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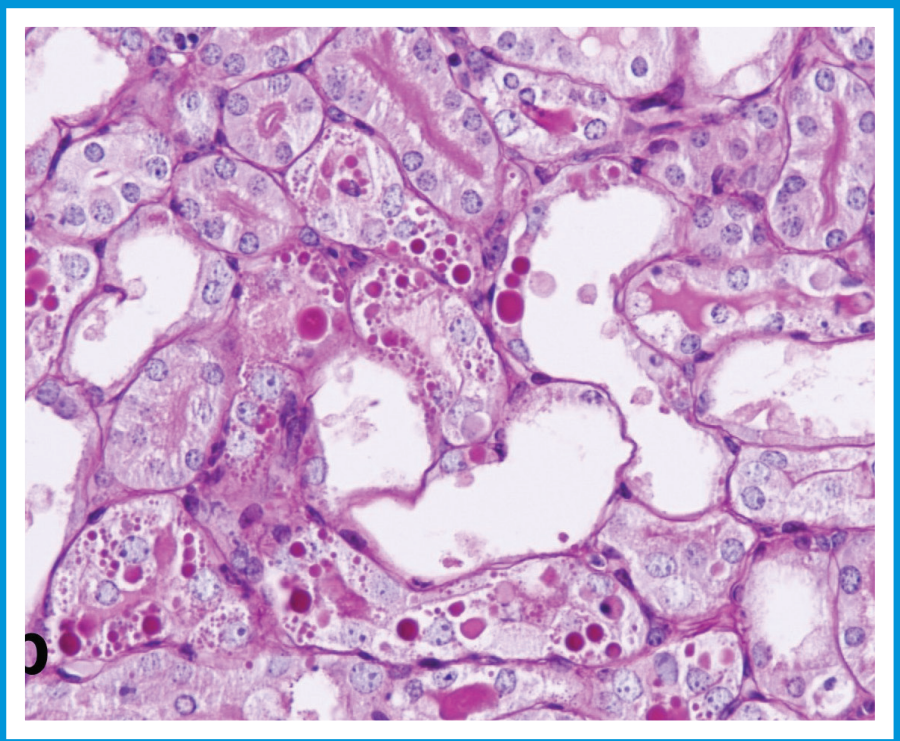

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Original Scientific Article

CLINICOPATHOLOGICAL SURVEY OF 56 CANINE MALIGNANT MAMMARY TUMOURS IN SLOVENIA - PROGNOSTIC VALUE OF CLINICAL STAGE AND HISTOLOGICAL GRADE

Mojca Cerovšek[1], Tanja Plavec[2], Petra Zrimšek[3], Milan Pogačnik[1], Jelka Zabavnik[4]

[1]Institute of Pathology, Administrative and Forensic Veterinary Medicine, [2]Clinic for Small Animals Medicine and Surgery, [3]Clinic for Reproduction and Horses, [4]Institute of Anatomy, Histology and Embryology, Veterinary Faculty, University in Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: mojca.cerovsek@vf.uni-lj.si

Summary: Mammary gland tumours are the most frequent class of neoplasm seen in female dogs; some breeds are reported to be at increased risk. This retrospective study describes the clinical and histopathological findings in 56 female dogs with mammary carcinomas that underwent surgery at the Clinic for Small Animal Medicine and Surgery of the Veterinary Faculty University in Ljubljana. Data relating to age, breed, spaying and history of pseudopregnancy were collected and survival analyses performed. The prognostic value of clinical stage and histological grade, based on 2 year survival after surgical removal of the tumour, was evaluated. Mammary carcinomas most often developed in dogs at 10 years or older (64.3 %), most falling into the 10 and 11 age group. Occurrence was the highest in Saluki (5.56 per 100 dogs), Miniature Schnauzer (0.44 per 100 dogs) and Medium Poodle (0.41 per 100 dogs) breeds. Survival times differed significantly for dogs with simple and complex tumours ($P<0.05$). Significant differences in survival were revealed between groups of dogs with different histological grades ($P<0.05$). Kaplan–Meier survival curves for dogs classified by clinical stage differed between dogs with stages I and IV and also II and IV ($P<0.05$). All dogs in subgroups with clinical stage I or II and histological grade I survived a 2-year follow up period (2-YFUP) after surgical removal of the tumours. In contrast, the average survival of dogs with clinical stage IV and histological grade III was less than 200 days. Our results reveal the high prognostic value of the combination of clinical stage and histological grade, based on survival of bitches after surgical removal of mammary gland tumour.

Key words: canine mammary gland tumour; clinical stage; histopathological diagnosis; histological grade; prognostic value

Introduction

Mammary gland tumours (MGTs) are the most frequent class of neoplasm seen in dogs (1, 2, 3). Early ovariectomy is a key element in decreasing the risk for MGTs (3). Genetic influence is apparent as the incidence of MGTs differs between breeds (4), although depending on the study, contradicting results can be found (3).

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The average age at diagnosis is between 10 and 11 years (5). Older dogs with MGTs have poorer prognosis, probably due to the fact that malignancy increases with age (6, 7, 8, 9, 10). However, age is not necessarily regarded as a prognostic factor (3, 8), given that older dogs are more likely to die from other causes. Dogs with tumours that ulcerate overlying skin and dogs with rapid and invasive growth of tumours also have shorter survival times (5, 6, 7, 11).

Surgical excision is the treatment of choice for most types of local MGTs (3, 12). The extent

of surgery is usually determined by the surgeon, the decision being based on whether treatment is seen as curative or palliative, taking into account the owner's consent. Many factors have been examined for their possible influence on the post-treatment survival of dogs and for their ability to predict recurrence and/or metastasis. Tumour size is considered one of the most important determinants of clinical staging in cancer (13). Additionally, vascular or lymphatic invasion and lymph node metastases have been associated with decreased survival and increased risk of tumour recurrence (3). Based on the clinical data of tumour size (T designation), presence of lymph node metastases (N category) and of distant metastases (M category), MGTs can be clinically staged according to the original World Health Organization's (WHO) TNM system (14) or the modified version (15). The TNM system is a prognostic factor for breast cancer in women that could also be used for canine MGTs (11, 13).

Histopathological examination shows that hyperplastic tissue, benign and malignant tumours can be present throughout the mammary glands of dogs in a variety of histologically defined forms, and with combinations of histogenetically different cells inside a single tumour (16, 17, 18). Tumours are classified as complex when they are composed of epithelial and myoepithelial cells, and simple when only one of these cell types is present (16). Inconsistencies between histopathological features and biological behavior or prognosis (19) have been documented, since approximately 10 % of the MGTs in the dog can be histologically misdiagnosed as benign (16). Due to these discrepancies, histological grade should be used for MGTs, as it could be helpful for classification and prognosis (20, 10). In human medicine, for the characterization of breast cancer, the grading system according to Elston and Ellis (20) is the most widely used (21). This method has also been applied for the grading of canine mammary carcinomas (22, 23, 24), and Karayannopoulou et al. (8) found it predictive for prognosis. In the prospective study of Pena et al. (10) the canine-adapted version of this method was evaluated and the authors identified it as significantly and independently associated with clinical outcome.

