometrial glands with endometrial fluid accumulated in their lumen. These conditions provide an excellent environment for bacterial growth, which can lead to the development of pyometra. Pyometra is often caused by entering the progesterone phase of the sexual cycle with an abnormal endometrium, which can result in an overgrowth of bacteria that is normally isolated from this area of the anatomy. Surgery is the preferred treatment for pyometra unless the owner adamantly wants to breed with the bitch. The medical treatment consists of administering antibiotics and prostaglandins. If the bitch is still in the luteal phase, progesterone-receptor blockers may also be administered to diminish the influence of progesterone (33).

Acromegaly occurs as a consequence of excess secretion of the growth hormone (GH). Progesterone-induced GH secretions originate from the foci of hyperplastic ductular epithelium of the mammary gland (34, 35). In contrast to the GH from the pituitary gland, GH from the mammary gland is not pulsatile and cannot be stimulated by the GH-releasing hormone (GHRH) and nor can it be inhibited by somatostatin (34, 36). The progesterone-induced GH excess may lead to insulin resistance, exhaustion of the pancreatic ?-cells and consequently diabetes mellitus (32). If diabetes mellitus is diagnosed while there is a high level of progesterone secretion, it might have a reversible nature. However, the source of progesterone must be removed as early as possible. Therefore, an ovariectomy is advised if diabetes mellitus occurs during the luteal phase, although it is difficult to predict whether the pancreatic insulin production will completely recover. In any case, supportive therapy with insulin is recommended after the surgery. In order to prevent hypoglycaemia and to achieve the right dosage of insulin, daily blood glucose measurements are needed and the insulin dose adjusted accordingly (37).

Pseudopregnancy is a syndrome that accompanies the extended luteal phase of all the non-pregnant ovarian cycles in the bitch (38). An important precipitating factor for pseudopregnancy appears to be a rapid decline in the plasma progesterone concentration, which is assumed to be the trigger for the release of prolactin, which in turn would give rise to pseudopregnancy (39). Correspondingly, an ovariectomy performed in the luteal phase often induces an overt pseudopregnancy. Studies using the progesterone-receptor antagonist aglépristone, have suggested that a sudden decline in the plasma progesterone concentration induces an increase in the concentration of prolactin (40, 41).

The development of mammary gland tumours in the bitch is clearly hormone dependent. The role of progestins in the pathology of the mammary gland was revealed in 1969, when Schneider et al. published a study about the protective effect of an ovariohysterectomy on mammary tumour development. They estimated that in comparison with intact dogs, bitches that had been spayed prior to their first oestrus had a 0.05 % risk of developing malignant tumours. This increased to 8 % if spayed following their first oestrus and rose to 26 % if spayed after their second oestrus. The spaying of older dogs does not reduce their risk of developing malignant tumours, although an ovariectomy does appear to reduce their risk of developing benign tumours (42). The protective effect of an early pregnancy, which is well known in the human, has not been demonstrated in the dog. As in the normal mammary gland, GH receptors have been demonstrated in neoplastic tissue (43). It has been speculated that the maximal effect of progestins on the mammary gland might be facilitated by the additional local action of GH. Yet, it must still be proven whether progestin-induced GH acts as an intermediate in the progestin-stimulated development of canine mammary tumours.

Conclusion

Being familiar with the endocrinological events associated with the oestrous cycle in the bitch could help the clinician to understand any complications that may eventuate during the luteal phase and to provide the best possible treatment for them. Applying this knowledge to breeding management, which is based on the hormonal changes in the oestrous cycle, will provide better results than those produced by using the empirical, behaviour-oriented approach. Canine female reproduction is a rapidly developing field in veterinary medicine and the pharmaceutical industry has provided us with several new possibilities to improve breeding programmes or to treat maladies associated with the oestrous cycle. Therefore it is of great importance that the small animal clinician keeps up to date with newly emerging information and developments regarding the endocrinology of the oestrous cycle in the bitch.

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POJATVENI CIKLUS PRI PSICI: PREGLEDNI ČLANEK

J. Butinar, E. Mujagić, S. Galac

Povzetek: Regualacija pojatvenega ciklusa pri psici, ki ga sestavljajo proestrus, estrus in metestrus in traja približno 3 mesece, sledi pa mu različno dolg anestrus, je med živalskimi vrstami v veterinarski medicini edinstvena. Trajanje in regulacija lutealne faze se ne razlikujeta pri ciklični in breji psici. V nasprotju z nekaterimi drugimi živalskimi vrstmi maternica ni vpletena v regulacijo cikličnega rumenega telesa. Prav tako je prvi del lutealne faze popolnoma avtonomen, v nasprotju z drugim, ki je odvisen od hipofiznih dejavnikov, predvsem prolaktina. Še vedno ni pojasnjeno, ali ima LH pri psici luteotropno vlogo ali ne. V zadnjem času so prišli do nekaterih novih spoznanj o hormonskih dogajanjih, na primer uveljavljanje koncepta praga FSH, ki lahko sprožijo pojatveni ciklus. Prav tako so bile nedavno v raziskavah z antagonisti progesteronskih receptorjev osvetljene nekatere fiziološke posledice lutealne faze, na primer navidezna brejost (psevdogravidnost). Dajanje aglépristona, antagonista progesteronskih receptorjev, v zgodnji lutealni fazi ni vplivalo na njeno trajanje. Članek predstavlja pregled novih znanstvenih spoznanj o pojatvenem ciklusu pri psici, s poudarkom na urejanju in zapletih lutealne faze, kot so cistična hiperplazija, sindrom endometrija – piometra, akromegalija, inzulinska odpornost in sladkorna bolezen ter povečano pojavljanje tumorjev mlečne žleze.

Ključne besede: estrus; lutealna faza; psica

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Original research paper

MORPHOMETRICAL ANALYSIS OF GASTRIN CELLS IN THE GASTRIC MUCOSA OF THREE-WEEK-OLD PIGS (*Sus scrofa domesticus*) AND A COMPARISON WITH OTHER GASTRIC ENTEROENDOCRINE CELLS

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Summary: The number and localisation of enteroendocrine cells from different parts of the gastric mucosa of three-week-old pigs were analysed. The basic method for the visualisation of enteroendocrine cells was immunohistochemistry and the results were compared to those derived through both the toluidine-blue staining for enteroendocrine cells and Grimelius silver staining methods. The greatest number of gastrin cells was in the pyloric mucosa with 124.66 ± 4.54 gastrin cells per mm^2 in the antrum and 140.32 ± 5.30 per mm^2 in the pyloric canal. The highest density of gastrin cells in relation to the thickness of the gastric mucosa was in the middle third of the mucosa with approximately 50 % of all gastrin cells. The remaining gastrin cells were evenly distributed in the upper and lower thirds of the mucosa. Cutaneous mucosa was negative for gastrin and other enteroendocrine cells. We found that the area of the small curvature, which has cutaneous and pyloric types of mucosa, had an average of 29.21 ± 2.97 gastrin cells per mm^2 and that the areas with cardial and fundic mucosa had lower averages of 0.61 ± 0.14 cells per mm^2 and 1.14 ± 0.11 gastrin cells per mm^2, respectively. Morphometric analyses of the serotonin and somatostatin cells from the pyloric area, where there was the highest density of gastric cells, were performed. The numbers of both types of cells were much lower compared to the gastrin cells, with 17.7 ± 2.30 serotonin cells and 50.7 ± 2.76 somatostatin cells per mm^2 of mucosa. The results of the silver staining and the toluidine-blue staining only partially correlated with the results of the immunohistochemical reaction. While many more cells reacted to the Grimelius silver staining (153.5 ± 0.32 cells per mm^2) than to the toluidine-blue staining (51.15 ± 2.20 cells per mm^2), there were still considerably less of them in comparison with the number of cells that reacted immunohistochemically.

Key words: veterinary medicine; stomach; enteroendocrine cells; gastrin; somatostatin; serotonin; immunohistochemistry; Grimelius silver staining; toluidin blue; pig

Introduction

There are four distinct regions of gastric mucosa in pigs: the oesophageal, or proventricular part (*pars oesophagealis s. proventricularis),* which is located at the gastric entrance and has cutaneous mucosa, and three parts that have glandular mucosa (*pars glandularis*). The anterior third of glandular mucosa, which is located in the anterior part of ventricular body between the oesophageal part and the *genu* ventriculi and includes the gastric diverticulum, has cardial mucosa. The second part is behind the genu and it contains fundic mucosa with fundic glands, and the third area is the caudal part of the stomach, which has pyloric mucosa and begins at the larger antrum and continues into the pyloric canal that contains numerous sero-mucosal glands in the propria (1). Enteroendocrine cells can be found scattered among the epithelial mucosal cells in all parts of the gastric mucosa (2). They belong to the diffuse endocrine system or the APUD system (amino precursor uptake and decarboxylation). In this sys-

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tem byogene amines and peptides are synthesised – neurohormones and neurotransmitters, suggesting their relation to neurones (2, 3). There are 6 types of enteroendocrine cells in the stomach: A, G, D, EC, P and ECL cells. The A cells synthesise gastro-glucagon, the G cells (argyrophil cells) produce gastrin, D cells produce somatostatin and the EC cells (enterochromaffin or argentaffin cells) produce 5-hydroxytryptamine (5-HT). The P cells produce both prostaglandin and motilin and the ECL cells (enterochromaffin-like cells), which can be visualised with the Grimelius silver staining, produce histamine (4, 5, 6).

Gastrin is a heptadecapeptide with 17 amino acids produced by gastrin cells (G cells). Gastrin cells are located within the gastric epithelium and in humans, where they have a characteristically conical or oval form with microvilli on their surface (7), the highest concentrations are found in the antrum. Their ultrastructure depends on their activity status (8) and they contain three types of granules (6, 9, 10). Gastrin molecules are synthesised from precursor molecules (10). There are two chemical forms of gastrin, gastrin I and gastrin II (7), and several biologically active forms: large gastrin G34, small gastrin G17, mini gastrin G14 and component I (11). All forms have at C-end of the molecule tetragastrin, a prolongation molecule, which is also biologically active (2). Gastrin has an exocrine function as it secrets into the gastric lumen, it has also an endocrine function and influences distant tissues by secreting into the blood, and as it regulates the activities of neighbouring cells, it also has a paracrine effect (2). Gastrin is not only important for the normal functioning of the stomach, but also for the normal functioning of other parts of the digestive system – the duodenum, pancreas, liver. In humans it has been shown to stimulate the excretion of gastric acid and pepsin, the growth of gastric mucosa, the secretion of pancreatic enzymes and the secretion of water and electrolytes in the stomach, pancreas, liver and Brunner glands. It increases the absorption of water and electrolytes from the small intestine, stimulates contractions of the stomach and gall bladder and relaxes the muscles of the pyloric and the ileocaecal sphincter. It also stimulates the secretion of insulin, acetylcholine, somatostatin, pancreatic polypeptide and calcitonin as well as affecting the proliferation of ECL cells (2, 12, 13, 14, 15).

There is already a lot of published data regarding gastrin cells of different animal species. Three types of gastric cells have been described in newborn rats, according to their level of differentiation - primitive, transitional and typical gastrin cells (9). However, very little data is available that describes at what age they appear and if there are any age-dependent variations in their numbers and localisation within the gastric mucosa and physiological characteristics of the animal species at certain ages. We found very few references relating to the gastrin cells of the pig, and even fewer pertaining to the different pre- and postnatal developmental stages and to the adaptation to the changes in the feeding practices after birth and weaning. The majority of gastrin in the stomach of pigs is small gastrin 17 (16) and there are equal quantities of gastrin 17 and its precursor, gastrin 34 (17). The secretion of gastrin depends of several factors: the composition of food, the quantities of releasing and inhibiting peptides (GRP, GIP) in the circulation, nervous factors (vagus), and paracrine and endocrine factors (prostaglandin, somatostatin, histamine etc.) (18, 19). In this study we analysed the gastric mucosa and gastrin cells of three-week-old pigs. At this stage the pigs are weaned and their gastric mucosa has to quickly adapt to new types of food. It is also a period when piglets experience frequent gastrointestinal disorders. For comparison we also analysed the localisation and distribution of the somatostatin and serotonin cells, both of which are involved in controlling gastrin secretion.

Material and methods

Materials

Ten weaned, three-week-old, farm pigs were killed and their stomachs, which were immediately removed from their abdominal cavity, were opened along the major curvature. The stomach content was removed and samples were taken from six parts of the gastric wall: from the cardia, fornix and gastric body, and from the antrum, pyloric canal and the minor curvature. The tissue was then fixed in buffered formalin for approximately 24 hours before being embedded in paraffin following standard laboratory procedures. The paraffin blocks were then cut into 7µm thick sections.

Histology

One tissue section from each of the gastric regions was stained with haematoxylin and eosin for the histological examinations.

Immunohistochemistry

The immunohistochemistry was performed using the PAP method (peroxidase - anti-peroxidase) described by Sternberger (20). We applied a 1:1500 dilution of polyclonal rabbit antihuman gastrin-17 as primary antibodies (DAKO, cat. no. A568) to slides that were then incubated in a humidified chamber overnight at 4 °C. These antibodies also react with gastrin-34. The slides were then treated with a 1:50 dilution of goat anti-rabbit immunoglobulins (Amersham) prior to a final treatment with a 1:50 dilution of PAP complex (Sigma, cat. no P1291). All the dilutions were made with a 2.5 % solution of bovine serum albumin (BSA) in a buffered phosphate saline. DAB (3'–diaminobenzidin tetrahydrochloride, Sigma, cat. no. D5905) was used as a substrate and the tissue sections were counterstained with either haematoxylin or 1 % methyl green, dehydrated and mounted with synthetic resin. After we had completed the morphometric analyses of the PAP tissue sections the DAKO LSAB®2 System, HRP product (Dako, cat. no. K0675) became available on the market. By using it in accordance with the manufacturer's instructions we were able to assess the quality of the product by making a comparison of the immunohistochemical reactions of the PAP method and this new system.

Grimelius silver staining (21)

We used the Grimelius silver stain to determine the numbers of gastrin cells and compared these results with those of the PAP method. This histochemical method was also used to determine the population of all the enteroendocrine cells in the stomach that react with this type of silver staining.

Toluidine-blue staining for enteroendocrine cells (22)

The toluidine-blue staining method, which is adjusted for the detection of enteroendocrine cells, supposedly enables a partial differentiation of these cells without the use of immunohistochemistry. Cells containing gastrin should stain in a metachromatic manner and other enterochromaffin cells in an orthochromatic manner, e.g. cells containing serotonin, after a pre-treatment in hot hydrochloric acid.

Treatment of serial tissue sections using all the methods and a double-PAP procedure for the localisation of gastrin and other enteroendocrine cells

Serial tissue sections were prepared using only the samples from the pyloric part of the stomach. One tissue section was treated with toluidine blue, on another tissue section we demonstrated gastrin cells using the PAP method and a third one was silver stained following the Grimelius silver-staining procedure. A fourth tissue section was immunohistochemically treated for a reaction to serotonin using the PAP method and a 1:1000 dilution of polyclonal rabbit anti-serotonin antibodies (Inc Chemical Credential). A fifth tissue section was used for a double-immunohistochemical reaction - the first layer for gastrin and the second layer for somatostatin (Dako, cat. no. A 566, diluted 1:1500). A sixth tissue section was used for a double-immunostaining procedure to determine serotonin (first layer) using a nickel-ammonium-sulphate-enhanced DAB substrate (23) that gives either a dark brown or a black insoluble-reaction product; and somatostatin (second layer) using a DAB substrate that gives an insoluble brown-reaction product. We also localised somatostatin-immunoreactive cells on another tissue section. As a comparative measure, the LSAB technique was also used later to detect serotonin, somatostatin and gastrin.

The double-immunohistochemical reactions were achieved using the steps of the PAP method with the application of the first primary antibody and the incubation in the first substrate. However, instead of being dehydrated, the tissue sections were incubated with a second primary antibody in a humidified chamber overnight at 4 °C. The next day the procedure was concluded with repeating of all the consecutive steps of the PAP method that are usual after incubation with primary antibodies. The only change was the use of the different substrate. During the optimisation of the procedure we tested different combinations of antibodies, as this was found to be an impor-

Table 1: Mean values (± SE) of the thickness of gastric mucosa in different parts of the stomach of a three weeks old pig and mean number of gastin cells per mm^2 (± SE) of mucosa

Area	Mucosal thickness in µm ± SE per mm^2 of mucosa	Mean number (± SE) of gastrin cells
1	243,50 ± 0,81	0,00 ± 0,00
2	280,86 ± 1,04	0,61 ± 0,14
3	501,19 ± 1,33	1,14 ± 0,11
4	329,96 ± 1,38	124,66 ± 4,54
5	410,05 ± 0,94	140,32 ± 5,30
6	313,11 ± 1,12	29,21 ± 2,97

Legend:
1 - area with cutaneous mucosa (oesophageal part, cardia)
2 - area with cardial mucosa (fornix)
3 - area with fundic mucosa (gastric body)
4 - area with pyloric mucosa (antrum)
5 - area with pyloric mucosa (pyloric canal)
6 - area with cutaneous and pyloric mucosa (small curvature)

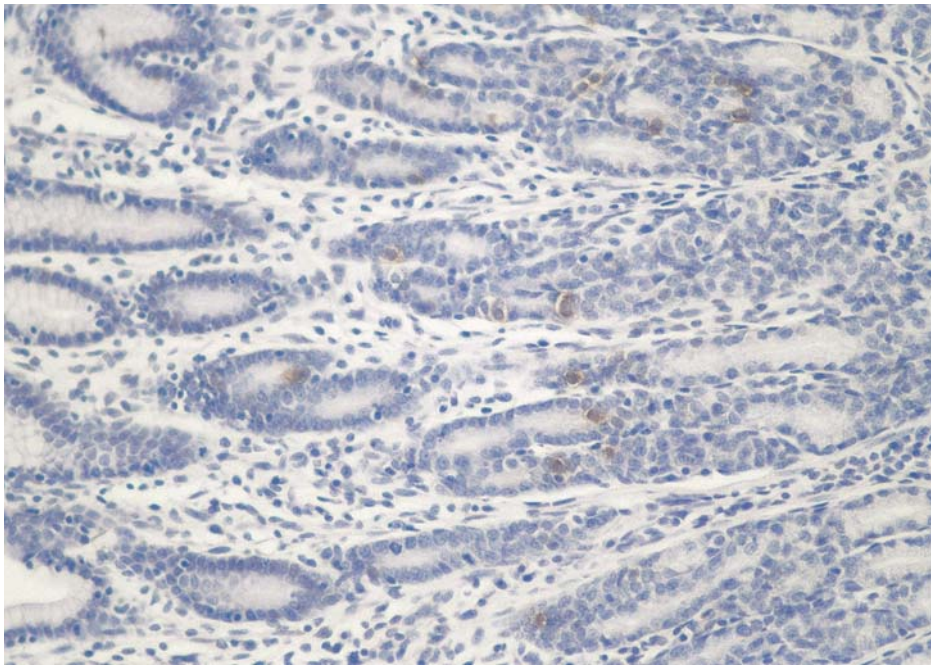

Figure 1: Cardial mucosa of a three-week-old pig, a few gastric cells. PAP, DAB, counterstained with haematoxylin, x 20

tant factor in the quality of the reaction. We achieved the best results using the aforementioned combination.

Determination of the thickness of the gastric mucosa

The average thickness of the gastric mucosa samples from the five pigs was measured using an ocular micrometer at a magnification of 100x. Ten measurements, from five locations on two consecutive tissue sections, were taken and the distances between the units on the ocular micrometer were calculated with an object micrometer.