The aim of our study, therefore, was to evaluate the clinical and pathological characteristics of 56 mammary carcinomas with respect to the breed, age, spaying and history of pseudopregnancy, clinical stage, histopathological diagnosis and histological grade. Factors potentially associated with 2 year survival following surgical removal of the tumour were also evaluated.

Material and methods

Dogs and data collection

For this retrospective study fifty-six tissue samples were obtained from fifty-six bitches with malignant primary tumours that underwent surgery at Clinic for Small Animal Medicine and Surgery of the Veterinary Faculty University in Ljubljana (CSAMS VF, UL) between 2003 and 2009. In the case of the presence of multiple tumours, data were recorded but only the most malignant tumour, according to histopathological estimation, was included in the study. Data about age, breed, spaying and history of pseudopregnancy, surgical procedures and survival were collected from the medical records of the bitches, written questionnaires and interviews with owners. Clinical staging according to the TNM system was made at the time of surgeries. For every bitch a radiograph of the thorax was obtained before surgery. Routine 3-view (2 laterolateral projections and a dorsoventral projection) thoracic radiographs were taken. Re-check on 3 months, 6 months and then once a year was recommended. In the case of regional lymph node involvement, re-check was recommended 1 month after surgery. Follow-up data were collected over a 2 year follow-up period (2-YFUP) and expressed as survival time (the time between surgery and death due to the tumour or death from other causes or euthanasia). In the case of the recurrence of the disease the disease free time was recorded.

Histopathological and clinical evaluation

Histological type. Tissue samples were fixed in 10 % buffered formalin immediately after surgery and processed routinely. Histopathological diagnoses were based on haematoxylin and eosin-stained sections according to the WHO criteria (16).

Histological grading. Histological grading was evaluated in accordance with Elston and Ellis (20). Criteria of tubule formation, nuclear pleomorphism and mitotic counts were scored on scales from 1 to 3. The scores for each category

were added together and the total score converted to give the histological grade: 3 - 5 points: grade I (well-differentiated carcinoma), 6 - 7 points: grade II (moderately-differentiated carcinoma) and 8 - 9 points: grade III (poorly differentiated carcinoma). Histological grades were determined by at least two histopathologists.

Clinical staging. Dogs without regional lymph node or distant metastases were categorized as stage I, II or III, depending on tumour diameter (3 cm; 3 to 5 cm; more than 5 cm respectively). Dogs with regional lymph node involvement were classified as stage IV and dogs with distant metastasis as stage V, regardless of the tumour size (15). Metastases in regional lymph nodes and the presence of tumour cells in the lymphatic vessels of the primary lesion were confirmed by histopathological analysis.

Statistical analysis

Given the small consistency of the groups of age and breed, these two parameters were not taken into account for the statistical evaluation.

Survival time was defined as the time from surgical removal of the tumour to the date of death or recurrence of the disease (two years follow up period). For dogs that died of causes unrelated to the MGTs or had recurrence of the disease, the date of death/recurrence was defined as the censored date for calculating survival time (13, 25). Survival curves were constructed using the Kaplan–Meier method and the differences in survival between groups assessed using the log rank test for the following potential prognostic factors: clinical stage, histological grade, spaying, multiple tumours present, history of pseudopregnancy, histological tumour type (simple/complex) and combinations of clinical stage/histological grade. Histopathological classification was not included in the preparation of the survival curves because of the small number of cases in individual subgroups. The only dog with clinical stage V was included in the group of dogs with clinical stage IV. Variables for which the difference between survival curves was significant or near statistical significance were included in a multivariate model. The Cox proportional hazard model was used for multivariate analysis on factors potentially associated with survival 2-YFUP (26). For a categorized variable, a hazard ratio (HR, also called ''relative risk'') shows the hazard of a category compared with the reference category. For a continuous variable, HR shows the hazard ratio of two individuals that differ by one unit for the variable in question. An HR greater than 1.0 corresponds to an increase in risk and an HR less than 1.0 to a decreased risk.

Values of $P<0.05$ were considered significant for all analyses; data were analysed using software IBM SPSS Statistics 17.0.

Results

Of the 56 bitches included in our study, 12 (21.4 %) were spayed and 9 (16.1 %) had histories of pseudopregnancy. All the ovariectomies were made late in life. Multiple tumours developed in 18 dogs (32.1 %). 36 dogs (64.3 %) were 10 years or more, with most falling into the group of age 10 or 11.

The most frequently presented breed with MGT was English Cocker Spaniel (19.6 % from all dogs with MGTs), followed by mixed breeds (16.1 %) and Medium Poodle (7.1 %). Comparing the number of the dogs of an individual breed with the number of that breed in Slovenia (according to data from the Veterinary Administration of Slovenia, December, 2011), mammary carcinomas developed most frequently in Saluki (2/36 dogs; 5.56 %), followed by Miniature Schnauzer (3/459; 0.44 %) and Medium Poodle (4/976; 0.41 %) (*Table 1*). The prevalence of MGTs regarding the breed were not taken into account for the statistical evaluation given the small consistency of the groups.

Histopathological diagnosis included 4 carcinomas in situ, 29 simple type carcinomas (17 tubulopapillary carcinomas, 10 solid carcinomas and 2 anaplastic carcinomas), 19 complex type carcinomas and 1 case of malignant mixed tumour, mucinus carcinoma, squamous cell carcinoma and malignant myoepithelioma. From the 50 carcinomas 22 (44 %) were grade I, 13 (26 %) were grade II and 15 (30 %) were grade III.

Regarding clinical stage, 23 (41.1 %) dogs were stage I, 11 (19.6 %) stage II, 7 (12.5 %) stage III, 14 (25 %) stage IV and 1 (1.8 %) was stage V.

Of the 56 dogs, 29 (51.8 %) were still alive 2 years after surgical removal of MGTs, 14 (25 %) died within this period, in 7 dogs (12.5 %) recurrence of the disease was recorded, and 6 (10.7%) dogs died of causes unrelated to MGTs.

Table 1: Number and proportion of dogs with mammary gland tumours in separate breeds presented to Clinic for Small Animal Medicine and Surgery of the Veterinary Faculty University in Ljubljana between 2003 and 2009 and included in our study

BREED	n° DOGS WITH TUMOURS	n° DOGS IN SLOVENIA*	THE PROPORTION OF PRESENTED DOGS WITH MGTs**
Saluki	2	36	5.56
Miniature Schnauzer	2	459	0.44
Medium Poodle	4	976	0.41
English Cocker Spaniel	11	3157	0.35
Doberman Pinscher	2	746	0.27
Samoyed	2	1513	0.13
Pekingese	3	3030	0.10
Maltese	2	5231	0.04
Golden Retriever	2	9093	0.02
German Sheperd Dog	3	22589	0.01
Mixed	9	92934	0.01

*According to data from the Veterinary Administration of Slovenia (December, 2011).
** (n° of dogs in our study of the given breed with MGT/n° of dogs of this breed in Slovenia) x100.

Table 2: Kaplan–Meier survival for variables possibly associated with survival 2 years after surgical removal of mammary gland tumours

Variable	Categories	n of dogs (%)	Survival time (days) (mean ± SE)	P Value
Clinical stage	I II III IV/V	23 (41.1) 11 (19.6) 7 (12.5) 15 (26.8)	700.0 ± 30.1 730.0 ± 0.0 553.0 ± 123.5 303.1 ± 76.9	<0.05
Histological grade	I II III	22 (44.0) 13 (26.0) 15 (30.0)	730 ± 0.0 602.0 ± 70.8 332.5 ± 82.0	<0.05
Ovariectomy	Yes No	12 (21.4) 44 (78.6)	594.5 ± 73.5 576.9 ± 41.9	0.930
Multiple tumours	Yes No	21 (37.5) 35 (62.5)	560.3 ± 63.0 592.1 ± 44.7	0.707
Pseudopregnancy	Yes No	9 (16.1) 47 (83.9)	576.7 ± 103.3 580.5 ± 39.0	0.813
Histological type	Simple Complex	29 (60.4) 19 (39.6)	507.9 ± 56.4 704.4 ± 25.9	<0.05

Table 3: Results of multivariate analysis of variables associated with survival 2 years after surgery in dogs that had undergone surgical removal of mammary gland tumours

Variable	Categories	Relative Hazard	95% Confidence Limits for HR	P Value
Clinical stage	I II III IV/V	0.179 0.237 0.353 Reference	0.071 – 0.448 0.082 – 0.683 0.101 – 1.235	<0.001 0.008 0.103
Histological grade	I II III	0.338 0.467 Reference	0.145 – 0.787 0.171 – 1.276	0.012 0.138

Total. 48; event: 36; censored: 12

Table 4: Number of survived and censored dogs in subgroups with different clinical stage and histological grade

Clinical stage	Histological grade	n	n censored (%)	n survived (%)
I	I	**11**	**2 (18.2)**	**9 (100.0)**
I	II	3	1 (33.3)	2 (100.0)
I	III	3	0 (0.0)	3 (100.0)
II	I	**7**	**1 (14.3)**	**6 (100.0)**
II	II	4	2 (50.0)	2 (100.0)
II	III	0	0	0
III	I	2	0 (0.0)	1 (50.0)
III	II	3	1 (33.3)	1 (50.0)
III	III	2	1 (50.0)	1 (100.0)
IV	I	2	1 (50.0)	1 (100.0)
IV	II	3	1 (33.3)	0 (0.0)
IV	III	**10**	**1 (10.0)**	**0 (0.0)**

*Groups which are included in further evaluation are bolded

Table 5: Kaplan-Meier survival for combination of clinical stage and histological grade associated with survival 2 years after surgery in dogs that had undergone surgical removal of mammary gland tumours

Clinical stage	Histological grade	n (%)	Survival time (days) (mean ± SE)	P Value
I	I	11 (39.3)	730.0 ± 0.0	<0.001
II	I	7 (25.0)	730.0 ± 0.0	0.001
IV	III	10 (35.7)	184.0 ± 58.8	

Figure 5 shows Kaplan-Meier survival curves for compared subgroups.

Factors associated with prognosis

Of all the variables included in the univariate study, histological grade, histological type (simple/complex) and clinical stage were associated significantly with survival at 2-YFUP of dogs that had undergone surgical removal of MGTs (P<0.05).

Kaplan–Meier survival curves differed for dogs with clinical stages I and IV, and also II and IV (P<0.05), whereas the difference between curves for dogs with stages I and III, and II and III, were near the level of significance (P=0.06 and P=0.053, respectively). There was no significant difference between the survival curves for dogs with stages I and II or for dogs with stages III and IV (P=0.489 and P=0.115, respectively) (*Figure 1*). Significant differences in survival were revealed between groups of dogs with different histological grades (P<0.05); mean survival time was calculated as 730 ± 0 days for grade I, 602 ± 70.8 days for grade II and 332 ± 82 days for grade III (*Figure 2*). Significant difference was observed between each pair of survival curves according to histological grade (P<0.05).

The difference in survival time between dogs with simple (mean survival time 507.9 ± 56.4) and complex (704.4 ± 25.9) tumours was significant (P<0.05) (*Figure 3*).

The survival curve for dogs with multiple MGTs did not differ from that for dogs with only one tumour (P=0.707). No association was found between survival of ovariectomized and non-ovariectomized dogs (P=0.930), or between groups of dogs with and without a history of pseudopregnancy (P=0.813) (Table 2).

Variables included in the multivariate analysis were clinical stage, histological grade and histological tumour type (simple/complex). The histological tumour type is shown not to be significant (P>0.05), therefore, in the final model, only clinical stage and histological grade were included.

Relative risk for clinical stage III was not significantly lower than that for stage IV/V (P>0.05), whereas dogs with stages I and II exhibit 5.6 and 4.2 times lower relative risk than dogs with stage IV/V. Relative risks for histological grades II and III did not differ significantly, whereas that for grade I was 3-fold less than that for grade III (Table 3).

Table 4 shows subgroups of dogs with different combinations of clinical stage and histological grade. Because of the low number of cases in several subgroups, only those with n≥6 were further evaluated.

Kaplan-Meier survival curves revealed significant differences when comparing groups 1 (clinical stage I/histological grade I) and 2 (clinical stage II/histological grade I) with group 3 (clinical stage IV/histological grade III) (P<0,001 and P=0.001 respectively), whereas groups 1 and 2 did not differ significantly (P>0.05) (Figure 4).