Determination of the number of gastrin cells

The number of gastric cells was determined with a light microscope as were all the gastrin-positive cells on two tissue sections from each of the selected gastric regions. We counted the positive cells across the ten visual fields of each tissue section, i.e. 20 visual fields from each gastric

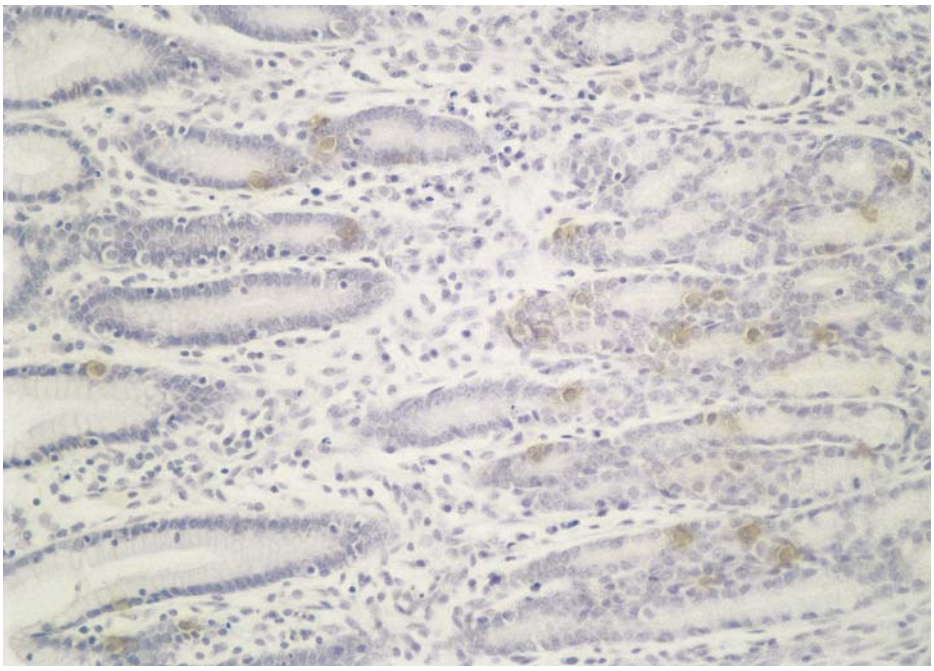

Figure 2: Cardial mucosa of a three-week-old pig, a few gastrin cells but more numerous than in figure 1 - comparative immunostaining to the PAP method (figure 1); DAKO LSAB®2 System, HRP; DAB, counterstained with haematoxylin, x 20

region. A visual field was determined as the height of gastric mucosa by the width of the field of view at a magnification of 200x. At that magnification the visual field was 530 μm wide. The area of a visual field (VFA) was calculated with the formula:

VFA = height of the mucosa x 530 (24)

The number of cells was then calculated per mm^2 of mucosa.

Statistics

The results were statistically evaluated using the Batch System software programme and evaluated with an analysis of variance. The calculations were made with a probability of 95 % (P = 0.05).

Results and discussion

A few references have been published in which the authors discuss the distribution of gastrin cells in the stomach of the pig (25) and their physiological significance at different ages (26, 27, 28). At three weeks the growth rate of a pig's stomach decreases in comparison with that of its body, which coincides with the adaptation of the gastric mucosa to the effects of active peptides, as was reported in a study on the effects of pentagastrin (29). It has also been reported that at the same age the speed of gastrin degradation in the gastric and intestinal lumen is higher in comparison with newborn and adult pigs, although it is not quite clear why (26).

In the samples of the gastric mucosa of three-week-old pigs used in our study, the thickness of the mucosa from the different regions of the stomach varied significantly, except between the antral mucosa and the mucosa from the small curvature (average thickness 329.96 ± 1.38 μm and 313.11 ± 1.12 μm, respectively). The average thickness of cutaneous mucosa was 243.50 ± 0.81 μm, of the cardial mucosa 280.86 ± 1.04 μm and the fundic mucosa 501.19 ± 1.33 μm. The average thickness of the mucosa in the pyloric canal was 410.05 ± 0.94 μm, which was significantly more than it was in the antrum, which was 313.11 ± 1.12 μm thick (Table 1).

The Grimelius silver staining revealed enteroendocrine cells in all parts of gastric mucosa, except in the areas with cutaneous mucosa. That correlated with both the immunohistochemical results and those of the toluidine-blue staining. Based on their staining properties, two types of cells were found, which could be representative of two types of enteroendocrine cells. One type of cells gave a light-brown reaction product with the silver staining while the others were dark brown. The highest concentrations of positive cells revealed by the Grimelius silver staining method were in the fundic mucosa, the antral mucosa and in the mucosa of the pyloric canal.

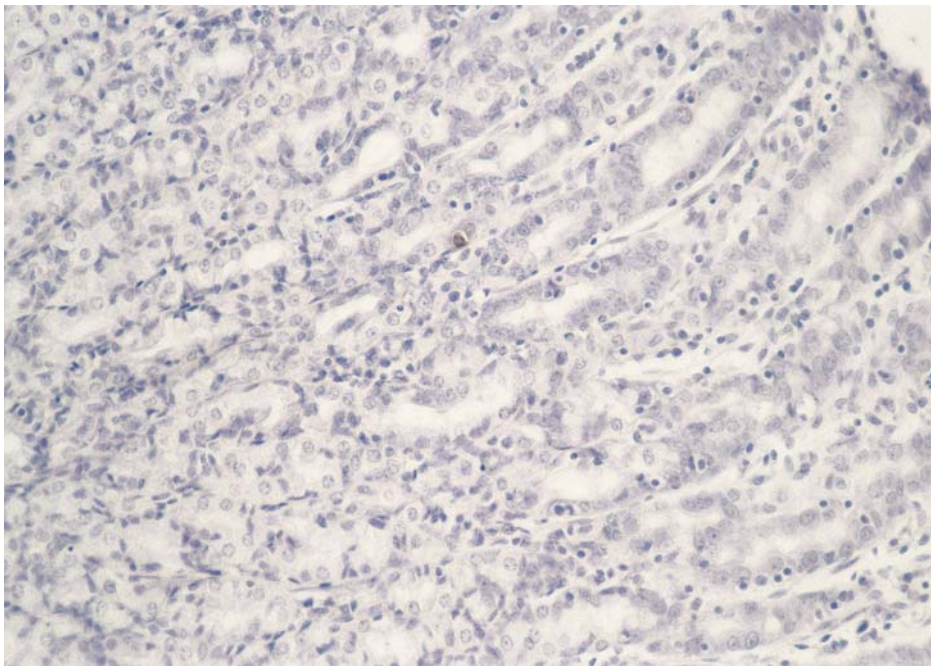

Figure 3: Fundic mucosa of a three-week-old pig, only one cell positive for gastrin; PAP, DAB, counterstained with haematoxylin, x 20

The cells were distributed throughout the mucosa with the highest concentrations being at the base of the glands with an intra-epithelial localisation.

The toluidine-blue stain reacted with cells in all areas of the gastric mucosa, with the exception of the cutaneous mucosa. The cells stained either orthochromatically or metachromatically. While the orthochromatic cells were localised among epithelial cells (intra-epithelial localisation), similar to the silver-stained cells, the metachromatic cells were extra-epithelial. As with the silver-stained cells, the greatest number of orthochromatic cells was in the mucosa of the fundus and the pyloric canal, and the least in the cardial mucosa. In all the regions, they were evenly distributed from the base to the luminal part of mucosa. Metachromatic cells were also found in all parts of gastric mucosa, with the exception of the cutaneous mucosa. Based on the localisation of the orthochromatic cells we believe that they are not gastrin cells as there was a very low number of immunohistochemically-positive gastrin cells in the fundic mucosa.

Gastrin cells revealed immunohistochemically were found in all parts of the gastric mucosa, except in the oesophageal part with cutaneous mucosa. However, there was great variation between the quantities determined in the different parts of the stomach: from just a few positive cells in the cardial and fundic mucosa to the large number of positive cells found in the pyloric canal. The highest concentration of gastrin cells was in the mid-third of the mucosa and only partly correlated with the localisation and number of cells revealed with the non-specific methods, Grimelius silver staining and the toluidine-blue staining. The intensity of the positive immunohistochemical reactions varied from light to dark brown. Although similar distributions of gastrin and other enteroendocrine cells were detected by both immunohistochemical methods, the PAP and the DAKO LSAB®2 System, it was subjectively assessed that the latter revealed more positive cells than the former (Figures 1 and 2). The distribution of gastrin cells in the gastric mucosa of the three-week-old pigs was similar to that determined by Bussolati (25) using immunofluorescence. However, as we do not know the ages of the pigs used in his study we cannot say that these results can be applied to pigs of all ages.

The distribution of the other types of enteroendocrine cells in parts of the stomach was distinctly different from the distribution of gastrin cells, with the exception of the cutaneous mucosa, which was completely negative for all enteroendocrine cells. In the mucosa of the pyloric canal we found a small number of serotonin-positive cells and unlike the gastrin cells, which were concentrated in the middle third of the mucosa, the serotonin-positive cells were evenly dispersed throughout the mucosa. In the tissue sections with double-immunohistochemical reactions, the

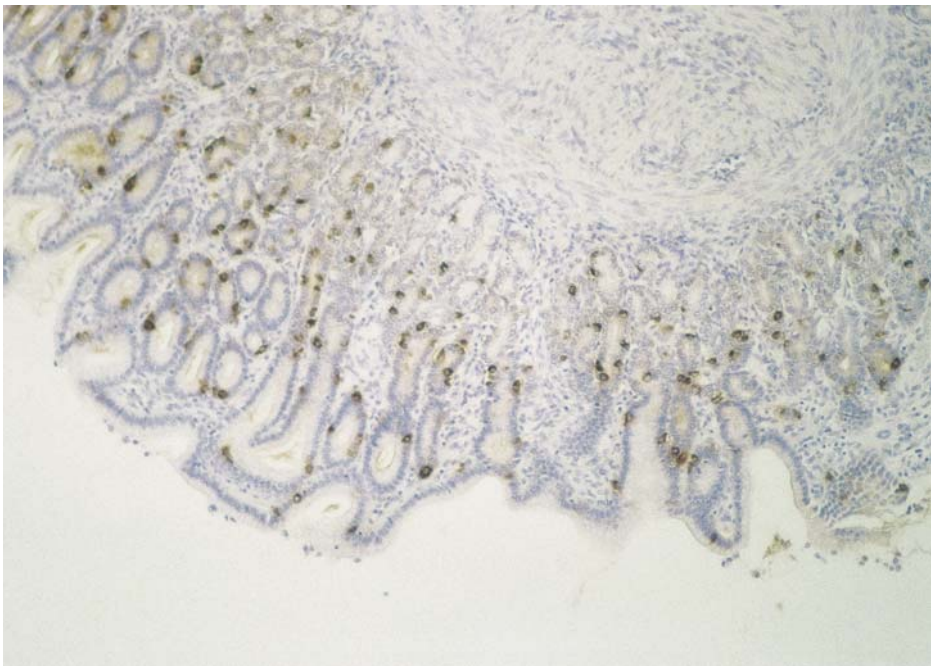

Figure 4: Pyloric mucosa of a three-week-old pig near the transition to duodenal mucosa. Numerous gastrin cells (DAKO LSAB®2 System, HRP; DAB, counterstained with haematoxylin, x 40)

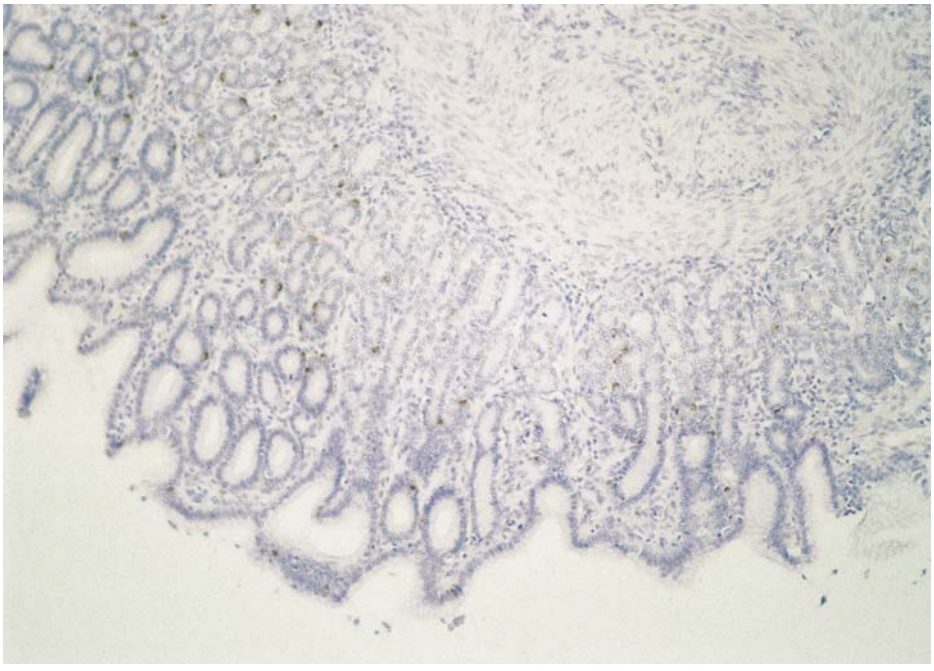

Figure 5: Pyloric mucosa of a three-week-old pig near the transition to duodenal mucosa - cells positive for somatostatin. DAKO LSAB®2 System, HRP; DAB, counterstained with haematoxylin, x 40

combinations of somatostatin with either gastrin or serotonin, we found both light and dark brown cells. This was similar to the variability in intensity of the gastrin-cell reaction product and could, in our opinion, be representative of different forms of the gastrin molecule.

The number of gastrin cells in the cardiac and fundic mucosa were statistically insignificant, averaging 0.61 ± 0.14 cells per mm^2 and 1.14 ± 0.11 cells per mm^2, respectively (Figures 1, 2 and 3). A subjective assessment of the numbers of gastrin cells revealed by both the PAP and DAKO LSAB®2 System immunohistochemical methods and a comparison of them indicated that the number of positive cells revealed by the latter was somewhat higher. This is entirely understandable, as it is much more sensitive of the two systems (Figures 1 and 2). The number of gastrin cells was significantly higher in the pyloric mucosa, both in the antrum and the canal, and

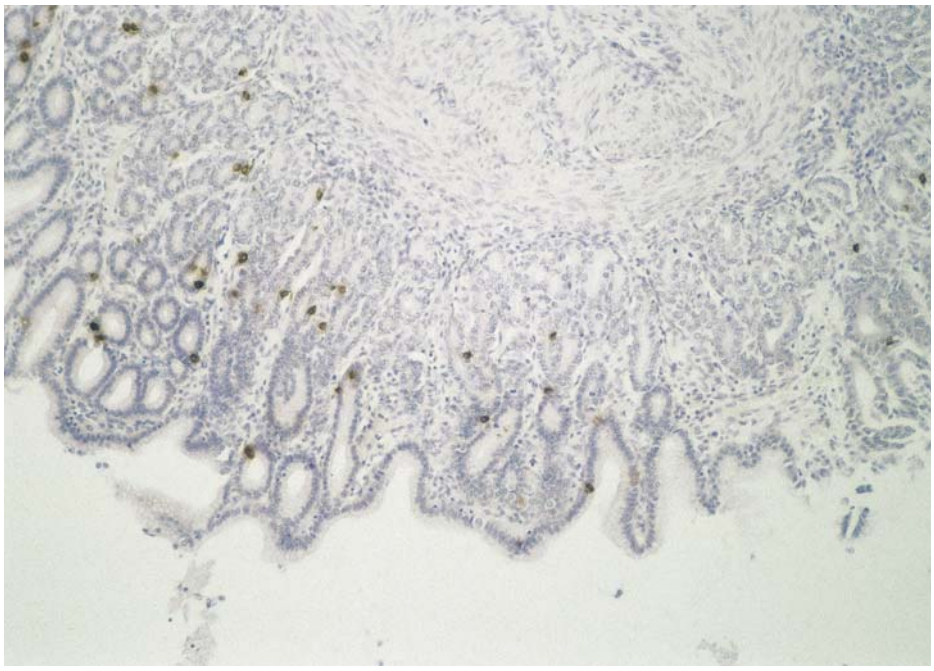

Figure 6: Pyloric mucosa of a three-week-old pig near the transition to duodenal mucosa - cells positive for serotonin. DAKO LSAB®2 System, HRP; DAB, counterstained with haematoxylin, x 40

Table 2: Mean values (± SE) for the number of cells, positive for gastrin, serotonin or somatostatin per unit of gastric mucosa (visual field) in pyloric canal of three weeks old pig, counted on consecutive serial sections, stained with toluidin blue (1), in Grimelius silver staining (3) and immunohistochemical reaction (IHC) for gastrin (2), serotonin (4) and somatostatin (5)

Number of serial section	Method / reaction for	Number of cells per mm² of mucosa ± SE
1	toluidin blue	51,15 ± 2,30
2	gastrin (IHC)	141,94 ± 5,53
3	Grimelius silver staining	153,5 ± 0,32
4	serotonin (IHC)	17,7 ± 2,30
5	somatostatin (IHC)	50,7 ± 2,76

in the area of the small curvature where cutaneous mucosa merges with the pyloric types of mucosa. The average values for the gastrin cells in these areas differed significantly. In the antral mucosa the average number of cells was 124.66 ± 4.54 per mm², whereas the average number of gastrin cells in the pyloric canal was 140.32 ± 5.30 cells per mm² and in the mucosa of the small curvature the average was 29.21 ± 2.97 cells per mm² (Table 1).

The highest concentration of gastrin cells was in the middle third of the pyloric mucosa, where the necks of the glands and the deep parts of gastric pits are located. The number of gastric cells in this part of the antral mucosa averaged 89.31 ± 7±.58 cells per mm² and 214.30 ± 8.61 cells per mm² in the mucosa of the canal. In relative terms that meant that 28.08 % of the gastrin cells in the antrum and 26.21 % of the gastrin cells in the canal were located in the basal part of the pyloric mucosa. The middle third of mucosa contained 50.62 % of all the gastrin cells in the antrum and 50.67 % of the gastrin cells in the canal; and the remaining 21.30 % of cells in the antral mucosa and 23.15 % of gastrin cells in the canal where located in the luminal third. This distribution pattern dissipated towards the duodenum where the cells were evenly distributed throughout all parts of the mucosa (Figure 4). In this area we also found cells that were immunoreactive to serotonin and somatostatin, which were evenly distributed among the gastrin cells (Figures 5 and 6).

With a few exceptions the numbers of enteroendocrine cells revealed by the different staining methods used on the serial tissue sections of pyloric mucosa differed significantly. The exceptions were the numbers of serotonin-immunoreactive cells and the number of

orthochromatic cells revealed by the toluidine-blue staining, and between the numbers of metachromatic cells in the toluidine-blue staining and in most, but not all, of the number of dark-brown cells revealed by the silver staining (Table 2).

With a comparison of the pictures taken from the serial sections it became obvious that the dark cells revealed by the silver staining were the same as the serotonin-immuno-positive cells. We were unable to confirm the same for the light-brown silver-stained cells and the gastrin cells. Orthochromatic cells were the same in both the toluidine-blue and the silver-stained cells.

The results of the morphometric analysis of the distribution of gastrin cells in the gastric mucosa of the three-week-old pigs and the comparison with the localisation and distribution of other enteroendocrine cells of the gastric mucosa added new information to the existing body of data (25). The results could also be compared with data from some publications discussing the physiological role of gastrin in the postnatal development of gastric mucosa in pigs (25, 26, 27, 28, 29). They also provide an insight into the comparitive sensitivities of some of the older non-specific methods for demonstrating enteroendocrine cells (Grimelius silver staining, toluidine blue for enterochromaffin cells) and the more specific immunohistochemical methods. The results of this study could provide a strong basis for further studies of the physiological and pathological processes of the digestive tract of the pig, given by the intensive postnatal morphological and functional changes in the digestive tract of this animal species. These are controlled and influenced by many factors (30) that could negatively affect the development of some or most of the segments of the digestive tract and enable conditions that are suitable for the development of pathological lesions in the stomach and intestines.

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MORFOMETRIČNA ANALIZA GASTRINSKIH CELIC V ŽELODČNI SLUZNICI TRI TEDNE STARIH PRAŠIČEV *(Sus scrofa domesticus)* TER PRIMERJAVA Z NEKATERIMI DRUGIMI ENTEROENDOKRINIMI CELICAMI V ŽELODCU

P. Juntes, M. Demšar

Povzetek: V sluznici želodcev tri tedne starih prašičev smo določali razporeditev in število enteroendokrinih celic v posameznih delih želodca. Izhodiščna za prikaz enteroendokrinih celic je bila imunohistokemična metoda, primerjalno pa smo enteroendokrine celice barvali s toluidinskim modrilom za tovrstne celice in jih srebrili po Grimeliusu. Največ gastrinskih celic smo ugotovili v vratarjevi sluznici, v antrumu 124,66 ± 4,54 gastrinske celice na mm^2 in v kanalu 140,32 ± 5,30 na mm^2. Največja gostota gastrinskih celic, glede na višino sluznice, je bila v srednji tretjini, kjer je bilo približno 50 % vseh tovrstnih celic, preostale pa so bile dokaj enakomerno razporejene v zgornji in spodnji tretjini sluznice. Na področjih s kutano sluznico ni bilo niti gastrinskih niti drugih enteroendokrinih celic. Na mali krivini želodca, kjer se prekrivajo področja brezžlezne in vratarjeve sluznice, je bilo v povprečju 29,21 ± 2,97 gastrinske celice na mm^2, na področjih s kardialno sluznico in sluznico pravih želodčnih ali fundusnih žlez pa je bilo njihovo število še manjše: 0,61 ± 0,14 celice na mm^2 kardialne sluznice ter povprečno 1,14 ± 0,11 gastrinske celice na mm^2 fundusne sluznice. Na področju vratarja, kjer je največ gastrinskih celic, smo morfometrično določili tudi število serotoninskih in somatostatinskih celic. Obeh vrst celic je bilo v sluznici tega področja značilno manj kot gastrinskih celic, in sicer serotoninskih 17,7 ± 2,30 na mm^2 sluznice ter somatostatinskih 50,7 ± 2,76. Rezultati srebrenja in barvanja s toluidinskim modrilom so se le deloma ujemali z rezultati imunohistokemične reakcije. Celic, ki so se v vratarjevi sluznici pobarvale s toluidinskim modrilom, je bilo bistveno manj kot smo jih ugotovili z imunohistokemično metodo (51,15 ± 2,20 celice na mm^2), medtem ko je v postopku srebrenja reagiralo bistveno več celic, čeprav še vedno manj kot pri imunohistokemični reakciji (153,5 ± 0,32 celice na mm^2).