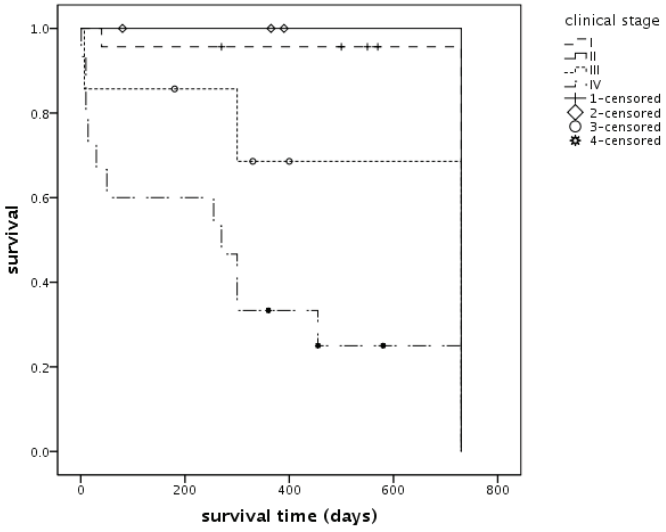

Figure 1: Kaplan–Meier survival curve for dogs with mammary gland tumours classified by clinical stage (P<0.05) (n=56)

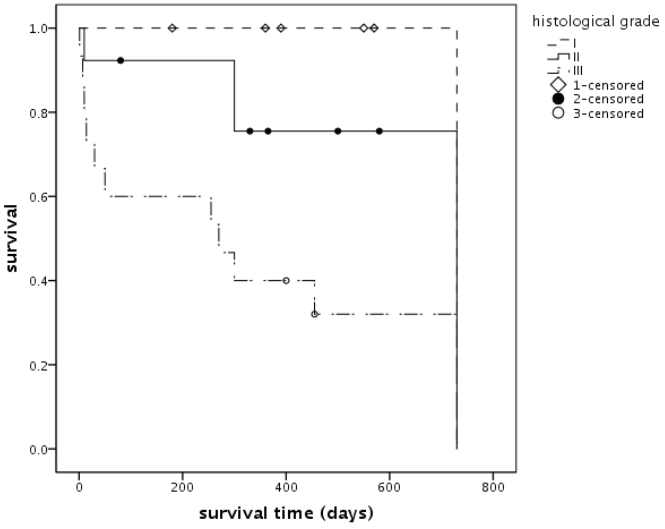

Figure 2: Kaplan–Meier survival curve for dogs with mammary gland tumours classified by histological grade (P<0.05) (n=50)

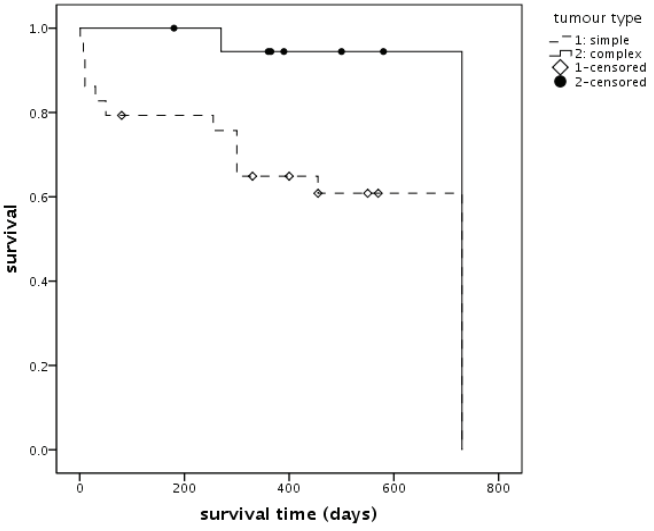

Figure 3: Kaplan–Meier survival curve for dogs with mammary gland tumours classified by tumour type – simple or complex (P=0.05) (n=48)

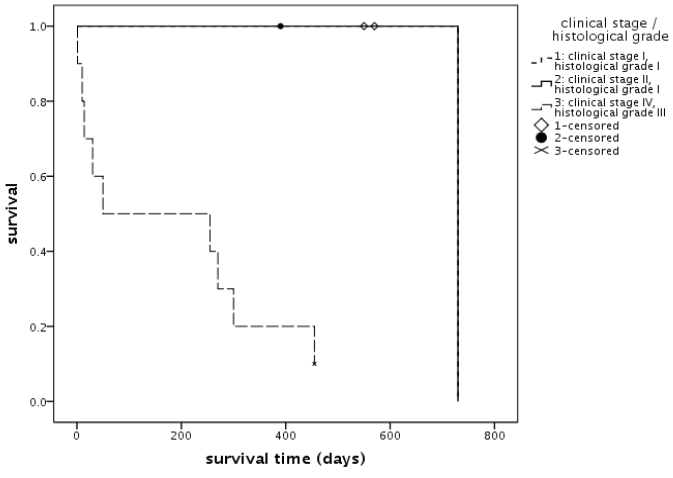

Figure 4: Kaplan–Meier survival curve for dogs with mammary gland tumours classified by combining clinical stage and histological grade (P≤0.01) (n=28)

Discussion

The most frequent neoplasms in intact female dogs are MGTs. It is therefore very important to have some protective actions to prevent the emergence of MGTs and, if they are diagnosed, to determine the prognosis. Neither of these is simple because of the many factors that can influence the biological behavior of the MGTs.

Steroid hormones play an important role in the etiology of MGT in dogs and ovariectomy of a bitch at an early age, modifying their levels is therefore the most effective way of preventing MGTs (3, 4). Some authors report that late spaying does not reduce the risk of malignant tumours (15), while others conclude that ovariectomy at any age may be beneficial to survival of dogs that develop MGTs (18, 3). In our patients with MGTs, none of the ovariectomies were made before the second estrous and 78.6 % dogs were intact, pointing to the protective effect of early ovariectomy. We found no association of survival in ovariectomized versus non-ovariectomized dogs with MGTs. In addition, the effect of pseudopregnancy on the development of MGTs is still the subject of debate (3, 27). In our study, 5 out of 9 dogs with a history of pseudopregnancy developed multiple MGTs. No association was found between survival of dogs with and without a history of pseudopregnancy (P=0.813). However, the small number of dogs with a history of pseudopregnancy makes it difficult to draw firm conclusions.

Contradictory results have been reported regarding breed predisposition towards occurrence of MGTs. MGTs occur more frequently in purebred dogs (3, 11, 26, 28) and the same was found in our study.

MGTs develop mostly in middle-aged and old dogs (3, 11). In our study most dogs with diagnosed MGTs were age 10 or 11, and the youngest was 5 years old, which fits well with the reported data (3, 5). Given the small number of cases in several subgroups of age and breed we did not evaluate the possible influence of these two factors on survival. Furthermore, dogs of different breeds differ in their life span.

Survival after MGT surgery varies significantly, depending on different tumour and dog characteristics, and different parameters therefore need to be evaluated in order to obtain a significant prognosis. Significant differences in the survival of dogs with different histological tumour types have been reported (25, 29, 30, 31). Malignancy increases from non-infiltrating *in situ* carcinoma over anaplastic carcinoma to sarcoma (16). Moreover, simple carcinomas have a poorer prognosis than complex carcinomas (28), and in some authors' opinion myoepithelial cells could act as tumour suppressors in regulating the transition from *in situ* to invasive carcinoma in humans (32). In our study the difference in survival time between dogs with simple and complex tumours was significant ($P<0.05$). However, by multivariate analysis, histological tumour type was revealed as not being significant ($P>0.05$).