Ključne besede: veterinarska medicina; želodec; enteroendokrine celice; gastrin; somatostatin, serotonin; imunohistokemija; srebrenje po Grimeliusu; toluidinsko modrilo; prašič

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Original research paper

THE ROLE OF *AEROMONAS HYDROPHILA* BACTERIUM AS A CAUSATIVE AGENT OF SEPTICAEMIA IN DOGS

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Summary: *Aeromonas hydrophila* is an organism commonly found in water, particularly during the warm season, though less commonly isolated as a pathogen in domestic animals and humans. The course of septicaemia caused by *Aeromonas hydrophila* in puppies is described to illustrate its bacterial pathogenic activity and the diagnostic procedures used. Seven of eight puppies, all clinically healthy at birth, died within ten days of birth, after receiving the same clinical course. Beside the gross pathology and histopathology, the internal organs were also bacteriologically examined. The puppies had diffuse acute fibrinous and necrotic bronchopneumonia. Infection with *A. hydrophila*, which was isolated from the liver, spleen, lungs and intestines, was determined as the cause of the sepsis and the consequent death. To establish the origin of the infection, cultures of milk, vaginal and rectal swabs taken from the dam were made and they were all negative for this bacterium. While the origin of the infection remains unknown, the underdevelopment of the puppies is thought to be a predisposing factor.

Key words: dog; septicaemia; bacterial pneumonia; *Aeromonas hydrophila*

Introduction

Aeromonas hydrophila is a non-fastidious, gram-negative rod-shaped, motile bacterium. The 1974 edition of Bergey's Manual includes it in the family *Vibrionaceae* (1). Its morphological and cultural characteristics are the same as is indicated for *Aeromonas caviae*, therefore the differentiation is difficult. As the species' name hydrophila ("water lover") indicates, the natural habitat of the microorganism is both fresh and sea water. It has been observed in numerous species of freshwater fish, the occasional salt-water fish, and in amphibians, reptiles, cattle and humans all over the world. *Aeromonas hydrophila* is recognized as an opportunistic pathogen or a secondary invader (2, 3, 4). There are some reports that describe the role of *Aeromonas hydrophila* in the pathology of mammals, however, most of them relate to humans (5, 6, 7) and there only a few describing its role in dogs (8, 9).

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The aim of this work is to show that infection with *A. hydrophila* can be severe enough to be considered a cause of death in mammals with weak immune responses.

Material and methods

Eight puppies were examined at birth and, at first, all appeared to be normal, healthy and strong. About 30 hours later, the first puppy showed signs of illness: it stopped sucking, became hypothermic and debilitated and died within 24 hours. All the puppies, except for one, developed the same symptoms and despite intensive care – warming, oxygen supplementation, supplemental feeding and glucose infusions – successively died within ten days.

The bitch, a five-year-old Doberman pinscher, in good general condition was admitted to the veterinary clinic due to difficulties in parturition. The act began on the 60th day of pregnancy and was run with caesarean section because of dystocia. During the operation the bitch was treated

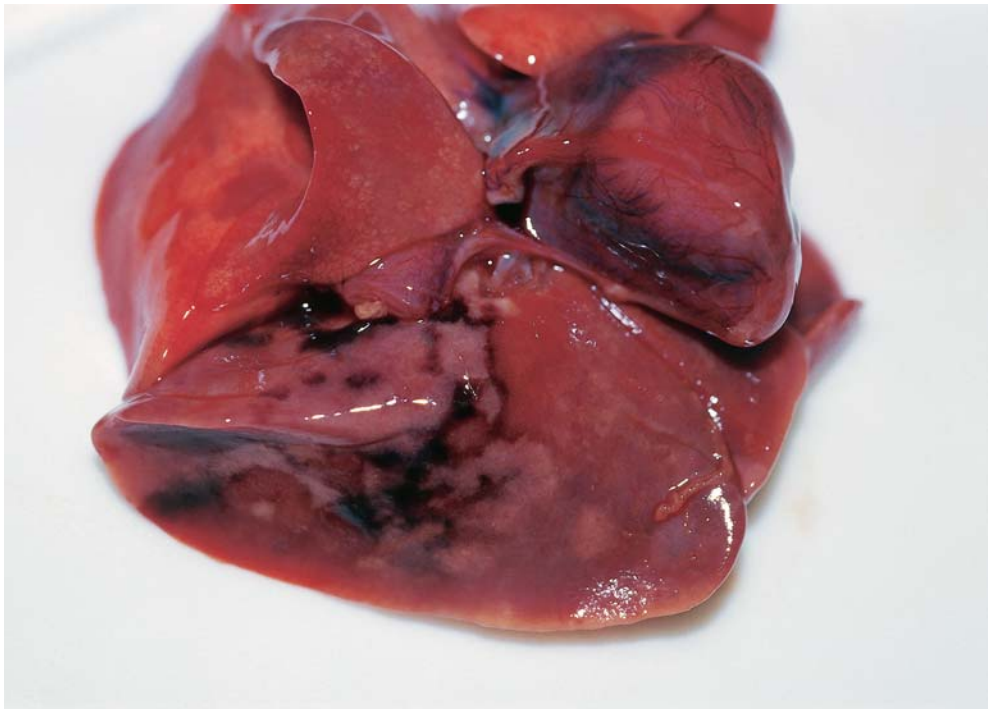

Figure 1: Lung. Acute diffuse fibrinous bronchopneumonia with multifocal areas of necrosis and haemorrhages

with antibiotics amoxycillin+clavulanic acid (Amoksiklav, Lek) and gentamicin (Gentamicin, Lek).

Two puppies were submitted for post-mortem examinations immediately after death: a female (body weight 400 g) and a male (body weight 500 g). A necropsy was performed and several tissue specimens were taken for further laboratory examinations. For the histological examinations, tissue samples from the brain, kidneys, heart, liver, lungs, spleen and intestines were fixed in a 10 % buffered formalin, routinely processed in paraffin and then stained with haematoxylin and eosin (HE). The lung and spleen samples were also treated with Grocott's methenamine silver staining and periodic acid Schiff reaction (to exclude mycotic infections), as well as Goodpasture's stain method (gram staining for tissues). Cryostatic tissue sections of the lungs, liver and kidneys were stained with Sudan III for fat. Imprints of the pleural surface were prepared for cytology, air dried, fixed in methanol and stained with Giemsa.

Samples for bacteriological examinations were taken from the liver, spleen, lungs and intestines. The material was inoculated on nutrient agar (Oxoid) supplemented with 5 % of ovine blood (BA) and Drigalski agar (DA) and incubated at 37 °C for 24 h, and on Sabouraud Dextrose Agar (SDA, bioMerieux, France) at 37 °C for five days. Subcultures for *A. hydrophila* identification were made on the BA. Simultaneously, the milk of the dam was examined and a few days later rectal and vaginal swabs were also taken from the dam. Bacteria that grew on the culture media were Gram stained (Difco-BBL) and tested for catalase and oxidase activity (Difco-BBL). The biochemical characteristics of the isolated bacteria were tested using classical biochemical tests and the Api 20NE, Api 20E and API Staph commercial systems (bioMerieux, France) in accordance with the manufacturer's instructions.

Results

The post-mortem findings of both animals were similar. The thoracic cavity was filled with a small amount of a serofibrinous, opaque inflammatory exudate. There were pulmonary lesions characteristic of acute fibrinous and necrotic bronchopneumonia with acute fibrinous pleuritis (Fig. 1). The pleura was covered with a thick layer of fibrin, the lung texture was firm, and the majority of the pulmonary tissue was heavily congested and oedematous with multifocal grey areas of necrosis on the pleural and cut surfaces. Both the liver and the kidneys were enlarged and congested, the spleen was enlarged and the intestines displayed acute catarrhal enterocolitis.

Microscopic lesions of the lung were consistent with the gross pathology findings – acute fib-

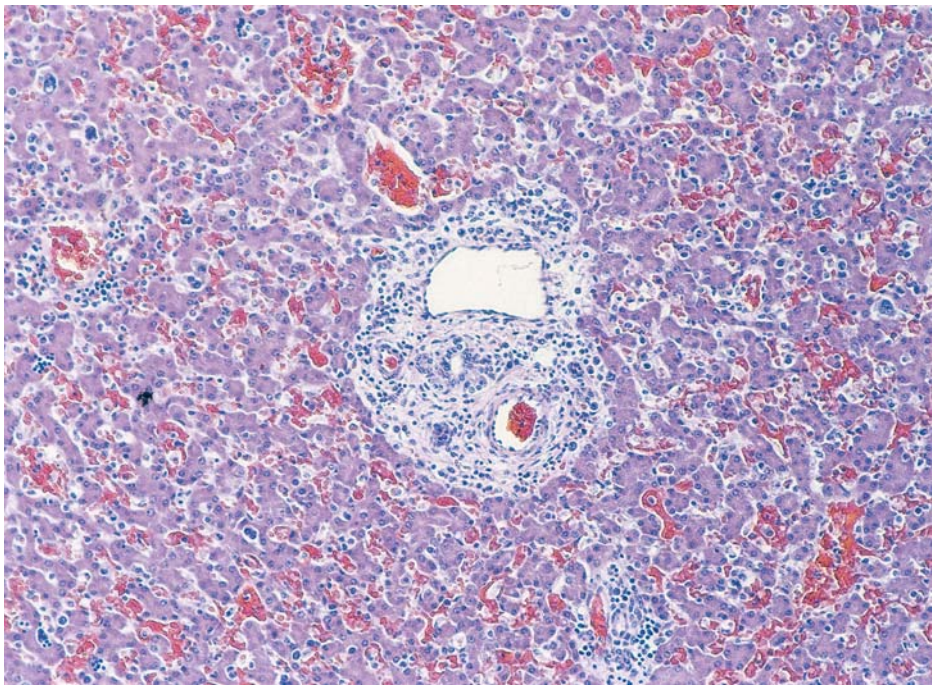

Figure 2: Liver. Many extramedullary haematopoietic foci and few macrophages located throughout the liver tissue and in portal areas. Haematoxylin & eosin, x 10

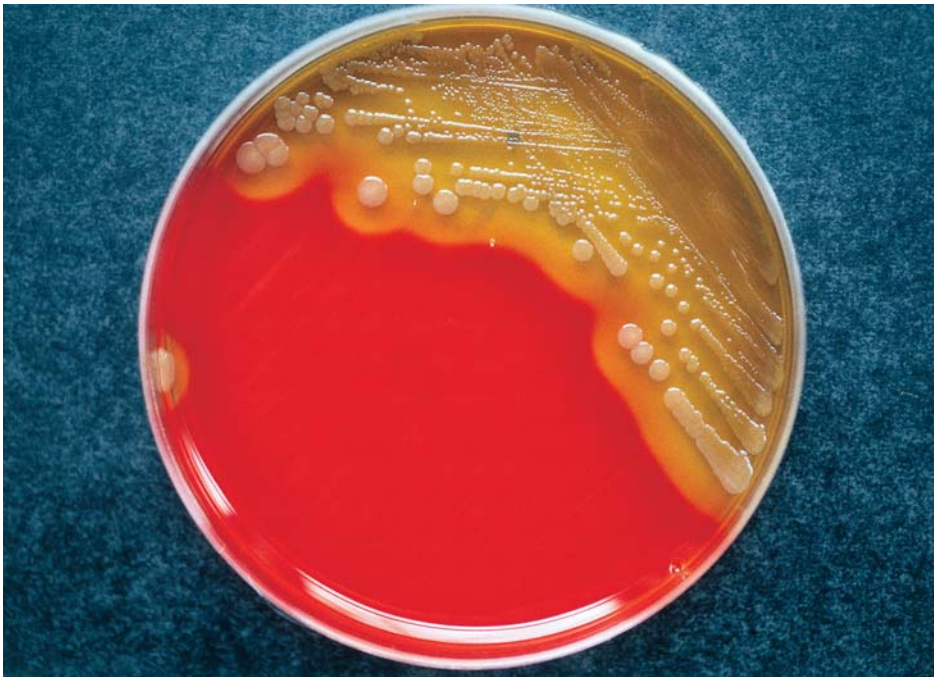

Figure 3: Culture of *Aeromonas hydrophila* with a large zone of haemolysis on 5 % ovine blood agar after 48 h incubation at 37 °C

rinous bronchopneumonia with multifocal areas of coagulative necrosis and acute fibrinous pleuritis. Several bronchi and bronchiole had necrotic walls and were filled with desquamated epithelial cells, numerous macrophages and neutrophils. The alveoli were distended and filled with large quantities of fibrin and inflammatory cells; mostly macrophages and some neutrophils. There were also large areas of multifocal coagulative necrosis. In many parts of the lung, we found numerous small rod-shaped gram-negative bacteria. Bacterial colonies of gram-negative rods were especially numerous and large in necrotic areas and were growing towards the periphery of such areas. In one puppy there were also large areas of multifocal haemorrhages. The interstitium around blood vessels was distended by oedema and contained a few macrophages, monocytes and neutrophils. Grocott's and PAS staining of the lungs and spleen established that they were negative for mycotic infectious agents. Goodpasture staining also established that the

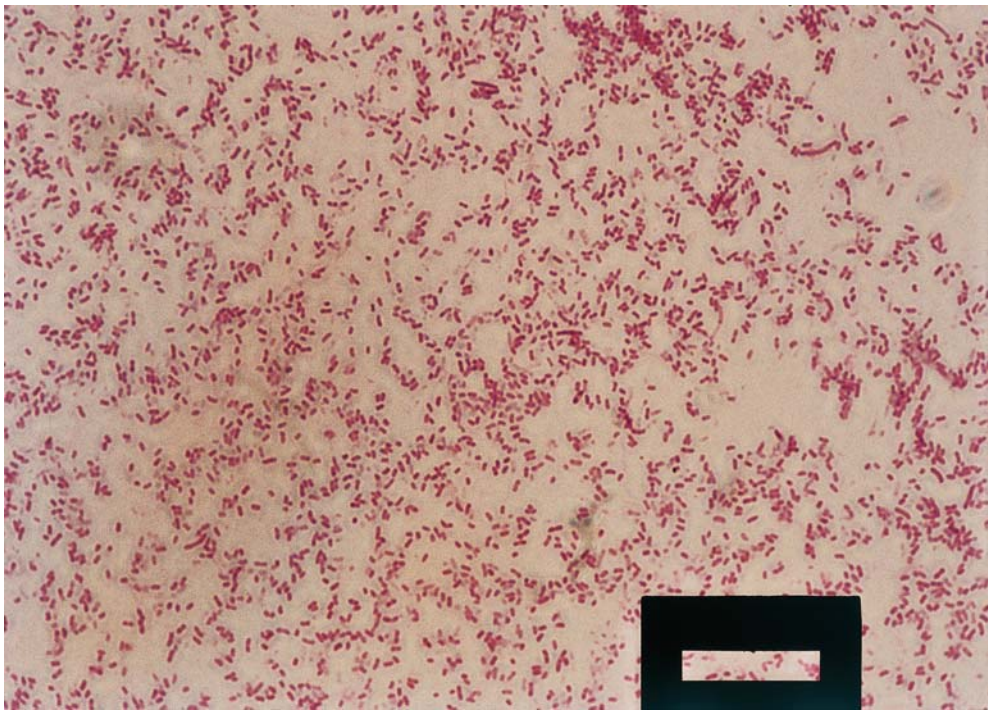

Figure 4: Gram stain of Aeromonas hydrophila ilustrating gram-negative rods

spleen was negative. The portal areas of the liver and interstitium around hepatic veins were oedematous, lymph vessels in the portal areas were distended, and portal tissues were infiltrated with numerous macrophages and a few eosinophils. Macrophages were also found scattered within the sinusoids. There were swollen hepatocytes as well as some disseminated necrotic hepatocytes, and karyolysis and cholestasis were also present. The Kupffer cells were swollen and many of them were in mitosis. There were many extramedullary haematopoietic foci with erythropoietic cells and megakaryocytes located throughout the liver tissue (Fig. 2). The kidneys were underdeveloped for the age of animals. They had foetal cortices with numerous mitoses of epithelial cells in the nephrons, and small, hyperchromatic glomerular cells forming palisades. The tubular cells were vacuolated and many revealed small accumulations of calcium salts within their cytoplasm due to tubular degeneration. The interstitium was distended by oedema. Erythrocytes in small vessels formed hyalinized cylindrical structures without obvious boundaries between cells, which was diagnosed as disseminated intravascular coagulation. The white and the red pulp of the spleen were not clearly separated. Lymphopenia was evident and a few necrotic lymphocytes were found in some follicles. The red pulp was highly cellular with diffuse extramedullary haematopoiesis, and the red pulp was congested. The epithelial lining of the small and large intestine was largely desquamated and the ratio between the intestinal villa and crypts in jejunum was approximately 4:1. Between the epithelial cells and the lamina propria in non-desquamated areas of mucosa there was a vacuolated layer of severe oedema; the propria was also oedematous and infiltrated with a small number of eosinophils. The intestinal lumen contained desquamated cells, an amorphous mass with several haemosiderin granules, and many bacteria forming colonies (mostly short rods and small cocci). In one puppy, the intestinal mucosa was strongly congested with evidence of haemorrhaging into the intestinal lumen. Lesions in other tissues were mostly congestion. Imprints of the pulmonary surface contained numerous short rod-shaped bacteria, degenerate macrophages – some of them containing bacteria in their cytoplasm, and a few neutrophilic granulocytes, erythrocytes and mesothelial cells (acute septic pleuritis).

Bacteriological cultures of the organs yielded abundant growth of large colonies (2-3 mm) that were flat, greyish, circular and convex with an entire margin and surrounded by a large zone of beta-haemolysis (Fig. 3). Gram-stained cultures demonstrated gram-negative rod-shaped bacteria (Fig. 4). A presumptive diagnosis of an *Aeromonas*

species was based initially on a positive oxidase reaction and additionally on the fermentation of carbohydrates. Our isolate produced both acid and gas from glucose and acid from arabinose, manitol, sucrose and maltose, but not from inositol or lactose. The numerical profile of the biochemical reactions in the Api 20NE system was 7577754. *Aeromonas hydrophila* was grown in an entirely pure culture, except with some rare colonies of non-haemolytic Escherichia coli in the intestines.

Bacteriological examinations of all the samples taken from the dam were negative for *A. hydrophila*. The vaginal swab yielded only a few colonies of *Staphylococcus haemolyticus*, which belongs to the group of coagulase-negative staphylococci. The bacteriological culture of the dam's rectal swab yielded non-haemolytic *Escherichia coli* and alpha-haemolytic streptococci.

Discussion

The sudden death of seven, out of eight, newborn puppies in such a short period after parturition can have many causes but an intensive infection with *Aeromonas hydrophila* was probably the fatal one. The gross pathology, the histological determination of numerous short rod-shaped bacteria in many tissues and the isolation of this bacterium in a pure culture indicated an acute septic condition.

Aeromonas hydrophila is a part of the normal flora of freshwater fish and is commonly present in fish ponds and tanks (10). Occasionally it can cause infections in humans, which range from soft-tissue infections, pneumonia, endocarditis and gastroenteritis to septicaemia (4, 11, 12). Cases of hospital infections with *A. hydrophila* were reported in humans as well (13). In dogs, *A. hydrophila* was demonstrated in a few cases as an aetiologic agent of disease, usually in young adults where it was considered an opportunistic pathogen (8, 9). That means that some other stressful factor should have been present at first. Animals can be faecal carriers of *Aeromonas* spp. (14). Ghenghes and others presented an interesting study on the presence of the *Aeromonas* species in domestic dogs and cats. They found that *Aeromonas* are not uncommon in healthy dogs and cats. Furthermore, they found this organism occurred in the Doberman breed to a higher degree than in other breeds. They emphasized that haemolysin-producing *Aeromonas* species in the faeces of healthy domestic dogs and cats may present a public health problem for humans who came into contact with them.

Numerous opportunistic bacteria can cause septicaemia in susceptible neonates. There are a few reports of neonatal *A. hydrophila* septicaemia in children that are comparable to our case. In these reported cases, the children were born at term and no signs of immaturity were present (15, 16). The aetiopathogenesis of infection in our case allows some speculation. Which factor facilitated the development of the extensive pneumonia and sudden death involving a microorganism of inherently low pathogenicity for mammals? The owner initially reported that the puppies were born at term. According to the structure of the kidney cortex and the diffuse, extensive extramedullary haemopoiesis in the spleen and liver at the age of seven days, we estimated that they were underdeveloped. Therefore immaturity could be a possible factor contributing to susceptibility for the infection and the fatal exit (17). Common canine viral infections, which can be an underlying factor for the secondary infection were ruled out as a predisposing factor because there were no morphological or histological lesions characteristic of parvoviral, distemper or herpes virus infections, and besides that the dam was vaccinated against them on a regular basis.

The pathomorphological lesions in both puppies were predominantly located in lungs. The diagnoses of pneumonia in children with *A. hydrophila* septicaemia were made after clinical and x-ray examinations only. There is no information on pathomorphological and histopathological changes in the lungs of children with septicaemia (15, 16). Pneumonia is frequently diagnosed in cases of *A. hydrophila* septicaemia (11, 18). In a study of fifteen cases of *A. hydrophila* septicaemia, extensive bilateral pulmonary lesions were found in more than half of the patients (13). The pneumonia in our case is, according to the histopathological lesions, similar to *A. hydrophila* pneumonia described in man (11). No pulmonary lesions were noted in dogs with *A. hydrophila* septicaemia (8, 9). Histopathological lesions in the lung can be partly the consequence of the aspiration of milk that occurs during supplemental feeding. But in our case that happened after the puppies devel-

oped clinical signs and was just a factor contributing to the quicker course of the disease.

Infections of the neonate may be acquired from a vaginal flora during parturition, through penetrated skin, a contaminated umbilicus or from the environment. No skin or umbilical lesions were found in any of puppies. Due to the *A. hydrophila*-negative results from all the samples taken from the dam, we don't believe that the dam was a carrier of this bacterium. On the other hand, it should be noted that the dam was on a prolonged antibiotic therapy, which could have changed intestinal bacterial flora by the time the rectal swabs were taken for bacteriology. This could explain why the *A. hydrophila* cultural examinations were unable to determine the presence of the bacterium.

Looking for other sources of infection we checked for the presence of other animals in their household that are known as carriers of *A. hydrophila*. The owners had no other animals or reptiles at all. Contaminated water or food, or even the hospital environment might have been the source of infection in this case but unfortunately we were not able to check into these possibilities. *Aeromonas hydrophila* has been implicated as a cause of gastroenteritis in humans and ingestion of contaminated water is another possible point of access for *A. hydrophila* into the intestine. The source of the puppies infection seems unlikely to be determined now since the material, taken from the dam, was negative to *A. hydrophila*. But the reason for the negative results could also be the preventative antibiotic treatment of the bitch after the caesarean section. The dam (but not the puppies) was treated with a combination of amoxycillin + clavulanic acid and gentamicin, the combination that is usually successfully used when dealing with *A. hydrophila* in fish (19). But in the present case the rapid progress of the disease was unfortunately fatal for all the puppies. According to the owner's data the dam had no further problems with infection. Six months after parturition, another rectal swab was taken and was also negative for *Aeromonas*.

To the best of our knowledge this is the first well document case of severe pneumonia caused by *Aeromonas hydrophila* in newborn puppies and their consequential sudden death with almost no chance of a successful treatment. Infection or contamination of domestic animals with *A. hydrophila* can be considered as a health risk for animals as well as humans, especially those in the early (neonatal and perinatal) and most-sensitive periods of life, particularly underdeveloped or premature individuals, and those with an impaired immune system.

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VLOGA BAKTERIJE *AEROMONAS HYDROPHILA* KOT POVZROČITELJICE SEPTIKEMIJE PRI PSIH

I. Zdovc, T. Švara, P. Juntes, T. Kotnik, M. Ocepek, M. Černe, M. Pogačnik

Povzetek: *Aeromonas hydrophila* je mikroorganizem, ki ga običajno najdemo v vodi, predvsem v toplejših obdobjih, zelo redko pa ga izoliramo kot povzročitelja bolezni pri domačih živalih in ljudeh. V prispevku je prvič natančno predstavljen primer patogenega delovanja bakterije *Aeromonas hydrophila* pri pasjih mladičih. Opisani so klinični potek septikemije, spremembe na notranjih organih in diagnostični postopki v vseh fazah preiskave. Osem novorojenih mladičev, ki so bili po porodu klinično zdravi, je v naslednjih desetih dneh postopoma poginilo z enakimi kliničnimi znaki bolezni. Poginule živali smo pregledali patoanatomsko in patohistološko, notranje organe pa tudi bakteriološko. Ugotovlili smo difuzno akutno fibrinozno in nekrotično bronhopneumonijo. V notranjih organih (jetrih, vranici, pljučih in črevesju) smo z mikroskopsko preiskavo ugotovlili številne identične gramsko negativne paličke, z gojiščno preiskavo pa je bila izolirana bakterija *Aeromonas hydrophila*. Izolacija čiste kulture, difuzna rast iz vseh pregledanih notranjih organov ter ujemanje izolata s histološkimi in kliničnimi spremembami pomeni potrditev diagnoze, da je bila bakterija povzročiteljica septikemije in posledičnega pogina mladičev. Zaradi ugotavljanja vira okužbe smo pregledali še vaginalni in rekatalni bris ter vzorec mleka psice. V nobenem vzorcu nismo ugotovili bakterije *Aeromonas hydrophila*, kar pripisujemo dejstvu, da je bila psica po porodu zdravljena z antibiotikom, za katerega je bila bakterija zelo dobro občutljiva. Najbolj verjeten vzrok za razvoj sepse pri mladičih je bila njihova nerazvitost ob porodu in velika dovzetnost za okužbo v zgodnjem obdobju, ko imunski sistem še ni opravljal svoje vloge.

Ključne besede: pes; septikemija; bakterijska pljučnica; *Aeromonas hydrophila*

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Original research paper

TREATMENT OF SUBCLINICAL STAPHYLOCOCCAL MASTITIS

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Summary: The purpose of this study was to evaluate the efficacy of a treatment of subclinical mastitis in dairy cows, caused by the *Staphylococcus aureus* bacteria strain. In both Europe and the USA *S. aureus* is the most frequently isolated pathogen present in dairy cows suffering from subclinical mastitis. Studies have shown that amoxycillin, in its own right, is not sufficiently effective in combating S. *aureus*, however, when used in conjunction with clavulanic acid its efficacy improves significantly. The infected animals were treated with Synulox®, which contains amoxycillin and clavulanic acid. In accordance with the manufacturer's instructions, the treatment was applied intramuscularly and intramammarily. In total, 61 mammary glands of 37 cows were treated. On average, the bacteriological efficacy of the treatment was 51.3 %. In animals with only one infected mammary gland the efficacy was 69.9 %. Considering that such animals represented 56.7 % of all the animals included in the study, we suggest that treating animals with only one or two infected mammary glands with Synulox® is sensible and economically justifiable.

Key words: veterinary medicine; mastitis-treatment; *Staphylococcus aureus;* amoxycillin; clavulanic acid

Introduction

The subclinical form of mastitis in dairy cows represents a significant problem in contemporary milk production, as it is associated with lower productivity and an increase in the somatic-cell count (SCC) in milk. Milk with an elevated SCC is of a lower quality due to an alteration in the quantity of the single ingredients (fat, protein, lactose, and minerals). The efficacy of subclinical-udder-infection treatments mainly depends on the species of the infectious agent and the duration of the infection. Other factors, such as the age of the animal, the preparation of the udder before milking and nutrition, also play an important role in the outcome of a mastitis treatment (2, 4).

While it is true that the percentage of animals suffering from subclinical mastitis in Slovenia is decreasing (31.3 % in 1990 compared to 21.9 % in 1997), the efficacy of treatments of such infections is also diminishing. This is as a result of the increasing resistance of microorganisms to antibiotics, and to the weaker immune systems of the animals due to increased milk production and management failures (1).

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Staphylococcus aureus (S. aureus) is, besides coagulase–negative *Staphylococcus* species, the most frequently isolated pathogen present in dairy cows suffering from subclinical mastitis in both the USA and Europe (2). On average, such infections in Slovenia account for half of all cases, however, this varies significantly within individual herds (1). In this study the *S. aureus* bacteria was more prevalent in the mammary glands of the older animals.

The outcome of a treatment of a mammary gland infection caused by *S. aureus* is very uncertain and is influenced by the age of the animal, the clinical type of infection (acute vs. chronic), the sensitivity of the infectious agent to certain antibiotics and the duration of the treatment.

In cases of chronic infection, the ability of the antibiotics to penetrate the affected tissue is poor. The fact that *S. aureus* can also be present intracellularly and in micro-abscesses represents an additional problem. In those areas it is particularly hard to achieve the appropriate concentrations of the antibiotic (3, 4).

The sensitivity of *S. aureus* to various antibiotics has diminished over time (1, 5), while the share of β-lactamase-positive strains has increased. The percentages vary significantly from country to country and ranges from 4 % in Norway to 76 % in Ireland. Very early in the devel-

opment of antibiotics an enzyme, which destroys penicillin, was described. The enzyme was termed "penicillinase" (now referred to as β-lactamase) and was found to be produced by a wide variety of bacteria. This still remains the most important method of bacterial defence to the β-lactam antibiotics (penicillins, cephalosporins etc.) (5, 6, 7, 8).

The duration of a therapy can also significantly influence the efficacy of the treatment. Acceptable results are achieved through a combination of intramuscular and intramammary applications of appropriate antibiotics over a 3 to 5 day period (9, 10).

According to other authors, the rate of success of bacteriological cures for clinical and subclinical mastitis caused by *S. aureus*, ranges from 15 % to 70 % (2, 7, 9, 10), which indicates the level of difficulty and complexity involved in the approach to treatment.

Amoxycillin in combination with clavulanic acid, which is a β-lactamase inhibitor, is one of the antibiotics that are being used with increasing regularity in the treatment of subclinical bovine mastitis. This combination was first successfully applied in human medicine in the treatment of infections caused by β-lactamase-positive strains of *S. aureus.*

Authors agree that amoxycillin in its own right is not sufficiently effective in combating *S. aureus* (7, 10, 12), however, when used in combination with clavulanic acid the efficacy of a treatment improves significantly.

Considering all the aforementioned facts we decided to test the efficacy of the amoxycillin-clavulanic acid combination in the treatment of subclinical mastitis in dairy cows caused by *S. aureus.*

Material and methods

Selection of Animals

The study included 37 dairy cows from 11 different herds, of different ages (Graph 1) and breeds, each with an increased SCC in their milk. In all cases, a microbiological test of the milk sample revealed the presence of *S. aureus.* The average SCC in milk from the infected udder quarters of the selected animals was $1428x10^3$/ml before treatment, and ranged from $210x10^3$/ml to $4057x10^3$/ml. Twenty-one animals had one infected mammary gland, 9 had two, 6 had three and in one case all four mammary glands were infected. In total, 61 mammary glands of 37 cows were included in the study.

Treatment

Synulox®, which is manufactured by Pfizer Animal Health, was used to treat the infection as follows:

- an injector with an intramammary solution containing 50 mg of clavulanic acid in the form of potassium clavulanate, 200 mg of amoxycillin in the form of Amoxycillin-Trihydrate and 10 mg of prednisolone.
- a solution for an intramammary application where 1 ml contains 35 mg of clavulanic acid in the form of potassium clavulanate and 140 mg of amoxycillin in the form of Amoxycillin-Trihydrate.

All the animals included in the study were treated in accordance with the following predetermined protocol:

- an application of the Synulox® injector into the affected udder quarter every 12 hours at 6 consecutive milkings;
- a parenteral application of the Synulox® solution, in the amount of 8.75 mg/kg, on the first and second days of the treatment with an interval of 24 hours between the doses.

Eleven days after the final application of the drug, another milk sample was collected from each of the animals. These samples were subjected to microbiological analyses and used as a control of the treatment's efficacy.

Treatment Efficacy Assessment Criteria

The efficacy of the treatment was assessed on the basis of a bacteriological examination. For the purpose of the study, a treatment was regarded as having been successful when the result of the bacteriological examination was negative for *S. aureus*.

Results

In Table 1 the distribution of cows in relation to the number of *S. aureus* infected udder quarters is presented. In 57 % (n = 21) of the selected cows only one udder quarter was infected. In 9 cows the infection was present in two, and in 6 cows in three udder quarters. There was only one case where all four quarters were infected.

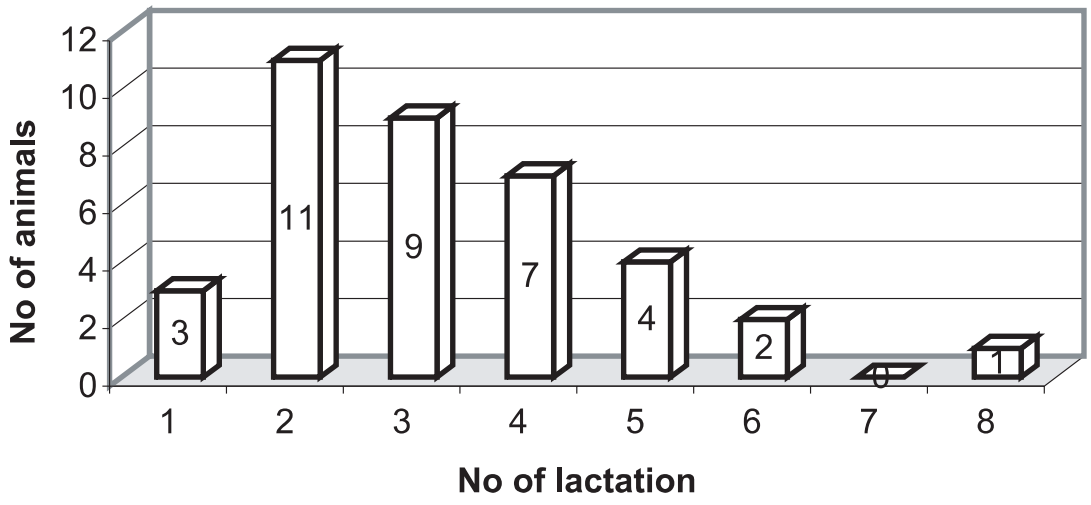
In Table 2 the results of the treatment are pre-

Table 1: Distribution of animals in relation to the number of infected udder quarters before treatment

No. of infected quarters per animal	1	2	3	4	TOTAL
No. of animals	21	9	6	1	37
No. of infected quarters	21	18	18	4	61

Table 2: Distribution of bacteriologically negative (successfully treated) animals in relation to the number of infected udder quarters before treatment

No. of infected quarters per animal	1 n = 21	2 n = 9	3 n = 6	4 n = 1	TOTAL	Bacteriological cure rate
No. of cured animals	13	5	1	0	19	51.3
No. of cured quarters	14	13	9	0	36	59.0
% of cured animals	61.9	55.5	16.7	0		
% of cured quarters quarters m.g	66.6	72.2	50.0	0		



Graph 1: Distribution of infected cows per lactation

sented in relation to the number of initially infected udder quarters. In our study we found a significant correlation between the number of successfully treated animals and the number of infected quarters. However, the difference in the percentages of successfully treated udder quarters was less significant.

Discussion

Synulox® was used in the treatment of 37 animals with one or more mammary glands infected by *S. aureus.*

The treatment protocol described above was selected in accordance with the claims of most authors that an efficient and effective treatment of subclinical and clinical mastitis caused by *S. aureus* takes at least 3 to 5 days (9.10).

The amoxycillin-clavulanic acid combination in the therapy was selected due to the high probability of the presence of ß-lactamase-positive strains *of S. aureus* in the infected animals (5, 6, 7).

There were differing opinions amongst authors regarding the best number of control samples to collect and when to collect them (7, 9, 10), hence, given our circumstances, we settled on one sample collection 14 days following the first application of the drug.

While the overall level of success using this bacteriological treatment (51.3 %) does not deviate substantially from claims made in other publications, it is clearly among the more successful methods, particularly as only subclinical types of bovine mastitis were treated (2, 7, 9, 10). The level of success that was achieved in treating animals with only one infected mammary quarter

(61.9 %) was very promising as they represented the majority (56.7 %) of all the treated animals. The comparative success of the treatment in our case is slightly diminished by the fact that in certain cases (n = 4), despite a successful bacteriological cure, the SCC did not fall below the level set down in the regulations governing the health and hygiene of milk (< 400,000 cells/ml).

While most of the infected animals were in their 2nd or 3rd lactation, which represents a lower average compared to some authors (2), we could not confirm a link between the number of lactations and the efficacy of the treatment.

Given our results we believe that the use of a combination of amoxycillin and clavulanic acid lived up to our expectations, and that the therapy of animals with only one or two infected mammary glands is sensible and economically justifiable. In our opinion however, it makes no sense to treat animals with three or four infected udder quarters irrespective of the type of therapy. In such cases culling should be seriously considered.

So far only a few studies of this type have been conducted in Slovenia. However, these previous studies considered both the clinical and subclinical forms of bovine mastitis together and therefore the results are not comparable (13, 14).

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ZDRAVLJENJE SUBKLINIČNIH STAFILOKOKNIH MASTITISOV

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Povzetek: Namen raziskave je bil oceniti uspešnost zdravljenja subkliničnih mastitisov pri kravah molznicah, povzročenih z bakterijsko vrsto *Staphylococcus aureus*. *S. aureu*s je najpogosteje izolirana patogena bakterija pri živalih s subkliničnim mastitisom v ZDA in v Evropi. Podatki iz literature kažejo, da amoksicilin sam ni dovolj učinkovit proti S. aureus, v kombinaciji s klavulansko kislino pa se učinek zdravljenja bistveno izboljša. Okužene živali smo zdravili s preparatom Synulox®, ki vsebuje amoksicilin in klavulansko kislino. Preparat smo po navodilu proizvajalca aplicirali v mišico in v mlečno žlezo. Skupno je bilo zdravljenih 61 vimenskih četrti pri 37 kravah. Uspešnost bakteriološke ozdravitve je bila v povprečju 51,3 %, pri živalih z eno okuženo vimensko četrtjo pa 61,9 %, in sicer ob dejstvu, da so le-te predstavljale 56,7 % vseh živali v raziskavi. Glede na rezultate menimo, da je uporaba kombinacije amoksicila in klavulanske kisline upravičila naša pričakovanja in da je zdravljenje živali z eno ali dvema okuženima četrtma smiselno in ekonomsko opravičljivo.

Ključne besede: veterinarska medicina; mastitis - zdravljenje; *Staphylococcus aureus;* amoksicilin; klavulanska kislina

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Original research paper

USING OXALIC ACID FOR VARROA MITE CONTROL IN HONEYBEE COLONIES DURING THE BEEKEEPING SEASON

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Summary: Twenty-one *Apis mellifera carnica* honeybee colonies were used to record the levels of Varroa mite mortality in the periods before and after oxalic acid (OA) treatments, which were conducted after the honey harvesting. The colonies each received from four to seven OA treatments. During the pre-treatment period, the daily natural mite mortality was estimated at 0.56 (±0.74). A high correlation was established between the daily pre-treatment mite mortality and the cumulative total of dead mites after each of the consecutive OA treatments (R = 0.92387). In the colony with the lowest daily mite mortality (0.08 ±0.05) there was no correlation with the number of mites that fell after the initial OA treatments conducted on August 1. The relative mite mortality ranged from 7.78 % (±1.68) during the brood period to 88.87 % (±8.41) in colonies without brood. Reducing a colony's mite population by employing OA treatments and a mite-control programme are discussed in this study.

Key words: honeybee; *Apis mellifera;* varroa control; oxalic acid

Introduction

Varroa destructor, a parasite of *Apis mellifera,* has to be controlled by the regular use of acaricides in order to maintain honeybee colonies. These are usually synthetic and their lipophilic and persistent characteristics result in a build-up of their residues in the wax and honey (1). Acaricide resistant mites have appeared in several European countries (2, 3). Natural, non-toxic substances to control varroa mites, such as organic acids (4) and essential oils (5, 6), have been developed and are increasingly being used by beekeepers (7).

Oxalic acid is a natural constituent of honey and EU regulations permit its use in biological beekeeping (EU Council Regulation, No. 1804/1999). Because of its high efficacy, OA is widely used in most Western European countries (7). Research has been conducted into the efficacy of oxalic acid applications (OA) as a method for controlling the mite in colonies, both with and without brood (8, 9, 10, 11).