With regard to clinical stage, dogs with more advanced tumour stage exhibit significantly shorter survival than dogs with low-stage disease (3, 30). In our study there was no significant difference between survival for dogs with stages I and II and those with stages III and IV/V, while survival for dogs with stages I and IV/V differs significantly from those with II and IV/V. The same trend was shown by multivariate analysis, where dogs with stages I and II have 5 and 4.3 times lower relative risk than dogs with stage IV/V. While there is general agreement that tumour size is an important prognostic factor, there is conflicting evidence as to the size category, and therefore clinical stage, at which prognosis changes significantly for the worse (13, 15). Similarly to our observations, it has been reported (13, 25) that dogs with tumours larger than 5 cm (stage III) exhibit a significantly poorer survival than those with smaller tumours. On the other hand, Philibert et al. (30) found a significant difference in survival between dogs with stage I and those with stages II and III. In addition to large tumour size, lymph node status has been reported to be a poor prognostic factor in canine MGTs (11, 25). In our study, dogs with metastases in lymph nodes were associated with the shortest postoperative survival of all groups of clinical staging. Similarly, Karayannopoulou et al. (8) linked dogs with stage IV with poorer outcome, since 24 of 28 dogs with stage IV in their study died within 2-YFUP.

Numerous reports (3, 8, 33, 34) show that histological grading of malignant MGTs is significantly related to prognosis, with higher grade tumours having worse prognosis. The method of Elston and Ellis used in our study, was primarily developed for invasive adenocarcinomas of breast regardless of tumour type (20). Since

in veterinary medicine there are no generally accepted guidelines about which histological types of canine MGTs could be graded, the decision is left to the authors. Consequently, there are great variations in grading of canine MGTs between individual studies. Dutra et al (35) and Manuali et al. (24) have graded only simple and complex type carcinomas, Clemente et al (22) and Karayannopoulou et al (8) also carcinomas of special types, while Santos et al (23) have also graded carcinosarcomas and in situ carcinoma. Tumor samples in the study of Manuali et al. (24) and Clemente et al. (22) included carcinosarcoma, malignant mioepithelioma and sarcoma, but from their results it is not evident whether the tumors of this histological type were graded. The disadvantage of the Elston and Ellis method, when adapted to grading of canine MGTs, is that it does not include the evaluation of myoepithelial proliferation or mesenchymal areas (10). Namely, unlike in woman, complex and mixed carcinomas frequently occur in the dog (34) and if grading is restricted to simple type carcinomas, important prognostic information could be lost (20). Pena et al (10) therefore recommended some modifications of this method in relation to the evaluation of myoepithelial proliferation areas, mixed neoplasms and the evaluation of nuclear features. When these recommendations are taken into account, different histological tumour types can be graded. In our study, simple, complex and special type carcinomas were graded. Like Pena et al (10) we carefully graded complex tumours scoring the degree of tubule formation only in the epithelial parts of the tumours while the nuclear pleomorphism was evaluated throughout the tumour. There were significant differences in survival curves among groups of dogs with different histological grade ($P<0.05$). Karayannopoulou et al. (8) showed a 21-fold higher risk of death in the group of dogs with grade III carcinomas than in those with grade I and II carcinomas. We found that relative risks did not differ significantly between histological grades II and III, whereas dogs with grade I exhibited a 3-fold lower risk than dogs with grade III.

Further, we have studied the prognostic value of the combination of clinical stage and histological grade. Complete survival was recorded in subgroups of stage IV/grade I and stage I/grade III. However, due to the low number of cases, these two subgroups were not included in comparison. All dogs in subgroups with clinical stage I or II and histological grade I survived, in contrast to dogs with clinical stage IV and histological grade III, where average survival time after surgical removal of tumours was less than 200 days. Our results have demonstrated that a combination of clinical staging and histological grading is of high prognostic value, although corroboration is required on account of the small number of dogs in our study. Since MGTs are histologically very heterogeneous, clinical staging and histological grading could improve the estimation of their malignancy and therefore prognosis of dogs with MGTs.

Conclusion

In our study canine mammary carcinomas were most frequently recorded in Saluki, Miniature Schnauzer and Medium Poodle breeds. All dogs in subgroups with clinical stage I or II and histological grade I survived 2-years after surgical removal of the tumours. In contrast, average survival of dogs with clinical stage IV and histological grade III was less than 200 days. Our results show that the combination of clinical staging and histological grading provides a high prognostic value and should therefore be included in the diagnosis of every MGT.

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KLINIČNO PATOLOŠKA ŠTUDIJA 56 MALIGNIH TUMORJEV MLEČNE ŽLEZE PRI PSICAH V SLOVENIJI - PROGNOSTIČNA VREDNOST KLINIČNEGA STADIJA IN STOPNJE DIFERENCIACIJE TUMORJA

M. Cerovšek, T. Plavec, P. Zrimšek, M. Pogačnik, J. Zabavnik

Povzetek: Tumor mlečne žleze (TMŽ) je najpogosteje diagnosticirana oblika tumorja pri psicah in po poročilih avtorjev so posamezne pasme bolj nagnjene k nastanku te vrste tumorjev. Naša retrospektivna študija opisuje klinične in histopatološke ugotovitve pri 56 psicah z malignimi TMŽ, katerim so tumorje odstranili na Kliniki za kirurgijo in male živali Veterinarske fakultete Univerze v Ljubljani. Zbrali smo podatke o starosti, pasmi, sterilizaciji in morebitni, v preteklosti diagnosticirani, navidezni brejosti psic in izvedli analizo preživetja. Na podlagi 2-letnega preživetja po kirurški odstranitvi tumorjev, smo ocenili napovedno vrednost kliničnega stadija in stopnje diferenciacije tumorja. Maligni TMŽ so bili najpogostejši pri psicah starih 10 let ali več (64,3 %), z največjim deležem psic starosti 10 in 11 let. Maligni TMŽ so bili najpogostejši pri psicah pasme Saluki (5,56 na 100 psov), pritlikavem šnavcerju (0,44 na 100 psov) in srednjem kodru (0,41 na 100 psov). Razlika med preživetjem psic z malignimi TMŽ kompleksnega in enostavnega tipa je statistično značilna ($P<0.05$). Med skupinami psov z malignimi TMŽ različne stopnje diferenciacije je statistično značilna razlika v preživetju. Statistično značilna je razlika med Kaplan–Meierjevimi krivuljami preživetja psic s kliničnim stadijem I in IV in tudi II in IV ($P<0.05$). Vse psice s kliničnim stadijem I ali II in stopnjo diferenciacije I so preživele 2-letno obdobje po kirurški odstranitvi tumorjev. Povprečen čas preživetja psic s kliničnim stadijem IV in stopnjo diferenciacije III je bil krajši od 200 dni. Iz rezultatov naše študije je razvidno, da ima kombinirana ocena kliničnega stadija in stopnje diferenciacije tumorja visoko napovedno vrednost za preživetje psic po kirurški odstranitvi malignega TMŽ.