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Experiments have been conducted in honeybee colonies by spraying (9), evaporating (12) and trickling an OA-water solution into the hive (13, 14, 15). During broodless periods, Radetzki (16), Nanetti et al. (17) and Imdorf et al. (18) found it to be highly effective in killing the mite and they estimated the elimination level at 97.3 %, 98.3 % and 99.5 %, respectively. When a capped brood was present, Mutinelli et al. (15) achieved 95 % efficacy after three treatments of a 5-%-OA solution and Brřdsgaard et al. (10) reported a 24 % efficacy of one spring treatment administered by trickling.

In our previous experiments 50 millilitres of an OA solution was used to treat one normally developed colony. Three OA treatments had an efficacy of 39.2 % when a brood was present and 99.4 % when there was no brood (13). This paper presents data from the periodic checking of the number of mites that had fallen onto the bottom of hives to determine the natural mite-fall. The aim was to establish the effectiveness of OA as a single substance for controlling varroa in honeybee colonies by using a sucrose-in-water solution (14). We also aimed to establish the optimal strategy for using oxalic acid applications to control mites during the

2002 season in colonies with capped broods and for winter treatments of broodless colonies.

Materials and methods

Twenty-one *Apis mellifera carnica* honeybee colonies, populated in national standard AŽ "back load" hives (19) with nine combs (41 x 26 cm) in each brood and honey compartment, were located at one site near Vipava. In the spring of 2002, metal sheets (38 x 29.8 cm) were placed on the floor of each of the hives in order to record the hives' natural mite mortality. On the sampling dates, the numbers of mites were recorded. The pre-treatment natural mite fall of each of the colonies was recorded on 6 different occasions for those colonies whose initial OA treatment was on August 1 and on 9 different occasions for those colonies whose treatment began on August 8. The mite mortality after each of the consecutive OA treatments was also recorded.

The number of OA treatments each colony received was determined after establishing the mite mortality before and after each treatment. The treatments were performed as follows:

- Group A, which consisted of five colonies (Nos. 1, 2, 8, 14, 19), received 7 OA treatments; on August 1, 8 and 20, September 7, October 10, November 14 and December 24;
- Group B – four colonies (6, 9, 17, 31) – received 6 OA treatments; on August 1, 8 and 20, September 7, November 14, and December 24;
- Group C – four colonies (11, 20, 21, 24) – received five OA treatments; on August 1, 8 and 20, November 14, and December 24;
- Group D – three colonies (4, 23, 29) – received five OA treatments; on August 8 and 20, September 7, November 14, and December 24;
- Group E – five colonies (3, 5, 7, 27, 28) – received four OA treatments; on August 8 and 20, November 14, and December 24.

The treatments were applied to each colony by trickling the OA solution over the combs, *in situ*, and squirting the bees in the brood compartment using a syringe. Respiration masks, protective glasses and rubber gloves were worn while applying the solution. The mite fall during the treatment period was recorded after each OA application.

The outside temperatures during the August OA treatments ranged from 30 to 33 ^{0}C. On September 7 the outside temperature was 24 ^{0}C, on October 10 it was 22 ^{0}C, on November 14 it was between 10 and 12 ^{0}C and on December 24 it was 6 ^{0}C.

The colonies received 50 millilitres of a 2.9 % OA and 31.9 % sucrose-in-water solution (w/w), using oxalic acid dihydrate (Riedel-de Haën), sucrose (sugar) and de-mineralised water (Gregorc and Planinc, 2001).

The percentages of mites killed by the experimental treatments (FTB) were estimated using the formula: FTB = FOA1 / (FOA1 + FOA2) x 100 (Gregorc and Planinc, 2001). FOA1 is the total number of mites that dropped during the consecutive treatments of colonies with capped broods and FOA2 is the number of mites that fell during the December treatment of the broodless colonies.

The efficacy of the treatments was also estimated by comparing the numbers of mites that fell before and after the treatments and the mite mortality between the consecutive OA treatments. The data analyses were performed by ANOVA (analysis of variance) with the use of the Statgraphic (20) programme.

Results

During the pre-treatment observation periods, a total of 39 days performed in intervals between May 2 and August 1, 2002, the average daily natural mite-death was estimated at 0.56 (±0.74). The average mortality per colony during the total observation period was estimated at 21.71 (±29.01) mites. In this period 1.45 % (±0.83 %) of the total varroa mite population died naturally. The average numbers of mites that dropped onto the bottom boards of each group are shown in Table 1.

Mite mortality after the first OA treatment was significantly higher ($P<0.01$) in the colonies of group A than it was in groups B, C, D and E. Statistically significant differences were also found between these groups. The number of mites that fell after the first OA treatment of the highly-infested colonies of group A correlated with the daily mite mortality prior to treatment (R = 0.81398) (Fig. 1). A high correlation was found when the total number of dead mites (R = 0.8851387) and the daily mite mortality (R = 0.92387) during pre-treatment period were compared to the cumulative total of dead mites observed after each of the consecutive OA treatments.

Table 1: The average (±SD) mite mortality after consecutive OA treatments. The number of mites that fell naturally prior to treatment and the % of natural mite mortality prior to the treatments compared to the total number of mites that fell during the experiment. The data relate to the five groups of colonies, each of which were exposed to a different number of OA treatments

Group	Mite mortality after the OA treatments (±SD)	Mite mortality prior to the OA treatments (±SD)	Mite mortality (%) prior to the OA treatments (±SD)
A	3107.80 (±1622.57)	60.00 (±40.84)	1.94 (±0.81)
B	1248.00 (±553.12)	1.00 (±1.15)	0.06 (±0.08)
C	754.25 (±331.82)	13.25 (±5.56)	1.77 (±0.66)
D	831.00 (±176.55)	14.33 (±4.04)	1.68 (±0.13)
E	520.40 (±271.14)	0.00 (±0.00)	0.00 (±0.00)

The cumulative mite mortality after the OA treatments of the different groups and the natural daily mite mortality prior to the OA treatments are shown in Figure 2. In the colony that had the lowest average daily mite mortality (0.08 ±0.05) there was no statistically significant correlation with the number of mites that fell after the initial OA treatment conducted on August 1.

In the colonies of group A, the average mite mortality after the first OA treatment was 204.4 (±81.60). The mite mortality after each OA treatment ranged from 114.4 (±150.80) mites after the seventh treatment conducted on December 24 to 1065 (±605.38) mites after the fifth treatment on October 5 (Figure 3). The number of mites that fell after the first OA treatment on August 1 was, on average, 341.48 (±352.17) times higher than the average daily natural mite mortality (0.55 ±0.78) monitored in the pre-treatment period.

The relative mite mortality during the brood period ranged from 7.78 % (±1.68) recorded after the first OA treatment conducted on August 1 to 88.87 % (±8.41), which was recorded after the November 11 OA treatment when the colonies were without brood. The relative values of mites that fell during the OA treatments are shown in Figure 4.

All the colonies had queens throughout the experiment and the normal death rate of the worker bees was not altered by the treatments. The development of the colonies over winter and the spring of 2003 were normal and comparable to the development of other colonies in the region.

Discussion

Counting the mites that drop onto the bottom board is a reliable diagnostic method (21). The correlation between the high natural mite mortality and the number of mites that fell after the OA treatments indicates the importance of measuring the natural mite mortality prior to treatments in order to accurately establish the degree of a colony's infestation.

The natural mite-mortality per day, which until the August OA treatments was estimated at an average of 0.56, increased up to a maximum of 1.54 mites per day. The colonies of the experimental apiary showed variations in the levels of mite infestation. The high daily natural mite-mortality correlates with the numbers of mites that fell after the first and subsequent OA treatments. The total mite mortality after several OA treatments also correlates with the pre-treatment natural mite mortality. A relationship between the mites in the hive debris and the mite population (22) is evident in colonies with approximately one mite "drop down" per day. In colonies with a low mite mortality (approximately 0.5 mite per day), the correlation is not as evident and considerable differences in mite mortality after the OA treatments are found. In these colonies the cumulative mite mortality during the OA treatments averaged 701 (±161) (Fig. 2).

It is important to continuously monitor the number of mites dropping in bee colonies in order to establish the appropriate timing and sequence of the summertime OA treatments. It seems that for colonies with approximately 1.5 natural mite-deaths per day a suitable programme of OA treatments should be established. In our experiment the reduction of the mite population by 7.78 % after the first, and 9.2 % after the second OA treatment ensured that the colonies remained viable. The efficacy of further OA applications increased up to 53.4 % and 88.9 % after the October and November OA treatments, respectively. The results of our experiment confirmed that using OA to treat

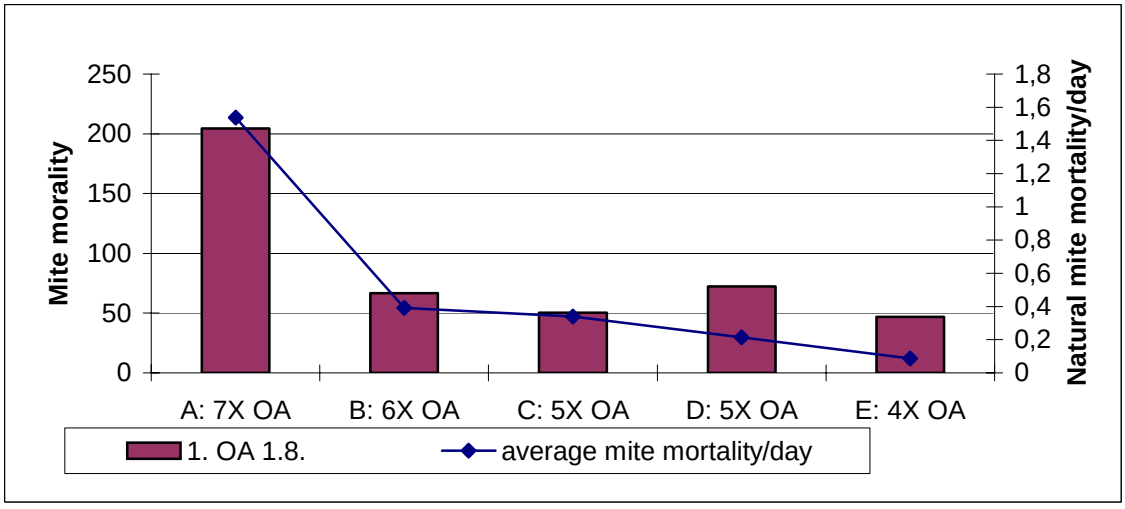

Figure 1: Diagram showing treatments groups (A-E) and the number of mites that fell after the first OA treatment conducted on August 1. The average natural mite mortality/day during the pre-treatment observation period is also shown to illustrate the high correlation between the two sets of figures (R = 0.81398)

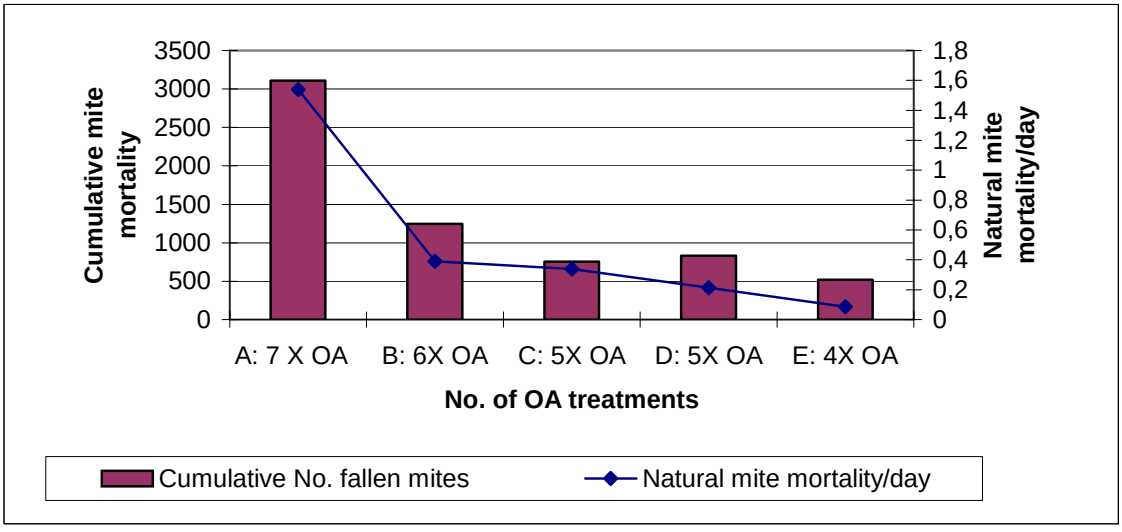

Figure 2: The cumulative mite mortality following the oxalic acid applications during the experiment is shown separately for each group. The average daily natural mite mortality during the pre-treatment observation period is also shown. As indicated, the colonies in each group received between four and seven treatments

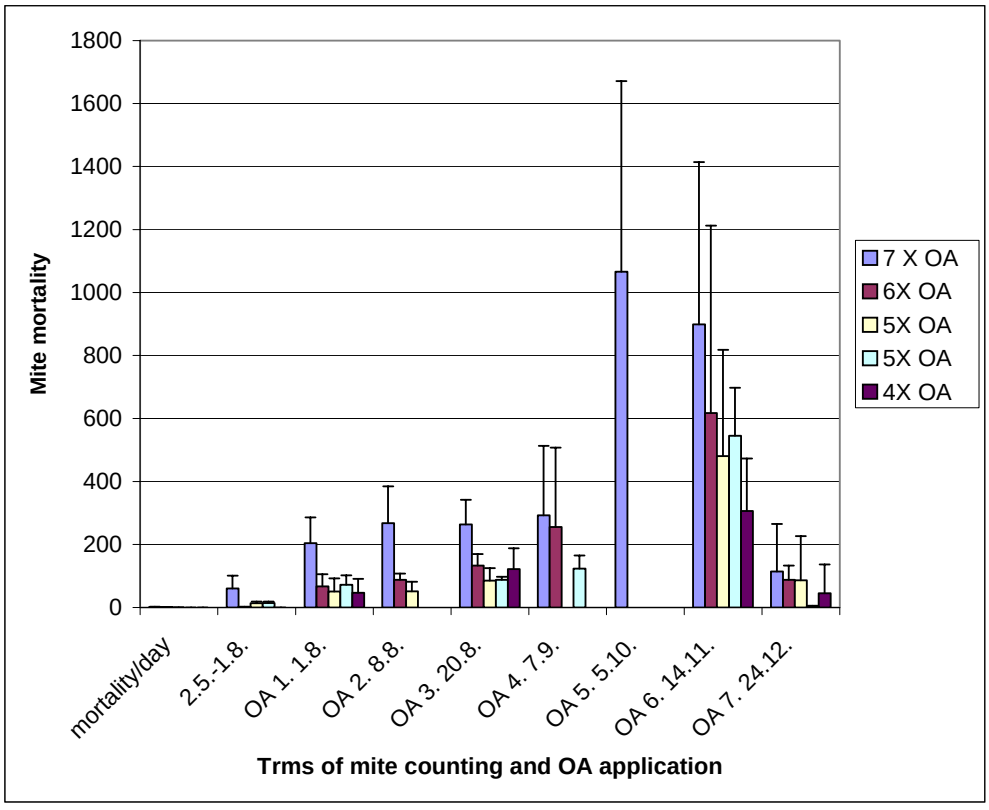

Figure 3: Mite mortality after each of the OA applications. All colonies received OA treatments on August 8, August 20, November 14 and December 24. The colonies in group A, which had a higher mite mortality during the experiment, were additionally treated during the brood period on August 1, September 7 and October 5. The results achieved by the final OA treatment, conducted on December 24, indicate that there were very few mites remaining in the colonies that were by then broodless and preparing for overwintering. Bars indicate standard deviation

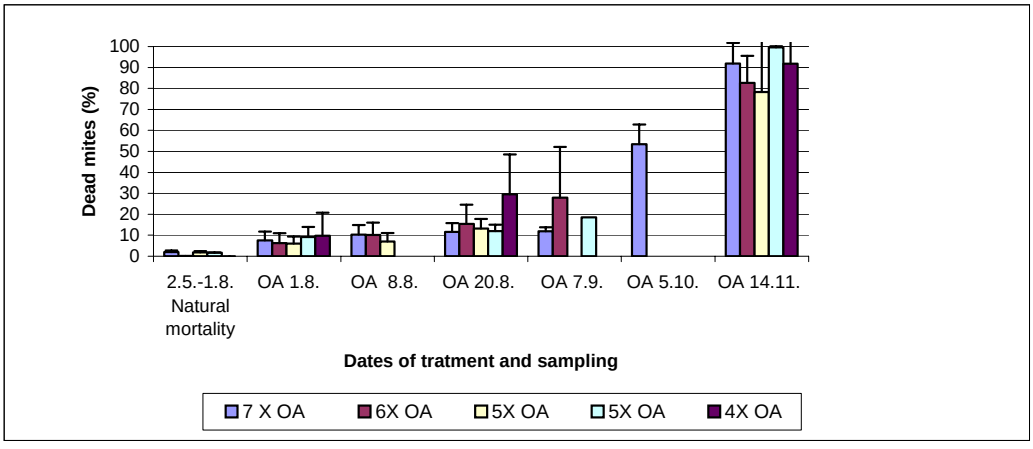

Figure 4: Relative OA efficacy expressed as a percentage of mites killed. The first set of columns labelled "2.5. - 1.8. Natural mortality" represents the percentage of mites that died naturally during the pre-treatment observation period. The other sets of columns represent the cumulative mite mortality after each OA treatment relative to the total mite mortality observed throughout the experiment. Bars indicate standard deviation

colonies without a capped brood is highly effective (13, 17). The consecutive OA treatments of colonies with brood initially had an efficacy of 12 % and 23 % (August), which rose to 51 % after the November treatment (13). Brødsgaard et al. (1999) established similar levels of OA efficacy against the mites and Gregorc and Poklukar (23) achieved a level of approximately 21 %.

The efficacy of the OA applications that we applied during the season are understated because mite reproduction within the colonies and mite re-invasion from both control and neighbouring colonies were not taken into account. Reducing a colony's mite population by employing OA treatments after the honey extraction is essential to ensure its normal development and wintertime survival. The use of an OA-only mite-control programme in a honeybee colony is an effective and useful method of reducing the mite population and helping the colony develop in specific climatic and geographical conditions.

Further experiments must be conducted in order to establish how to manage separate and specific treatments of highly-infested colonies within an apiary and to establish a method to evaluate the degree of a mite reinvasion.

Acknowledgements

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UPORABA OKSALNE KISLINE ZA ZATIRANJE VAROJ V ČEBELJIH DRUŽINAH V ČEBELARSKI SEZONI

A. Gregorc, I. Planinc

Povzetek: V enaindvajsetih čebeljih družinah smo ugotavljali naravni odpad varoj pred dajanjem oksalne kisline (OA) in po njem. V poskusu so bile čebelje družine v devetsatnih AŽ panjih. Kovinski testni vložki (38 x 29,8 cm) so bili vstavljeni v podnico panja spomladi 2002. Oksalna kislina je bila uporabljena po končanem pašnem obdobju in po iztočenju medu iz panjev. Družine so bile zdravljene od štiri- do sedemkrat. Vsaka družina je dobila 50 ml vodne raztopine oksalne kisline (2,9 % oksalne kisline in 32,9 % sladkorja v vodi). Odstotek mrtvih varoj na testnem vložku smo izračunali na osnovi števila odpadlih varoj po posameznem zdravljenju in ga primerjali s skupnim številom odpadlih varoj v posamezni družini v celotnem poskusu. Učinkovitost posameznega dajanja oksalne kisline smo ugotavljali s primerjavo odmiranja varoj po dodajanjih in analizirali varianco posameznih parametrov. Pred zdravljenjem je naravno odpadlo na dan 0,56 (±0,74) varoj. Korelacija je bila ugotovljena med dnevnim naravnim odpadom pred uporabo oksalne kisline in kumulativno vrednostjo odpadlih varoj po zaporednem dodajanju oksalne kisline (R = 0,92387). V družini z najnižjim naravnim odpadom varoj (0,08 ± 0,05) ni bilo korelacije s številom odpadlih varoj po prvem dodajanju oksalne kisline 1. avgusta. V času, ko je bila v družinah zalega, je bil relativni odpad varoj po uporabi oksalne kisline 7,78 % (±1,68), odpad v času brez zalege pa je bil 88,87 % (±8,41). V prispevku obravnavamo možnosti zmanjševanja populacije varoj v čebelji družini z uporabo oksalne kisline.

Ključne besede: medonosna čebela; *Apis mellifera;* zatiranje varoj; oksalna kislina

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Original research paper

CHANGES IN THE CLOTTING TIMES AND FIBRINOGEN CONCENTRATIONS IN HORSES DURING A SHOWJUMP

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Summary: Changes in the clotting time and fibrinolytic activity of 5 horses were assessed during a showjumping event. Venous blood samples were collected at rest prior to the trial, immediately after it and again 30 min. and 60 min. later. All the samples were immediately centrifuged at 2600 rpm for 10 min. and a Clot 2 coagulometer (SEAC, Italy) was used to assess the Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT) and the fibrinogen levels of the obtained plasma. The only statistically significant differences observed during the analysis of the results were in the aPTT and fibrinogen values. Bonferroni's multiple comparison test showed that there was a statistically significant decrease ($P < 0.05$) in the aPTT immediately after the trial. It also showed that there were statistically significant decreases in the levels of fibrinogen 30 min. ($P < 0.001$) and 60 min. ($P < 0.05$) after the trial compared to those at rest, and 30 min. ($P < 0.01$) after the trial in comparison with those immediately after the trial. The results suggest an increase in blood coagulability similar to the human athlete.

Key words: coagulation; clotting time; fibrinogen; horse; physical exercise

Introduction

The effects of exercise on blood clotting and fibrinolysis have been extensively investigated. However, the extent of fibrinolysis and fibrinogenolysis after the fibrinolytic activation that occurs with exercise is still unclear.

While some investigators found increased levels of fibrin degradation products (FDPs) and evidence of fibrinogen degradation associated with exercise (1), others did not (2, 3). This could be due to differences in the duration, strenuousness and types of exercise or in the different assay methodologies.

Hunter (4) was among the first to report that the blood of animals that had been run to death was incoagulable, suggesting that exercise affects haemostasis. Fibrinolytic activity is also enhanced during exercise in healthy human beings and the magnitude of this enhancement correlates with both the intensity and duration of the exercise. Increased fibrinolytic activity appears to counterbalance exercise-induced increases in coagulability (5).

The effects of physical conditioning on blood clotting and fibrinolysis have been investigated (6, 7, 8), however, how physical conditioning affects fibrinolytic activity while at rest and exercise-induced fibrinolysis remains unclear.

The purpose of this study was to determine whether clotting times and fibrinolysis in horses are affected by maximal exercise during showjumping events.

Material and methods

Five clinically healthy and traditionally trained Sella Italiana horses, with an average age and body weight of 7 years and 450 ±35 kg, were used in our study. They were fed three times a day: at 07:00 on hay, at 13:00 on concentrates and at 19:00 on both hay and concentrates.

All the subjects underwent one month of preagonistic training, composed of five 1-hourly sessions per week. These were spent walking (15 min.), trotting (25 min.), galloping (10 min.) and jumping (2 jumps). A further two sessions per

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week were dedicated to jumping, with each horse jumping 10 obstacles between 1 and 1.10 m high.

The contest was a 350-m jump trial with 14 obstacles. All the horses were subjected to a 20-minute warm-up before the trial (5 minutes of walking, 5 minutes of galloping and 10 minutes spent jumping 6 different jumps ranging from 80 to 120 cm in height).

The blood samples, which were all collected through jugular venepunctures, were taken at rest prior to the trial, immediately after the trial and again 30 and 60 minutes after the trial. Two types of vacutainer tubes (Terumo Corporation, Japan) were used to collect the samples. Using a Hemat 8 double-capillary automatic cell counter (SEAC, Italy), the blood samples collected in EDTA vacutainer tubes were assessed in order to establish the values of the following parameters: red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (PLT).

Blood samples that were collected in vacutainer tubes that contained 3.8 % sodium citrate were immediately centrifuged at 2600 rpm for 10 min. and the Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT) and the fibrinogen levels of the resulting plasma were assessed using a Clot 2 coagulometer (SEAC, Italy).

As the intragroup variance was not significant, the statistical elaboration of the data was carried out on the mean values of the clotting and haematological parameters studied.

An analysis of variance (one-way and repeated measures ANOVA) was applied in order to evaluate the statistically significant differences between the experimental conditions (at rest *vs* immediately after the trial, 30 min. after the trial and 60 min. after the trial; immediately after the trial *vs* both 30 min. and 60 min. after the trial; and 30 min. after the trial *vs* 60 min. after the trial). If ANOVA showed an acceptable level of significance ($P < 0.05$) then Bonferroni's test was applied for a post hoc comparison.

Results

Tables 1 and 2 show the mean values of both the clotting (PT, aPTT, TT and fibrinogen) and the haematological parameters (RBC, WBC, Hb, Hct, MCV, MCH, MCHC and PLT), as well as the relative standard deviations, standard errors and statistically significant differences, of blood samples that were obtained from 5 horses that participated in an official showjumping event. The samples were taken under four different experimental conditions: at rest, immediately after the trial, 30 min. after the trial and again 60 min. after the trial.

Figure 1 shows the variations in the clotting parameters (PT, aPTT and TT) observed under those conditions.

Figure 2 is a scatter graph that shows the fibrinogen mean values obtained under the four experimental conditions.

After analysing the results, the only statistically significant differences we observed among the clotting parameters considered were those for aPTT ($F(3, 12) = 5.59$, $P < 0.01$; ANOVA for repeated measures) and fibrinogen ($F(3, 12) = 14.04$, $P < 0.0003$; ANOVA for repeated measures).

Bonferroni's multiple comparison test showed that there was a statistically significant decrease in aPTT ($P < 0.05$) immediately after the trial. It also showed that there were statistically significant decreases in fibrinogen both at 30 min. ($P < 0.001$) and 60 min. ($P < 0.05$) after the trial when compared to the at-rest values, and at 30 min. ($P < 0.01$) after the trial when compared to the values immediately after the trial. We observed statistically significant differences in the following haematological parameters: RBC ($F(2, 10) = 63.66$, $P < 0.0001$; ANOVA for repeated measures), WBC ($F(2, 10) = 13.72$, $P < 0.001$; ANOVA for repeated measures), Hb ($F(2, 10) = 62.54$, $P < 0.0001$; ANOVA for repeated measures), Hct ($F(2, 10) = 80.28$, $P < 0.0001$; ANOVA for repeated measures), MCH ($F(2, 10) = 36.07$, $P < 0.0001$; ANOVA for repeated measures), MCHC ($F(2, 10) = 127.6$, $P < 0.0001$; ANOVA for repeated measures) and PLT ($F(2, 10) = 14.02$, $P < 0.001$; ANOVA for repeated measures).

When Bonferroni's multiple comparison test was applied, RBC showed a statistical increase immediately after the trial ($P < 0.001$) as well as 30 min. after the trial ($P < 0.05$) compared to the at-rest value, and a statistical decrease 30 min. after the trial ($P < 0.001$) compared to the value immediately after the trial. WBC showed a statistically significant increase immediately after the trial ($P < 0.0l$) compared to the at-rest value. Hb

Table 1: Mean values of the clotting parameters, together with the relative standard deviations, standard errors and statistically significant differences, of blood samples that were obtained under different experimental conditions from 5 horses that participated in an official showjumping event

PARAMETER	Experimental conditions											
	Rest			Trial			After 30 min.			After 60 min.		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
PT(sec)	10.90	0.43	0.19	10.45[a]	0.15	0.07	10.66	0.09	0.04	10.50	0.16	0.08
aPTT(sec)	42.76	3.39	1.52	34.44	4.67	2.01	38.58	3.30	1.47	40.50	1.52	0.68
TT(sec)	20.34	1.98	0.88	21.03	2.51	1.12	20.26	1.27	0.57	20.28	2.09	0.94
Fibrinogen (mg/dl)	174.90	4.79	2.14	168.40	4.93	2.20	155.80[b]	4.27	1.91	162.60[a]	3.36	1.50

Explanation: a = P < 0.05 *vs* rest
b = P < 0.001 *vs* rest

Table 2: Mean values of the haematological parameters, together with the relative standard deviations, standard errors and statistically significant differences, of blood samples that were obtained under different experimental conditions from 5 horses that participated in an official showjumping event

PARAMETER	Experimental conditions											
	Rest			Trial			After 30 min.			After 60 min.		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
RBC (M/fiL)	6.93	0.40	0.17	9.69[a]	0.62	0.27	7.37[c]	0.44	0.20	7.28[d]	0.44	0.20
WBC (K/|iL)	6.70	0.76	0.34	8.21[b]	0.97	0.43	7.47	0.90	0.40	7.63	0.96	0.43
Hb fe/dL)	10.66	1.02	0.45	15.04[a]	1.11	0.50	11.42[a]	0.52	0.23	11.22	0.54	0.24
Hct (%)	33.04	3.41	1.53	47.90[a]	4.32	1.93	34.06[a]	1.61	0.72	33.82[d]	1.79	0.80
MCV(£L)	47.40	2.51	1.12	47.80	1.48	0.66	46.20	2.49	1.11	46.60	2.19	0.98
MCH(pg)	15.34	0.64	0.29	15.04[a]	0.60	0.27	15.54[a]	0.89	0.40	15.42	0.87	0.39
MCHC (g/dL)	32.28	0.29	0.13	31.46[a]	0.71	0.32	33.54[c]	0.40	0.18	33.12	0.34	0.15
PLT(K/nL)	136.40	39.78	17.79	147.80[b]	22.90	10.24	133.20[a]	12.05	5.39	138.80	18.58	8.31

Explanation: a = P < 0.001 *vs* rest b = P < 0.01 *vs* rest
c = P < 0.05 *vs* rest d = P < 0.001 *vs* trial

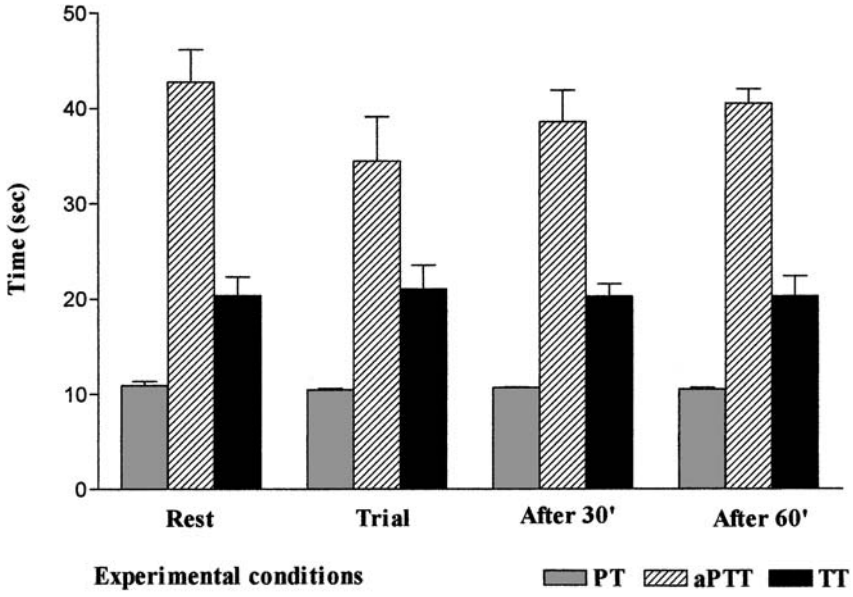

Figure 1: Variations in the clotting parameters (PT, aPTT and TT) of blood taken from 5 horses under different experimental conditions during an official showjumping event

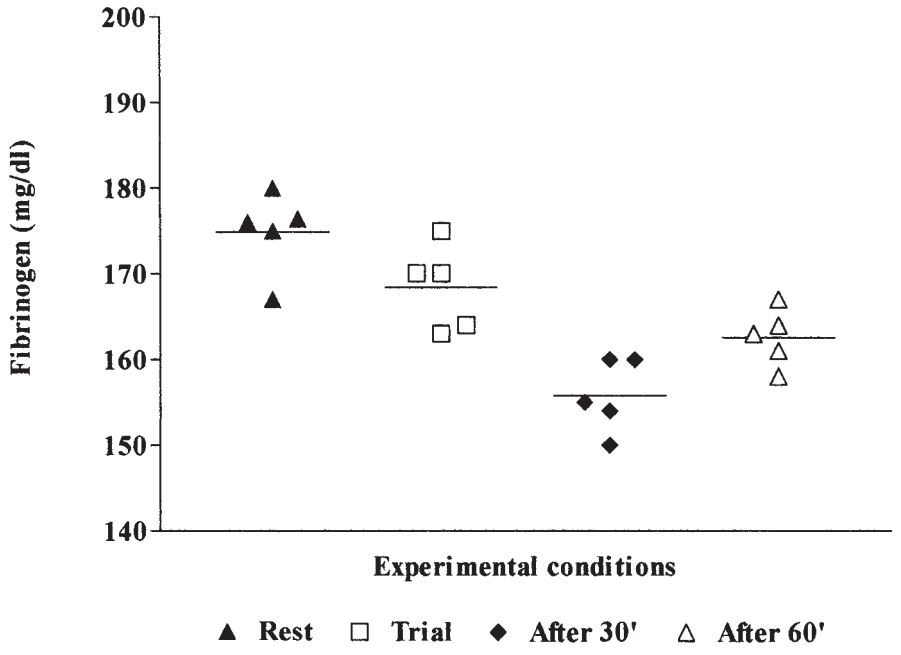

Figure 2: Scatter graph of the mean values of fibrinogen obtained from blood taken under different experimental conditions from 5 horses during an official showjumping event

showed a statistically significant increase immediately after the trial ($P < 0.001$) compared to the at-rest value and a statistical decrease 30 min. after the trial ($P < 0.001$) compared to the value immediately after the trial. Hct showed statistically significant increases immediately after the trial ($P < 0.001$) and again 30 minutes later ($P < 0.0l$) compared to the at-rest value, and a statistical decrease 30 min. after the trial ($P < 0.001$) compared to the value immediately after the trial. MCH showed statistically significant decreases immediately after the trial ($P < 0.001$) and 30 minutes later ($P < 0.001$) in comparison with the at-rest value. MCHC showed a statistically significant decrease both immediately ($P < 0.001$) and 30 min. after the trial ($P < 0.001$) compared to the at-rest value and a statistical increase 30 min. after the trial ($P < 0.01$) compared to the value immediately after the trial. PLT showed a statistical increase immediately after the trial ($P < 0.01$) and again 30 minutes later ($P < 0.05$) in comparison with the at-rest value.

Discussion

After analysing the results, the only statistically significant differences we observed among the clotting parameters considered were the aPTT and fibrinogen values obtained from the samples taken immediately after the trial; this decrease could have been caused by the type of exercise undertaken. Changes to the haemo-clotting balance have been recorded in the horse (9, 10, 11, 12), the cat (13), and in man (14).

In man it has been shown that, contrary to efforts of long duration, submaximal efforts do not involve changes in aPTT and PT (14).

Fibrinolytic activity is also enhanced during exercise in healthy human beings and the magnitude of this enhancement correlates with both the intensity and duration of the exercise (15, 16). However, the mechanism that shortens the blood clotting parameters after physical exercise remains unclear. The increased blood coagulability of horses, which is similar to that of human athletes, could represent the starting point of critical circulatory disorders such as disseminated intravascular coagulation (DIC), which is found frequently in athletic horses. Thus, the haemo-coagulative factors of athletic horses should be constantly monitored, particularly when they are subject to training and exacting competitions (9).

The haemochromatic changes were within the normal pattern for an athlete horse as has been described by several authors; these changes can be caused by exercise-induced splenic contractions (17). In fact, Persson et al. demonstrated that the spleen is the only reservoir for red blood

cells in the horse (18). Even though there were statistically significant differences between the before-and-after-trial Hct values, we did not take the influence of haemo-concentrations into account in our results as the Hct values were within the normal physiological range. The knowledge gained through this study of the clotting and haematological parameters in the athlete horse has enabled us to better understand, from a clinical point of view, the alterations in the haemostatic processes in horses during physical exercise.

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SPREMEMBE ČASA STRJEVANJA KRVI IN KONCENTRACIJE FIBRINOGENA PRI KONJIH MED PRESKAKOVANJEM OVIR

G. Piccione, A. Arcigli, A. Costa, F. Fazio, G. Caola

Povzetek: Proučili smo spremembe časa strjevanja krvi in fibrinolitične dejavnosti pri petih konjih med preskakovanjem ovir. Vzorce venozne krvi smo odvzeli pred tekmovanjem, takoj po tekmovanju, 30 minut po tekmovanju in 60 minut po tekmovanju. Vse vzorce smo takoj po odvzemu centrifugirali 10 minut pri 2600 obratih na minuto. Pri dobljeni plazmi smo s koagulometrom (SEAC Clot 2) določili protrombinski čas (PT), aktivirani parcialni tromboplastinski čas (aPTT), trombinski čas (TT) in vrednost fibrinogena. Pri analizi dobljenih rezultatov smo statistično značilne razlike zaznali samo za aPTT in fibrinogen. Bonferronijev test za primerjavo več vzorcev je pokazal statistično značilno znižanje ($P < 0,05$) aPTT takoj po tekmovanju. Fibrinogen je v primerjavi s časom pred tekmovanjem izrazito padel po 30 minutah ($P < 0,001$) in po 60 minutah ($P < 0,05$), v primerjavi s časom takoj po tekmovanju pa po 30 minutah ($P < 0,01$). Rezultati kažejo na povečano sposobnost koagulacije krvi, podobno kot je znano pri ljudeh - športnikih.

Ključne besede: koagulacija; čas strjevanja krvi; fibrinogen, konj, fizična aktivnost

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Case report

TREATMENT OF SCROTAL HERNIA IN FOALS

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Summary: Scrotal hernias in foals are usually congenital and not life threatening, whereas in adult stallions they are life threatening and require surgery. The treatment of hernias in newborn colts is normally conservative and surgical treatment is rarely indicated. Congenital indirect scrotal hernias are more common in foals than direct scrotal hernias. This paper is a review of the clinical signs and treatment of three foals with indirect scrotal hernias. A two-day-old foal had strangury and mild colic and a two-month-old trotter foal was surgically treated to correct a large non-reducible scrotal hernia. A three-day-old trotter foal had a reducible indirect scrotal hernia and colic, which was due to impacted meconium. There were no complications or hernial recurrences within the four months directly following the surgical procedures. The surgical treatment of congenital hernias is indicated when colic, incarceration of the intestinal loops, strangury or inguinal, scrotal or preputial oedema occur or when the hernia is so large that manual repositioning is impossible.

Key words: scrotal hernia; clinical signs; surgery; foal

Introduction

Scrotal hernias of foals are congenital and usually considered hereditary. They may be caused by an excessive outgrowth of the extra-abdominal part of the gubernaculum, which results in a vaginal process with an unusually wide neck (1). They are usually located in the left-hand side of the scrotum. Congenital hernias are either direct, which causes intermittent colic, or they are indirect - the intestine passes through the vaginal ring into the vaginal tunic - and usually asymptomatic.

Congenital indirect hernias, which are noticeable shortly after birth, are easily reduced when foals are rolled onto their back and usually resolve spontaneously within 3 to 6 months (2, 3).

Direct hernias occur when there is a rupture of the common vaginal tunic and the small intestine, and occasionally a testicle, escapes through into the subcutaneous space of the scrotum and prepuce (4). Direct or ruptured hernias in foals are evident within 4 to 48 hours of birth and cause colic, depression, severe scrotal and preputial swelling as well as oedema (5). These hernias are usually not reducible and are treated as surgical emergencies.

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Usually the jejunal loops are situated subcutaneously. Incarcerations of the large colon in congenital inguinal hernias have been reported. Abdominal compression during parturition may be responsible for ruptured inguinal hernias in foals (6, 7).

In congenital indirect scrotal hernias, intestinal loops can be palpated in the scrotum when the foal is standing and are generally unilateral. However, repositioning is difficult in the standing position, therefore, if the hernia is not too large, the foal is rolled onto its back, which makes the repositioning easy and painless. Indirect hernias should be monitored frequently due to the risk of incarceration. Incarceration should be suspected in a non-reducible umbilical hernia that increases in size and warmth, and is painful, firm, or oedematous.

Surgical corrections are recommended for direct hernias and for uncomplicated indirect hernias that do not resolve spontaneously within the first three to six months or that increase in size (4).

Surgical corrections may involve:

- an inguinal approach with castration;
- laparoscopic repair with castration (8);
- an inguinal approach without castration (9); or
- a midline laparotomy with closure of the vaginal ring.

The last two methods may cause atrophy of the testicle. In cases of direct or ruptured scrotal her-

Figure 1: Oedema of the prepuce caused by an indirect scrotal hernia

Figure 2: Indirect left scrotal hernia. Jejunal loops are in the distended vaginal sac

nia, the intestine is usually viable and no resection is necessary, although delayed necrosis is possible and has been reported (6).

History and clinical findings

Three foals with indirect scrotal hernias underwent surgery at the Veterinary Faculty's Clinic for Reproduction and Horses, in Ljubljana.