Ključne besede: tumor mlečne žleze pri psicah; klinični stadij; histopatološka diagnostika; stopnja diferenciacije; prognostična vrednost

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Original Scientific Article

COMPARATIVE STUDY OF IRON, MAGNESIUM AND ZINC AND DAILY INTAKES IN CERTAIN MEATS AND MEAT PRODUCTS

Nina Bilandžić[1]*, Marija Sedak[1], Maja Đokić[1], Ivana Varenina[1], Božica Solomun Kolanović[1], Đurđica Božić[1], Mate Brstilo[2], Darja Sokolić-Mihalak[3], Zorica Jurković[3]

[1]Laboratory for Residue Control, Department for Veterinary Public Health, [2]General Department, Croatian Veterinary Institute, Savska cesta 143, HR-10000 Zagreb, [3]Croatian Food Agency, I. Gundulića 36b, 31000 Osijek, Croatia

*Corresponding author, E-mail: bilandzic@veinst.hr

Summary: Different meats (beef, pork, horse, pork kidney) and meat products (bolognese meat sauce, ham in casing, liverwurst, beans with cooked bacon, beef tripe, smoked pork neck, pancetta, sausage, cooked bacon) were collected in Croatian markets during 2012. The concentrations of Fe, Mg and Zn were determined and were in the ranges (mg/kg): Fe 1.24–63.9, Mg 86.2–333 and Zn 7.08–64.6. The highest element contents measured in different meats and meat products were (mg/kg): Fe 53.2 in pork kidney, Mg 263 in beef and Zn 51.7 in beef. The lowest mean levels were (mg/kg): Fe 6.33 in pork, Mg 173 in pork kidney and Zn 13.2 in different meat products. Significant differences for all three elements were observed between the food groups tested. The estimated mean daily intake (EDI) of Fe, Mg and Zn in different types of food contributing to the recommended dietary allowance (RDA) for women and men were in the ranges (%): Fe 0.07–1.33; Mg 0.09–0.17 and Zn 0.40–1.29. The results obtained in food groups tested for Fe levels is in agreement with literature values reported, though differences were found for Mg and Zn.

Key words: Fe, Mg; Zn; meat; meat products; ICP-OES; Croatia

Introduction

Essential elements such as Cu, Co, Fe, Zn, Mg Se and Mn are required for adequate physiological functions and should be available through dietary intake. Insufficient intake of Fe and Zn causes fatigue, poor growth, anaemia, rickets and impaired cognitive performance in humans (1). Furthermore, elements such as Cu, Zn, Fe, Se and Mn are key for the enzymatic systems that counteract free radicals in the organism. Iron plays a major role as an oxygen carrier in haemoglobin in blood, or myoglobin in muscle, and it is also required for many metabolic processes (2). Zinc has multiple biochemical functions and is present in a large number of proteins, and also as a physiological constituent of the oxidant defence system (3). Magnesium is needed for more than 300 biochemical reactions in the body. It helps maintain normal muscle and nerve function, keeps heart rhythm steady, supports a healthy immune system, and keeps bones strong (4).

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Meat and meat products are important source of proteins, trace elements and B vitamins and greatly contribute to the daily intakes of these micronutrients in the human diet in many parts of the world (5). It is well known that meat is the one of the richest sources of Zn in the total diet and it also provides sufficient amounts of Cu (6, 7). Also, meat represents the primary source of heme Fe, the iron form having the highest bioavailability (8, 9).

There has been a general decrease in the amount of red meat consumed in developed countries, primarily attributed to the reduction in beef consumption in the late 1990s (2). Furthermore, pork is the most widely consumed meat in the EU with continuous increasing consumption. Processed meat includes meat that has been preserved by methods other than freezing, such as salting, smoking, marinating, air-drying or heating, e.g. ham, bacon, sausages, cooked bacon, salami, corned beef and tinned meat. For example with regard to processed meat intake, Sweden, Norway and Germany have the highest intakes, primarily due to the amount of sausages they consume (10).

Trace element contents in meat and meat products depend on different factors, such as environmental conditions, type of pasture, feed composition, genetic characteristics of animals, rearing practices, slaughtering method and aging. Furthermore, technological treatments and cooking conditions are important for levels of trace elements in meat products (2, 11). Total Fe content is not changed after heat processing of meat, though the heme:non-heme iron ratio is modified with a decrease of heme iron concentration depending on the severity of the heat treatment utilized (9). In previous studies, low iron contents were established in pork, poultry and rabbit meats (6). A variation in Fe content was found among different species. However, the Fe content of the same type of meat may vary due to the age of the animal at the time of slaughter, the diet of the animal and husbandry practices (2).

The objective of this study was the assessment of Fe, Mg and Zn concentrations in the meat of different species, minced meat and different processed meats consumed in Croatia. Results were assessed by comparing estimates of dietary exposures with the recommended dietary allowances (RDA) recommended by Food and Nutrition Board of the Institute of Medicine (12).

Materials and methods

Sample collection

In total, 27 meat products were collected in Croatian markets during 2012: 4 minced meat products; 9 different meat products (bolognese meat sauce, ham in casing, liverwurst, beans with cooked bacon, beef tripe, smoked pork neck, pancetta, sausages, cooked bacon), 5 beef meat, 3 pork meat, 3 horse meat and 3 pork kidney samples. Following collection, samples were labelled and stored in polyethylene bags and frozen at -18°C prior to analysis.

Sample preparation

All reagents were of analytical reagent grade, HNO_3 and H_2O_2 (Kemika, Croatia). Ultra high purity water processed through a purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Technologies, Osijek, Croatia) was used for all dilutions. Plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9, v/v) and by subsequent rinsing with double deionised water and drying prior to use. Calibrations were prepared with Fe, Mg and Zn standard solutions of 1 g/l (Perkin Elmer, USA). The stock solution was diluted in HNO_3 (0.5%).

Meat and meat product samples (0.5 g) were digested with 4 ml of HNO_3 (65% v/v) and 2 ml of H_2O_2 (30% v/v) in a microwave oven. A high-pressure laboratory microwave oven (Multiwave 3000, Anton Paar, Germany) was employed to perform the acid digestion of samples. The digestion program began at a potency of 500 W, then ramped for 1 min, after which samples were held for 4 minutes. The second step at a potency of 1000 W (ramp 5 min) was held for 5 minutes. The third step at a potency of 1400 W (ramp 5 min) was held for 10 minutes. A blank digest was carried out in the same way.

Digested samples were diluted to a final volume of 50 ml with double deionised water. Concentrations of Fe, Mg and Zn were determined on a wet weight basis as mg/kg. All samples were run in batches that included blanks, a standard calibration curve and two spiked specimens. Detection limits were determined as the concentration corresponding to three times the standard deviation of ten blanks. The quality

of data was checked by an analysis of the recovery rate using certified reference material: dogfish liver (DORT-4, National Research Council, Canada). The reference material was treated and analysed under the same conditions as the samples.

Quantification of Fe, Mg and Zn

An inductively coupled plasma optical emission spectrometer with axial and radial viewing plasma configuration (ICP-OES Model Optima 8000, Perkin-Elmer, USA) operating at a 40 MHz free-running ratio-frequency and provided with an S 10 autosampler (Perkin-Elmer) was utilized. The nebulization system was equipped with a chemical-resistant concentric glass nebulizer coupled to a glass cyclonic spray chamber. A torch with an alumina-made injector was used. The polychromator, equipped with an Echelle grating, had a spectral range of 160–900 nm and a resolution of 0.009 nm at 200 nm. A UV-sensitive dual backside illuminated Charge-Coupled Device (CDD) array detector was used. The CDD array detector collects both the analyte spectra and nearby background spectra, allowing for simultaneous background correction and providing improved precision and analytical speed. The instrumental operating conditions used are shown in Table 1.

Determination of daily intake

The estimated daily intake (EDI) was calculated by the equation (13):

EDI = [(Mean of mg per kg of food) multiplied by (Daily Intake of food)] divided by [Adult body weights (60 kg)].

The contributions of each food items to the average dietary intake of elements were provided by comparison of mean values to RDA values expressed for females and males, with the assumption that the average adult woman and the average adult man consumed the same diet, i.e. the average typical diet of this population group.

Statistical analysis

Statistical analysis was performed using the Statistica 6.1 software (StatSoft Inc., Tulsa, USA). Concentrations were expressed as mean ± standard deviation, median, minimum and maximum values. One-way analysis of variance was used to test for differences in element levels in samples. In addition, determination of differences in concentrations of the element concentrations (Zn, Mg and Fe) between different food items were analysed using the *t*-test. Results were considered significant at $p < 0.05$.

Table 1: Operating conditions for Optima 8000 ICP-OES

Parameter	Value
Plasma viewing mode	Axial
Read time	1–5 s
Measurement replicates	3
RF incident power	1300 W
Plasma argon flow rate	8 L/min
Nebulizer argon flow rate	0.55 L/min
Auxiliary argon flow rate	0.4 L/min
Sample uptake rate	1 mL/min
Inner diameter of the torch injector	2.0 mm
Nebulizer type	Concentric glass (Meinhard)
Spray chamber type	Glass cyclonic spray chamber

Results and Discussion

The accuracy of results were checked and showed good accuracy with the recovery rate for tested elements (%): Fe 99.6, Mg 104.9, Zn 106.9. The limits of detection (LODs, mg/kg) of elements in meat samples were: Fe 0.005, Mg 0.008 and Zn 0.0015. The mean content of these elements are presented in Table 2. Analysis of variance showed significant differences between the tested food groups for all three elements tested ($p < 0.001$).

Selected food items represent only a portion of the food items that contribute to the average daily intake of essential elements. Also, consumption of meat or meat products is dependent on culture and availability and varies considerably between individuals (14). In a recent study of dietary exposure in different countries, it was concluded that the food groups that most contributed to the intake of Zn were meat and poultry, followed by breads, cereals and dairy products (7, 14, 15, 16). It is generally known that iron rich foods are those of animal origin, while those of plant origin are rich in non-heme iron (17). Cereals and legumes often contain a high amount of inhibitors to mineral absorption, such as phytates and polyphenols, which inhibit which inhibit zinc and/or non-heme iron and creating insoluble complexes in the intestines. Therefore the bioavailability of these micronutrients from these foods is often poor (18). The food groups that contributed the most for Mg intake were fish and fish products, cereals and cereal products and meats and offal (19, 20).

Regarding the requirements of essential elements for a number of physiological functions, their deficiencies may play a negative role in children's development, pregnancy and elderly health in humans and animals (3, 21, 22). The US Food and Nutrition Board of the Institute of Medicine is in charge of estimation of nutritional deficiency problems and toxicity and establishing the quantities of various nutrients by setting the recommended dietary allowance (RDA), the estimated average requirement (EAR), the adequate intake (AI) and the tolerable upper intake level (UL) for essential trace elements (12). The recommended dietary allowance (RDA) and the adequate intake (AI) for the prevention of disease and as the sufficient recommended adequate intake of elements for females/males are (mg/kg): for Fe 18/8; for Mg 310/400 and for Zn 8/11 (12).

Table 3 shows the estimated daily intake (EDI) for Fe, Mg and Zn based on the concentrations found in this study calculated by taking into account the average consumption of 120 (g/day per adult) for meat and meat products in Croatia (23). The EDIs for Fe in the meat of tested species and pig kidney ranged from 0.0127 to 0.1064 mg/day, thus contributing 0.07–0.59% and 0.16–1.33% of the RDA values of 18 mg/day for adult women

Table 2: Contents of Fe, Mg and Zn (mg/kg) in meat and meat products collected from Croatian markets

		Fe[1]	Mg[2]	Zn[3]
Meat and meat product	N	Mean ± SD (ranges)	Mean ± SD (ranges)	Mean ± SD (ranges)
Meat product	4	7.20 ± 2.35 [a] 4.27-9.92	212 ± 30.9 [a] 173-256	13.4 ± 2.97 [abc] 10.7-18.1
Processed meat products	9	7.05 ± 6.85 [b] 1.24-23.5	195 ± 76.4 86.2-333	13.2 ± 5.01 [bcc] 7.08-24.4
Meat				
Pork	3	6.33 ± 2.76 [c] 3.40-8.88	220 ± 38.1 182-258	22.2 ± 9.31 [a] 12.1-30.4
Beef	5	24.9 ± 2.72 [abcc] 20.6-27.7	263 ± 29.7 [ab] 227-309	51.7 ± 14.3 [acc] 36.3-64.6
Horse	3	22.4 ± 2.02 [abcd] 20.1-23.9	213 ± 38.5 179-255	41.3 ± 18.4 [bcc] 24.9-61.3
Kidney, pork	3	53.2 ± 14.7 [abccd] 36.5-63.9	173 ± 20.7 [b] 153-194	26.8 ± 10.4 [ab] 19.4-34.1

Vertically, letters show statistically significant differences: 1 between: meat and meat products: [a] ($p < 0.001$); meat and different products: [b] ($p < 0.001$); meat: [c] ($p < 0.001$), [d] ($p < 0.01$); [2 a] ($p < 0.05$), [b] ($p < 0.001$); [3 a] ($p < 0.05$), [b] ($p < 0.01$), [c] ($p < 0.001$)

and 8 mg/day for adult men, respectively. For meat products and different products, EDIs values were only 0.014 mg/day, contributing 0.08% and 0.18% of the RDA values for women and men. For Mg, EDIs for different meat, pig kidney and both groups of meat products ranged from 0.39 to 0.53 mg/day, thus contributing in range 0.09–0.17% of the RDA of 310 mg/day for females and 400 mg/day for males. The similar EDIs of 0.026 mg/day for Zn in meat products and different products were measured, contributing less than 0.35% of the RDA of 8 mg/day for females and 11 mg/day for males. For meat and pig kidney, EDIs values were only in the range 0.044 to 0.103 mg/day, contributing 0.56–1.29% and 0.40–0.94% of the RDA values for women and men, respectively.