One 2-day-old Coldblood foal was brought in after the owner had noticed that it had difficulty urinating as well as intermittent mild colic. Its pulse rate (110/min), temperature (38.4 0C) and breath rate (20/min) were all normal during the clinical examination, however, the left-hand side of the scrotum was enlarged and intestinal loops were palpated. There was oedema on the prepuce (Figure 1) and the foal frequently attempted to urinate without success.

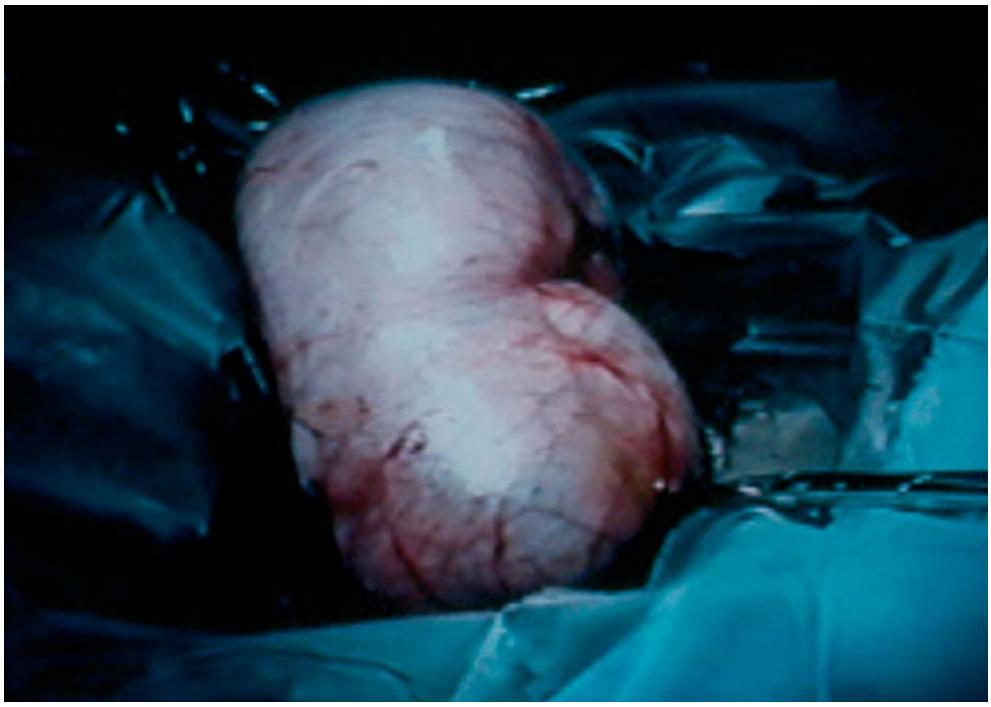

Figure 3: The dissected vaginal tunic was enlarged and very thin

Repositioning the loops was possible when the foal was in the dorsal position and 250 ml of urine was removed using a catheter. The faeces were yellow and normal. The diagnosis was an indirect left scrotal hernia with strangury.

The second foal was a two-month-old trotter. The owner had noticed a distension of the left-hand side of the foal's scrotum; otherwise the foal's behaviour was normal. During the clinical examination, the foal's pulse rate (72/min), temperature (38.0 0C) and breath rate (16/min) were normal. The intestinal loops were palpated in the enlarged left-hand side of the scrotum (Figure 2) and peristaltic movements could be seen beneath the skin.

There were no signs of discomfort or colic, however a manual repositioning was impossible. The diagnosis was a non-reducible indirect left scrotal hernia.

The third foal, a 3-day-old trotter, was depressed and lay down frequently and rolled onto his back. There were no faeces. During the clinical examination the foal's pulse rate (140/min), temperature (38.6 0C) and breath rate (40/min) were elevated. The abdominal wall was distended and there were signs of colic. Intestinal loops were present in the right-hand side of the scrotum. Repositioning was impossible because of the distended intestine. The diagnosis was an indirect scrotal hernia with meconium impaction.

Surgical procedures and results

The 2-day-old foal was sedated with 0.05 mg/100 kg of detomidine (DOMOSEDAN, Orion Pharma, Finland). 0.085 mg/kg of midazolam ((DORMICUM, Hoffman LaRoche, Swiss) and 1.5 mg/kg of ketamine HCl (KETAMINE, Veyx-Pharma GmbH, Germany) were used for the induction. We used 0.5 to 1 % halothane (FLUOTHANE, Zeneca, United Kingdom) with oxygen to maintain the anaesthesia. The skin was washed, cleaned and disinfected with chlorhexidine. A repositioning of the intestinal loops was performed before the operation. After the skin was incised over the inguinal ring, the vaginal tunic was bluntly transected. The vaginal tunic was then incised and the remaining loops (about 4 cm) were put back into the abdominal cavity. The ligament of the tail of the epididymis, which attaches the parietal tunic to the epididymis, was severed. By transecting the fold of the mesorchium and mesofuniculum, the testis, epididymis and the distal part of the spermatic cord were completely freed from the parietal tunic, bound and then removed. The external inguinal ring was closed using a continuous suture, and the skin with a Ford interlocking suture. The foal recovered 30 minutes after the operation. One hour later he had no strangury and urinated normally.

Because of the number of intestinal loops in

the vaginal cavity, we decided to treat the two-month-old trotter foal surgically. It was first sedated with 0.5 mg/100 kg of detomidine (DOMOSEDAN, Orion Pharma, Finland) and then anaesthesia was induced with midazolam (0.085 mg/kg; DORMICUM, Hoffman LaRoche Ltd, Swiss) and ketamine (2.2 mg/kg; KETAMINE, Veyx-Pharma, Germany). The anaesthesia was maintained using 1 % halothane (FLUOTHANE, Zeneca, United Kingdom) with oxygen. A large, approximately 10 cm, incision was made in the skin directly over the enlarged vaginal sac near the superficial inguinal ring. A digital dissection was used to bluntly dissect the parietal tunic from the surrounding fascia (Figure 3) and, through a thin, distended vaginal sac, peristalsis of small intestinal loops became obvious. These loops could not be forced back into the abdominal cavity when the vaginal sac was closed.

Therefore, an incision was made into the vaginal sac, the testicle and the vaginal sac were elevated and the jejunal loops (about 6 cm) were easily forced in with a finger through the vaginal ring and into the abdominal cavity. The testicle was removed as described in the first case. The parietal tunic was also removed to the level of the external inguinal ring. The ends of the parietal tunic, the subcutis and the skin were sutured, while the external inguinal ring was closed using continuous sutures. The foal recovered well from anaesthesia within 30 minutes and had neither complications nor any recurrence of the herniation during the 4 months immediately following the surgery.

The three-day-old trotter foal was operated because of an impaction of the meconium. The indirect scrotal hernia was a consequence of the impaction. For the sedation, premedication and anaesthesia we used the same drugs and doses as per the first case. After the laparotomy, the distended loops of the jejunum protruded through the wound. A needle was used to puncture the intestine to allow the gases to escape. The impaction was in the descending colon, cranial of the pelvis, and the meconium, which could not be forced into the rectum, was forced cranially into a loop that could be elevated out of the abdomen. The faecal matter was removed after an incision was made in the intestine, which was then sutured in two layers using a Cushing suture (Biosin, USP 3-0). The abdominal wall and the subcutis were closed with a continuous, absorbable suture. The skin was stitched with an interrupted non-absorbable suture. After the foal's hour-long recovery from the anaesthesia it no longer displayed signs of colic and normal, yellow faeces were voided two hours later. The indirect scrotal hernia was not surgically treated, because it was a consequence of the distension of the intestine. It disappeared after being manually manipulated. As with the other foals, there were no complications and no recurrence of the herniation during the four months immediately following the surgery.

Discussion

Congenital indirect scrotal hernias in foals are usually reducible, whereas acquired inguinal indirect hernias in adult horses commonly result in the strangulation of the small intestine and are surgical emergencies (10, 11). The surgical correction of a congenital indirect hernia is indicated if it does not resolve spontaneously, if the vaginal tunic ruptures (4) or if the owner is concerned because of an apparent increase in size of the hernia (4, 8). In acquired hernias, the intestine of an adult horse will become strangulated before it reaches the testis, whereas in foals a length of intestine can reach the fundus of the vaginal sac without strangulation. In the case of the two-month-old trotter foal, the owner noticed the enlargement of the left-hand side of the scrotum even though the foal had not displayed any discomfort since its birth. Despite the length of intestine in the vaginal sac, no strangulation had occurred. Some congenital inguinal hernias are caused by an excessive outgrowth of the extra-abdominal part of the gubernaculum. This results in a vaginal process with an unusually wide neck (1) and is the likely cause of the hernia in this instance. The vaginal ring in foals with a reducible indirect inguinal hernia is probably much wider than it is in foals with direct inguinal herniation where rupturing of the inner hernia sac occurs (6). In both our cases, the replacement of the intestine during the operation was easy, particularly as the vaginal ring of the two-month-old trotter was very wide.

In the first case, it was obvious that the indirect scrotal hernia had caused the strangury, preputial oedema and the foal's straining while trying to urinate. The mild colic was probably due to strangury as there was no evidence of a strangulation of the intestine present during the operation. The signs

disappeared immediately after the operation and the foal urinated normally without discomfort. An indirect hernia such as this can probably develop into a direct hernia due to the intermittent straining of the foal while trying to urinate. Van der Velden assumed that a direct inguinal hernia in newborn colts actually starts out as an indirect inguinal hernia; intestinal loops pass through the vaginal ring into the vaginal cavity and subsequently the parietal vaginal tunic ruptures. In foals with a direct inguinal hernia where the vaginal ring is still intact, it is very difficult to manoeuvre the prolapsed loops back through the narrow vaginal ring and into the abdomen and it is much easier to replace them after enlarging the rent up to the vaginal ring. Abdominal compression during parturition may also be responsible for ruptured inguinal herniation in foals.

In the third foal that we treated, the indirect scrotal hernia was a result of the meconium impaction. After surgically removing the impaction it was sufficient to just manually reposition the hernia, which then reduced within 5 days.

Although surgical treatment is occasionally required, most indirect scrotal hernias in foals usually reduce spontaneously with daily manual repositioning of the intestinal loops. Strangury is an indication for the surgical treatment of a congenital indirect scrotal hernia.

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ZDRAVLJENJE PRIROJENE MODNIKOVE KILE PRI ŽREBETU

V. Kadunc Kos

Povzetek: Modnikove kile so pri novorojenih žrebčkih pogosto prirojene in redko ogrožajo življenje, medtem ko so pri odraslih žrebcih pridobljene in življenje ogrožajoče. Večinoma so pri novorojenih žrebčkih prirojene kile ozdravljive konzervativno, le redko je potrebno kirurško zdravljenje. Pogostejše so posredne kot neposredne prirojene kile.

Članek opisuje klinično sliko in zdravljenje treh žrebčkov s posredno modnikovo kilo. Dva dni star hladnokrvni žrebček je kazal znake strangurije in blage kolike. Dva meseca star kasaški žrebček je bil kirurško zdravljen zaradi obsežne nereponibilne posredne kile. Tri dni star kasaški žrebček je kazal znake kolike zaradi zapeke (obstipacije mekonija), hkrati pa je imel reponibilno posredno kilo. Ob kontroli 4 mesece po operaciji ni bilo komplikacij ali ponovne kile.

Kirurško zdravljenje prirojenih kil je potrebno, kadar se pojavijo količne bolečine, vkleščenje vijug, težko uriniranje, oteklina v področju dimeljskega kanala, moda ali prepucija ali kadar je izpad črevesja tako obsežen, da ročna naravnava ni možna, kljub temu da žrebček nima znakov kolike.

Ključne besede: modnikova kila; klinična slika; kirurgija; žrebe

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Case report

CLINICAL ANALYSIS OF RECURRENT AIRWAY OBSTRUCTION IN HORSES

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Summary: Our research included 13 adult horses of different ages and breeds that had been referred to our clinic and suspected of having a pulmonary disease. The criteria for diagnosing recurrent airway obstruction (RAO) included having had, for a period longer than two months, clinical signs of a pulmonary disease likely to have been associated with the presence of hay and/or straw in the affected horse's environment. It also included finding pathological changes during a clinical examination, the presence of excessive respiratory secretions in the trachea during a bronchoscopic examination, a reduction in the partial pressure of the arterial oxygen and neutrophilia in the bronchoalveolar lavage fluid. All the horses used in our study met this criteria.

The most common clinical signs of RAO include high breathing frequency, a white serous or mucous nasal discharge, coughing and dyspnoea. We find that measuring the partial pressure of oxygen in the arterial blood is a reliable means of determining that a pulmonary disease is present and that a bronchoscopic examinationis is a reliable diagnostic method for examination of airways. We established that neutrophilic inflammation was in the bronchoalveolar lavage fluid and that two thirds of the examined horses were stabled most of the time.

Key words: veterinary medicine; obstructive lung diseases; diagnosis; horses

Introduction

Recurrent airway obstruction (RAO) is an inflammatory, obstructive airway disease that becomes clinically evident in middle-aged horses (1).

Attacks of airway obstruction are induced by the exposure of susceptible animals to organic dust (typically from hay or bedding). Following the exposure there is a massive influx of neutrophils into the airways, which is accompanied by bronchospasm and accumulation of mucus (2). The obstruction and inflammation are usually resolved when the source of the dust is eliminated (3).

In the early phases of the disease horses are alert and afebrile, although they usually cough during activity, feeding and/or the cleaning of the stable. There is reduced exercise tolerance and delayed recover from it. The frequency of coughing increases as the disease progresses. The nostrils may be flared and the horse may have a milky, serous or mucous nasal discharge. The horse may also use its abdominal muscles for exhalation to an exaggerated degree, and if the animal has had respiratory distress for some time, a heave line due to hypertrophy of the external abdominal oblique muscle may be obvious. Abnormal lung sounds are heard in varying degrees depending on the severity of the airway obstruction. Usually, the breathing sounds are louder throughout the airways, particularly over the peripheral lung fields, and wheezing is quite commonly heard. Percussion reveals an increase in the size of the lung field in severely affected animals (4).

In our research we wanted to determine which criteria are important for a clinical diagnosis of RAO. The research included horses that had come to our clinic during a one-year period with clinical signs and a history of having had a respiratory disease. All the horses were clinically examined, blood was taken for haematological and arterial gas analyses, a bronchoscopy was performed

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and the bronchoalveolar lavage fluid was cytologically analysed. The control group consisted of clinically healthy horses of different ages and breeds.

Material and methods

Animals

Thirteen horses of different breeds and ages were included in our research. They were referred to our clinic during a one-year period with a history and some clinical signs (coughing, nasal discharge, dyspnoea, reduced exercise performance) of lung disease. The control group consisted of 11 healthy horses of different ages and breeds.

Examination protocol

Each animal's history included details of its age, breed, sex, duration of disease, cough, nasal discharge (unilateral or bilateral), dyspnoea at rest, exercise, types of bedding and food used as well as the animal's general routine.

During the clinical examinations we collected data on submandibular lymphadenitis, rate of breathing, dyspnoea at rest, abnormal lung sounds both at rest and after a 30-second manual bilateral-nasal occlusion, abnormal sounds heard during a tracheal auscultation and any lung-field percussion abnormalities.

Bronchoscopy

The bronchoscopic examinations were performed after sedating the horses with detomidine and, if needed, with the use of a nose twitch. The tip of the endoscope was covered with a lidocaine-hydrochloride based local-anaesthetic gel. The examinations involved looking for the presence of erythema, checking the normally sharp carina for signs of bluntness and examining the tracheal and bronchial respiratory mucosae for evidence of inflammation. A local anaesthetic (lidocaine hydrochloride) was applied to the bronchial bifurcation and a fibroscope was inserted, distally, until it was felt to wedge. A plastic catheter was then passed through the fibroscope, through which 300 ml of buffered saline, warmed to 37 °C, was instilled and, immediately afterwards, gently aspirated using 60-ml syringes.

The respiratory secretions (RS) present in the cranial thoracic trachea were described according to volume, colour and nature. Some samples were bacteriologically and mycologically examined to confirm the absence of an infectious airway disease.

The bronchoalveolar lavage fluid was centrifuged. After the supernatant was poured off, smears were prepared from the remaining cell pellet and then air-dried. The dry smears were then stained following the method described by May–Grünwald-Giemsa and a differential count of 200 non-epithelial cells was performed using a light-microscope (40×). The results were given as a percentage of non-epithelial cells. The cells as well as any other non-cellular structures, microorganisms and/or foreign particles were described.

Haematology

An automatic haematological analyser was used to give a white-cell differential count and a complete blood count of blood taken from the jugular vein.

Arterial blood samples for a blood-gas analysis were collected from a. transversa faciei puncture and analysed with a gas analyser within 5 minutes of collection.

Statistics

All the data collected from the group of ill horses were compared to those from the group of healthy horses. The significance of differences between the two groups was analysed using a t–test for parametric data and a Mann–Whitney U test or an x^2 test for nonparametric data. The statistical analysis was performed using the SPSS (v.11) package for Windows. Differences of $p<0.05$ were considered statistically significant.

Results

Diagnostic criteria

According to the horses' owners, the horses had displayed clinical signs of a respiratory disease from between 3 to 60 months, or 19.54 months on average (SD = 5.4).

The owners also indicated that a cough was present in 12 of the horses (92.3 %) and that all

Table 1: Frequency of breathing, arterial blood gas measurement, bronchoalveolar lavage fluid analysis and haematology (arithmetic middle value ± SD; t-test)

	Control group	Ill horses	P
Breathing frequency	14.0 ± 0.8	23.1 ± 1.3	< 0.001
PaO_2 (mmHg)	106.03 ± 1.75	89.81 ± 2.96	< 0.001
$PaCO_2$ (mmHg)	39.72 ± 0.73	41.75 ± 0.59	0.040
pH	7.4322 ± 0.0044	7.4298 ± 0.0040	0.700
Macrophages (BAL) (%)	57.1 ± 6.0	18.1 ± 3.7	< 0.001
Lymphocytes (BAL) (%)	14.3 ± 2.0	7.2 ± 1.0	0.007
Neutrophils (BAL) (%)	25.1 ± 6.4	73.5 ± 4.8	< 0.001
Eosinophils (BAL) (%)	3.4 ± 1.0	1.0 ± 0.4	0.042
Basophils (BAL) (%)	0.2 ± 0.1	0.2 ± 0.1	0.863
Leukocytes (blood) (*10^9/L)	8.12 ± 0.53	10.87 ± 0.66	0.004
Erythrocytes (blood) (*10^{12}/L)	7.81 ± 0.30	7.80 ± 0.215	0.990
Haemoglobin (blood) (g/L)	128.5 ± 3.2	133.8 ± 2.95	0.233
Neutrophils (blood) (%)	50.17 ± 2.22	63.16 ± 2.00	< 0.001
Lymphocytes (blood) (%)	36.70 ± 2.18	27.68 ± 1.95	0.006
Monocytes (blood) (%)	6.14 ± 0.43	4.98 ± 0.37	0.054
Eosinophils (blood) (%)	4.52 ± 1.23	2.18 ± 0.53	0.103
Basophils (blood) (%)	0.68 ± 0.09	0.47 ± 0.06	0.056

Legend: PaO_2 - partial pressure of oxygen in arterial blood; $PaCO_2$ - partial pressure of carbon dioxide in arterial blood; pH - concentration of hydrogen ions in arterial blood; BAL - bronchoalveolar lavage fluid

horses had a nasal discharge, which we classified as being constant (15.4 %), frequent (69.2 %) or occasional (15.4 %).

Ten horses (76.9 %) displayed dyspnoea and were less tolerant of exercise according to their owners.

Age, breed and gender

The median age of the ill horses was 10.1 years (SD = 1.65), although most of the horses were aged between 7 and 8 years. The median age of horses in the control group was 9.7 years (SD = 1.24). There was no statistically significant difference between the two groups (t-test, p = 0.85).

There were five breeds represented in our study: Lipizzaner, Standardbred, Icelandic, Warmblood and Thoroughbred. There were no statistically significant differences among the ill and healthy groups of horses (x^2 test, p = 0.46).

There were no significant differences in the gender mix of the two groups (x^2 test, p = 0.48). The group of ill horses had 77 % females and 23 % males, while the control group had 63.6 % females and 36.4 % males.

Feeding, bedding, living in / outdoors

According to the owners 12 of the ill horses (92.3 %) and the entire control group were fed hay. One ill horse was fed grass silage. Straw was the most common bedding and was used by 46.2 % of the ill and 45.5 % of the control horses. The rest of the horses either had shavings for bedding or lived and slept outdoors. There were no statistically significant differences between the groups (x^2 test, p = 0.97).

About one third of the horses were kept outdoors (30.7 % of the ill horses and 36.3 % of the controls). The rest of the horses spent most of the time indoors, 38.5 % of the ill and 45.5 % of the control horses had only a few hours per day outside, while the remaining horses (30.8 % ill; 18.2 % control) spent less than 2 hours a day outside. There were no statistically significant differences between the groups (Mann-Whitney, p = 0.58).

Nasal discharge, dyspnoea, trachea auscultation, thoracic auscultation and percussion

Nearly half of the ill horses (46.2 %) displayed a nasal discharge during the examinations.

Generally it was bilateral, a milky white colour and its composition serous to mucous.

Dyspnoea at rest was evident in 30.7 % of the ill horses and 15.4 % of the ill horses had submandibular lymphadenitis, which was presenting as a bilateral enlargement of the submandibular lymph nodes.

Abnormal lung sounds were heard during the auscultation of each of the ill horses. In the two most serious cases there were no lung sounds at all during auscultation and biphasic expiration was evident.

Thoracic percussions revealed enlarged lung-field borders in 38.4 % of the ill horses and after a 30-second bilateral nose-closure 76.9 % of the ill horses developed abnormal tracheal sounds. None of these clinical sounds was heard in the control group.

Bronchoscopy

All the ill horses had some respiratory secretions (RS) present during the bronchoscopic examinations. There were a few droplets in 23.1 % of the horses, there was a small pool in 30.7 %, a large pool in 30.7 % and a very large pool in 15.5 % of the horses. Three of the control horses (27.2 %) had a small pool of respiratory secretions present in the trachea. The control group had far less respiratory secretions present in the trachea and this was statistically significant (Mann-Whitney, $p < 0.001$). The secretions of the ill horses were serous (53.8 %), mucous (30.8 %) and mucopurulent (15.4 %). The secretions of the control group were mucous. There was no statistically significant difference in the quality of the secretions of the groups of horses (Mann-Whitney, $p < 0.001$).

Frequency of breathing, arterial blood gas measurement, bronchoalveolar lavage fluid analysis and haematology

A t-test was used to analyse the results of comparisons between the medians of the two groups (Table 1). The differences between the average data were significant at $p < 0.001$.

There was a statistically significant difference between the breathing rate of the ill horses and that of the control group (Table 1).

The PaO_2 value of the ill horses, where 61.5 % had an Hg value lower than 95 mm, was statistically significantly lower than that of the control group (Table 1).

Statistically there were no significant differences between the $PaCO_2$ and pH of the ill horses and those of the control group.

The bronchoalveolar lavage fluid of the ill horses was a milky serous fluid, while the BAL fluid of the healthy horses was white and serous. There was a foamy surfactant present in both groups. Approximately one half of the instilled fluid was aspirated. The BAL fluid of the ill horses contained a significantly higher percentage of neutrophils and a lower percentage of alveolar macrophages than the group of healthy horses (Table 1). The percentages of lymphocytes, eosinophils and basophils did not differ between the two groups. There were also foreign structures such as plant particles present in the BAL fluid. Most of the BAL fluid contained Curschmann's spirals and macrophages.

There were no significant haematological differences between the two groups of horses (Table 1). While there was a statistically higher percentage of neutrophils in the group of ill horses, both groups fell within the limits of the normal range (Table 1).

Discussion

RAO as an inflammatory, obstructive airway disease becomes clinically relevant in middle-aged horses (1). It is one of the most common reasons for the retirement of sport and pleasure horses. In RAO there is an inflammatory reaction present in the airways that is responsible for bronchospasms, mucus accumulation, airway wall thickening and bronchial hyperreactivity. Clinical signs of the disease become evident at around 8 years of age (5, 6, 7, 8), which accords with our own experience and that of other authors. Most of the ill horses included in our research were aged between 7 and 9 years.

There was a significant difference in the breathing rates of the two groups of horses; a rapid breathing rate is a classic symptom of a respiratory disease. Lower airway obstructions in RAO-affected horses lead to changes in ventilation and blood flow, which in turn leads to inefficient blood-gas exchange and hypoxaemia. The body compensates for the insufficient gas exchange by breathing more rapidly (9).

During our research we found that measuring the level of PaO PaO_2 was useful in confirming the

presence of the respiratory disease. However, the PaO_2 value can be misleading. In RAO-affected horses that do not have an inflammation of the airways present at the time of examination, the PaO_2 value is likely to be within the normal limits (10, 11, 12), and we encountered this problem in 38.5 % of the ill horses. While we suspect that measuring the PaO_2 level of horse with RAO before and after treatment would be a good indicator of the efficacy of a treatment, we must rely on other tools to make a diagnosis of RAO.

The haematological values of the RAO-affected horses in our research group were within the normal ranges, which corresponds to the results from other published studies (5, 6, 13, 14).

The RAO-affected horses had significantly higher levels of respiratory secretions than the healthy horses. Three horses in the control group had a small pool of respiratory secretions present in their trachea. After consulting with their owners we discovered that these horses had had a viral respiratory infection during the previous month. The respiratory secretions of the ill horses in our study were mostly serous to mucous, which accords with reports from other authors (5, 8, 11). We found no signs of tracheal or bronchial respiratory mucosa inflammations in any of the horses. We consider a bronchoscopy to be an efficient method for the visualisation of airways and for confirming the presence of an airway disease. Some of the horses that had significant amounts of respiratory secretions present during the bronchoscopic examination, showed no signs of a respiratory disease during the clinical examination.

The average number of neutrophils was higher (25.1 %) in the control group than that reported in other studies (10, 15, 16). This was probably on account of the three horses that were in the recovery phase of a viral respiratory infection and the only way to distinguish between the two afflictions is through BAL cytology (11, 17). While the percentage of alveolar macrophages in our study was the same as that published by other authors, the lymphocyte count was lower, which was most probably due to the higher than normal neutrophil level. The most significant finding in the RAO-affected horses was the presence of neutrophilic inflammations (8, 15, 16, 17), where neutrophils represented 50 to 70 % of all cells. The percentages of neutrophils in our study was slightly higher than those reported in other studies. That could be due to the BAL-fluid aspiration technique. The length of the endoscope (2 m) didn't allow complete wedging of the endoscopic tube in bigger horses, so the samples were "contaminated" with cells from the upper airways, which contain higher concentrations of neutrophils (10, 15).

By the end of our study we were able to diagnose RAO in all the ill horses we examined. This could be because most of the horses in our ill population were adult Warmbloods (older than 5 years of age). The criteria we used for our diagnoses were as follows: the presence, for more than two months, of clinical signs of a respiratory disease together with the presence of hay and/or straw in the horses surroundings, pathological changes detected during the clinical examinations, the presence of respiratory secretions during the bronchoscopy, a lowered PaO_2 level and neutrophilia in the BAL fluid (50 to 70 % of neutrophils) (8, 15, 18). Other literature describes different sets of criteria for the diagnosis of RAO. These include the clinical presence of a respiratory disease (1, 7, 19, 20), neutrophilic airway disease – more than 10 % of neutrophils in the BAL fluid - connected to the presence of hay and/or straw in the horses' surroundings as well as a lowered PaO_2 level (1, 7, 17, 19, 20).

We found that coughing and a nasal discharge were reliable indicators that a respiratory disease was present. The coughing of RAO-affected horses is typically connected to higher concentrations of dust that are caused by feeding, the cleaning of the stable or through the activity of the horse (20, 21). An owner may also notice that the horse is performing poorly and that it has a nasal discharge and dyspnoea, which manifests as a bigger abdominal effort and flared nostrils during breathing (9, 20). In our research group 76.9 % of the ill horses were either performing poorly or were displaying signs of dyspnoea.

Most of the ill horses and all those in the control group were fed hay. One ill horse was fed grass forage as it had a history of RAO attacks whenever it was fed hay. Half of the horses had straw present in their surroundings, which was a second source of organic dust.

The manner in which a horse is kept is another factor influencing attacks of RAO. Two thirds of the ill horses were mainly kept indoors. The presence of RAO in Slovenia is probably due in part to climatic circumstances and to the tradition of

keeping horses indoors, especially during the wintertime. It is also partly due to a lack of awareness amongst horse owners and handlers of the correct ways to keep, feed and handle susceptible horses and of the causes and the manner of controlling the disease.

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KLINIČNA ANALIZA KONJ S PONAVLJAJOČO SE OBSTRUKCIJO DIHAL

P. Kramarič, A. Nemec, M. Fležar, I.Kern, Z. Pavlica

Povzetek: V klinično analizo smo vključili 13 konj različnih pasem in starosti, ki so na našo kliniko prihajali z anamnezo bolezni dihal. Merila za postavitev diagnoze ponavljajoče se obstrukcije dihal (POD) so bila: klinični znaki bolezni dihal dlje kot dva meseca v povezavi s prisotnostjo sena oziroma slame v okolici konja, patološke spremembe pri kliničnem pregledu, prisotnost respiratornega izločka pri bronhoskopiji, znižan parcialni tlak kisika v arterijski krvi in nevtrofilija v bronhoalveolarnem izpirku. Tem merilom so ustrezali vsi pregledani bolni konji.

Povišana frekvenca dihanja, belkast voden ali sluzast nosni izcedek, kašelj in oteženo dihanje so najznačilnejši klinični znaki POD. Ugotovili smo, da je parcialni tlak kisika dokaj zanesljiv kazalec obolelosti pljuč. Bronhoskopijo ocenjujemo kot učinkovito diagnostično metodo za pregled dihalnih poti in zanesljivo ugotavljanje bolezni dihal. V bronhoalveolarnem izpirku konj s POD smo ugotovili nevtrofilno vnetje. Kar dve tretjini bolnih konj je živelo pretežno v hlevu.

Ključne besede: veterinarska medicina; pljučne bolezni, obstruktivne – diagnostika; konji

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NEWS

New graduates of the Veterinary Faculty, University of Ljubljana

During 2002, the following 45 students graduated and were awarded the title »Doctor of Veterinary Medicine« (name, surname and the date of graduation):

Nataša Ajdič, January 30, Bašin Tea, June 18, Gašper Berginc, March 13, Tanja Bogataj, October 20, Jana Brankovič, June 26, Nina Cvirn, November 11, Andreja Čelak, January 30, Mirijam Černe, September 11, Aleš Činkole, September 11, Bojan Gašperič, May 15, Majda Golob, October 29, Aleksander Gros, October 20, Urška Henigman, March 21, Sebastijan Hobor, June 17, Sabina Jarc, November 11, Saša Juvančič, July 11, Jožica Kokalj Bernik, June 17, Rok Krajnik, January 29, Martina Lavrenčič, June 26, David Legiša, August 28, Marjeta Lisjak, June 26, Viktorija Lončar, January 30, Urška Luštrek, June 18, Alenka Metelko, May 28, Nina Mizerit, June 18, Katja Novak, September 11, Breda Osterveršnik, November 11, Manca Pavšič, June 26, Mojca Pečovnik, June 17, Rok Planovšek, January 29, Tanja Plavec, June 26, Kristina Porenta, December 8, Metod Praprotnik, January 23, Primož Rainer, March 12, Severine Sabine Sever, June 26, Andrej Škibin, January 29, Martina Škof (roj. Peklaj), March 13, Nataša Šuta, March 12, Igor Ujčič, August 28, Tanja Usar, June 26, Tjaša Vidmar, March 19, Irena Vračko, November 12, Matej Zupanc, March 13, Nina Zupanc, June 18, Ljiljana Žunjanin, January 30.

Matriculation at the Veterinary Faculty, University of Ljubljana

In the 2003/04 academic year, 439 students were enrolled in the study of veterinary science: 94 in the first year, 61 in the second year, 56 in the third year, 53 in the fourth year, 52 in the fifth year and 124 final year undergraduates.

Since the beginning of the veterinary study programme in the academic year 1956/57, 3061students have enrolled in the first year and of these 1381 graduated.

In the 2003/04 academic year, 15 students enrolled in the postgraduate course of biomedicine at the Veterinary Faculty: Ivona Dašič, Adrijana Dolinar, Neža Grgurevič, Primož Kern, David Legiša, Valter Mrak, Darja Pavlin, Tanja Plavec, Monika Ploj, Metod Praprotnik, Tina Roškar, Sara Suhadolc, Igor Ujčič, Oton Vidic in Diana Žele.

Student Jana Sterže enrolled in the postgraduate course of Environmental protection (this program started for the first time in 2003).

New masters of science

In 2003, the following doctors of veterinary medicine completed their Master's thesis:

Janez Kunc: Effects of holding time and packaging on the quality of frozen-thawed boar semen. Date of public defence: January 31. Mentor: Prof. Dr. Marjan Kosec.

Helena Dolajš: Residues of environmental pollutants and some veterinary drugs in raw milk. Date of public defence: February 4. Mentor: Prof. Dr. Darinka Zdenka Doganoc, co-mentor: Dr. Vesna Cerkvenik Flajs.

Nastja Jagličić Korpič: Listeriosis in chinchillas (Chinchilla laniger) and epidemiological situation in Slovenia. Date of public defence: April 18. Mentor: Prof. Dr. Ivan Mrzel.

Vladimira Erjavec: Evaluation of the influence of various drug carriers on the effect of hyperemic drugs applied on rat oral mucosa using electron paramagnetic resonance oximetry. Date of public defence: April 18. Mentor: Assist. Prof. Dr. Milan Petelin, co-mentor: Prof. Dr. Zlatko Pavlica.

Barbara Lukanc: Assessment of intestinal mucosa permeability with lactulose-mannitol test after haemorrhagic shock in dogs. Date of public defence: April 25. Mentor: Prof. Dr. Janoš Butinar, co-mentor: Dr. Alenka Seliškar.

Zlatka Podhostnik: Trace elements in Slovenian freshwater fish. Date of public defence: May 14. Mentor: Prof. Dr. Darinka Zdenka Doganoc

Darja Mihelčič: The assessment of inflammation in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction. Date of public defence: October 2. Mentor: Prof. Dr. Marinka Drobnič Košorok.

Tamara Čehovin: Enzyme-immunohistochemical properties of skeletal muscle fibres in brown bear (Ursus arctos). Date of public defence October 2. Mentor: Assist. Prof. Dr. Gregor Fazarinc.

Rok Pelc: Develpoment of hip dysplasia in police working dogs. Date of public defence: December 8. Mentor: Prof. Dr. Bojan Zorko, co-mentor: Assist. Prof. Dr. Teodora Ivanuša.

New doctors of science at the Veterinary Faculty in 2003

Marko Cotman: Intracellular transport of lanosterol 14-dimetilase (CYP51) and NADPH cytochrome reductase in mammalian cells. Date of public defence: April 22. Mentor: Prof. Dr. Damjana Rozman.

Leon Ščuka: The meta-analysis of clinical experiments of the use of salinomycin as a growth promotor in pigs . Date of public defence: June 27. Mentor: Prof. Dr. Štefan Drinovec.

Janez Posedi: Genetic markers for sheep gastrointestinal strongylids susceptible to benzimidazole antihelmintics. Date of public defence: July 8. Mentor: Prof. Dr. Andrej Bidovec.

Malan Štrbenc: Myosin heavy chain transitions during postnatal development of dog skeletal muscles. Date of public defence: October 28. Mentor: Assist. Prof. Dr. Gregor Fazarinc.

Vesna Kos Kadunc: Matrix metalloproteases in synovial fluid as diagnostic markers for detecting osteoarthritis in distal interphalangeal joint in horses. Date of public defence: December 9. Mentor: Prof. Dr. Marjan Kosec.

Awarded titles in 2003

Full time professor:

Marinka Drobnič Košorok, PhD, BChem, for phisiological chemistry

Associate professor:

Andrej Pengov, PhD, DVM, for microbiology and epizootiology

Assistant professor:

Nina Čebulj Kadunc, PhD, DVM, for physiology of domestic animals

Professional councillor:

Terezija Puškaš, MSc, DVM, for microbiology

Professional advisers:

Mateja Pate, DVM, for microbiology and molecular biology

Marija Seničar, MSc, DVM, for health care of fish

Nataša Žumer, DVM, for reproduction of domestic animals

Assistants:

Darja Mihelčič, MSc, DVM, dr. vet. med., for diseases and health care of pigs and ambulatory clinic

Tina Roškar, DVM, for for anaesthesiology, reanimation, intensive care and ambulatory clinic

Students' Prešeren awards

The Veterinary Faculty Students' Prešeren Awards were awarded to:

Nina Bremše for Catepsine b in mammary gland tumors and its use in evaluating disease outcome. Mentor: Ass. Prof. Dr. Polona Juntes.

Prepared by Malan Štrbenc

INSTRUCTIONS FOR AUTHORS

Slovenian Veterinary Research contains original articles which have not been published or considered for publication elsewhere. All statements in the articles are the responsibility of the authors. The editorial policy is to publish original research papers, review articles, case reports and abstracts of theses, as well as other items such as critical reviews of articles published in Slov Vet Res, shorter scientific contributions, letters to the editor, etc. Authors should send their contributions to the editorial board's address. All articles are subjected to both editorial review and review by an independent referee selected by the editorial board. The editorial board reserves the right to translate titles, summaries and keywords that have not been translated by the authors.

Contributions should be written in English and should not exceed 12 pages (27 lines per page, approx. 75 characters per line). They should be submitted electronically, written on any word processor for Windows, and accompanied by a hard copy. The text should be double-spaced and the lines should be numbered on the left-hand side. The margin on the left-hand side of the page should be 4 cm and the text should have no page breaks. Words should not be divided.

The front page of a contribution should start with the title, followed by the name and surname of the author(s). If there is more than one author, their names should be separated by commas. The next line ('Addresses of authors:') should contain the authors' full names and addresses (institution, street and number, postcode and place) after the colon. All the given data should be separated by commas. The name, address and E-mail and/or phone number of the responsible author should be supplied in the next line.

The Summary of 16-20 lines (1000-1500 characters) should follow on the next page. It should state the topic of the paper, the method used and the results. It should be clearly pointed out whether new methods were used, existing methods tested, or new ones introduced.

Under 'Keywords:' (after the colon), keywords should be given according to the 'Medical Subject Headings' (MeSH) standard. Individual words or word combinations should be separated by semicolons.

Scientific papers and papers which present the author's research and findings should also include the following obligatory headings assigned by the author to appropriate parts of the text: Introduction, Materials and methods, Results, Discussion, and References. Review articles should consist of an introduction, sections logically titled according to content, and references. Information on fund-providers and other matters important for the paper (e.g. technical assistance) should be supplied under 'Acknowledgements', which should be placed before references.

Tables, graphs, diagrams, figures, etc. may be enclosed separately or logically incorporated in the text file. They should be referred to by type and using Arabic numerals (e.g. Table 1:, Figure 1:, etc.). The colon should be followed by the text or title. On the reverse side of these items the name and surname of the first author, the title of the paper, and the name and number of the item should be supplied. Indicate, if necessary, where the item should be placed (top, bottom). All references cited in the text should appear in the References. They should be numbered in the text in the order in which they appear, marked with Arabic numerals placed in parenthesis. The first reference in the text should determine the number and order of the respective source in the References. If the author refers again to a source which has already been used in the text, he should cite the number the source had when it was referred to for the first time. Only works which have been published or are available to the public in any other way may be referred to. Unpublished data, unpublished lectures, personal communications and the like should be mentioned in references or notes at the end of the page on which they appear. Sources in the References should be listed in the order in which they appear in the text.

If the source referred to was written by six authors or less, all of them should be cited; in the case of seven or more authors, only the first three should be cited, followed by 'et al.'.

Any errata should be submitted to the editor-in-chief in good time after publication so that they may be published in the next issue.

Examples of references

Book: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

Chapterorar ticle in a book: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica 1995: 83-6.

NAVODILA AVTORJEM

Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo. Prav tako si uredništvo pridržuje pravico do prevodov naslovov, povzetkov in ključnih besed, ki jih avtorji ne bodo prevedli v slovenščino.

Prispevki naj bodo napisani v angleškem jeziku. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki morajo biti poslani v natisnjeni (en izvod) in elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilčene. Besedilo, ki naj bo na levi strani od roba oddaljeno 4 cm, naj ne bo razlomljeno v strani in besede ne deljene.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in/ali poštni naslov in/ali telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 16 do 20 vrstic (približno 1000 do 1500 znakov). V njem je treba povedati, kaj delo obravnava, katera metoda je bila uporabljena in kakšni so rezultati. Jasno je treba prikazati, da gre za nove metode, preverjanje in uvajanje metod ipd.

V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede po standardu Medical Subject Headings (MeSH). Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References.

Priloge so tabele, grafikoni, diagrami, slike ipd. Te avtorji priložijo posebej, lahko pa jih smiselno vključijo v datoteko z besedilom. Priloge morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Na hrbtni strani prilog naj bodo napisani ime in priimek prvega avtorja, naslov članka ter besede in številke, ki priloge opredeljujejo. Če je potrebno, se označi tudi položaj (zgoraj, spodaj). Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. Če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugače razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu.

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Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

Načini citiranja

Knjiga: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

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