The results obtained for three elements were compared with the literature data. Concentrations of Fe, Mg and Zn in different types of meat and products obtained in different countries in recent years are presented in Table 4.

Table 3: Estimation of daily intakes (EDIs) of Fe, Zn and Mg through consumption of meat and meat products and comparison to RDA values

Element	Fe		Mg		Zn	
Food	EDI (mg/kg BW/day)	Contribution of mean to RDA[a] (%)	EDI (mg/kg BW/day)	Contribution of mean to RDA[a] (%)	EDI (mg/kg BW/day)	Contribution of mean to RDA[a] (%)
Meat product	0.0144	0.08 (F) 0.18 (M)	0.42	0.14 (F) 0.11 (M)	0.0268	0.34 (F) 0.24 (M)
Processed meat products	0.0141	0.08 (F) 0.18 (M)	0.39	0.13 (F) 0.10 (M)	0.0264	0.33 (F) 0.24 (M)
Meat						
Pork	0.0127	0.07 (F) 0.16 (M)	0.44	0.14 (F) 0.11 (M)	0.0444	0.56 (F) 0.40 (M)
Beef	0.0498	0.28 (F) 0.62 (M)	0.53	0.17 (F) 0.13 (M)	0.1034	1.29 (F) 0.94 (M)
Horse	0.0448	0.25 (F) 0.56 (M)	0.43	0.14 (F) 0.11 (M)	0.0826	1.03 (F) 0.75 (M)
Kidney, pork	0.1064	0.59 (F) 1.33 (M)	0.35	0.11 (F) 0.09 (M)	0.0536	0.67 (F) 0.49 (M)

a RDA for female (F) and male (M): Fe 18 mg/day (F), 8 mg/day (M); Mg 310 mg/day (F), 400 mg/day (M); Zn 8 mg/day (F), 11 mg/day (M)

Table 4: Overview of the Fe, Zn and Mg contents in different meat products in previous studies

	Country Meat and meat products (reference)					
Element (mg/kg)	Denmark	France	Italy	Spain	UK	Turkey
Fe	BM 24 (2) PM 7 (2)	M 12.7 (19) O 60.8 (19)	BM 18-23.7 (6) PM 4.2-7 (6) HM 22.7 (6)		BM 18 (2) PM 7 (2)	M 1.36 (25) S 1.56; 1.07 (25)
Mg		M 286 (15) O 305 (15) M 309 (20) O 292 (20)		M 224 (26)		
Zn	BM 47 (2) PM 36 (2)	M 36.20 (7) M 36.76 (15) O 31.12 (15)	BM 39.4-47.5 (6) PM 9.8-22.8 (6) HM 19.5 (6)	PK 30.5 (27) M 42.3 (26)	BM 41 (2) PM 21 (2)	M 1.11 (25) S 0.49; 0.6; 1.59 (25)

BM- bovine, muscle; PM-pig, muscle; HM- horse, muscle; PK-pig, kidney; M-meat (without specification); O-offal (without specification); S-sausages

The concentrations of Fe in different type of meat and meat products ranged from 1.24 to 63.9 mg/kg. Among different meat products, a minimal mean Fe of 6.33 mg/kg level was measured in pork meat and maximal in liverwurst (23.5 mg/kg). Significantly higher Fe concentrations were found in pork kidney than those in meat products, different meat products, pork, beef and horse ($p<0.001$, all). However, significantly lower Fe levels were determined in meat products, different meat products and pork than in beef and horse meat ($p < 0.001$, all). The high levels of Fe measured in kidney may be explained by the role of the kidney in iron homeostasis. It is known that a significant amount of serum iron is available for glomerular ultrafiltration, and also that most of the filtered iron is reabsorbed by the renal tubules (24). The Fe contents reported in this study are in general agreement with the literature values reported for meat of different species (6, 19, 25). There are limited data regarding element levels in processed meat. In the very scarce data regarding element levels in meat products such as sausages, Fe concentrations lower than those measured in the present study were determined in Turkey (25).

The concentrations of Mg ranged from 86.2 to 333 mg/kg. The highest Mg concentration was found in the product beans with cooked bacon (333 mg/kg). Significantly higher Mg concentrations were determined in beef meat than in processed meat products and pig kidney ($p<0.05$; $p<0.001$). Relatively few data on the Mg content in meat products were available. Magnesium levels measured in meat samples in this study were lower that recently reported levels in France (15, 20) but similar to values from Spain (26). Also concentrations obtained in pig kidney were lower than those determined in offal samples from France (15, 20).

The concentrations of Zn were in the range 7.08–64.6 mg/kg. The highest zinc concentration was determined in smoked pork neck (24.4 mg/kg) in the group of processed meat products. Accordingly, the main findings were significantly higher Zn levels in: beef and horse meat than in meat products ($p<0.001$, both) and processed meat products ($p<0.001$, both); beef meat than in pig kidney ($p<0.05$); pig kidney than those in meat products ($p<0.05$) and processed meat products ($p<0.001$).

The concentrations of Zn in beef and pork meat presented are similar to results from other countries (2, 7, 15, 27, 28). However, results measured in horse meat were more than twice those from Italy (6). Also, Zn levels measured in both groups of meat products were more than 10-times higher than those measured in Turkey (25).

In conclusion, the results obtained for Fe levels are in agreement with literature values reported, while some differences were found for Mg and Zn.

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PRIMERJALNA ŠTUDIJA VNOSA ŽELEZA, MAGNEZIJA IN CINKA TER DNEVNIH VNOSOV NEKATERIH VRST MESA IN MESNIH IZDELKOV

N. Bilandžić, M. Sedak, M. Đokić, I. Varenina, B. Solomun Kolanović, Đ. Božić, M. Brstilo, D. Sokolić-Mihalak, Z. Jurković

Povzetek: V letu 2012 so na hrvaških trgih zbrali različne vrste mesa (govedina, svinjina, konjsko meso, ledvice svinj) ter mesnih izdelkov (bolonjska omaka, šunka v ovoju, jetrne klobase, fižol s kuhano slanino, goveji vampi, dimljena svinjska vratovina, panceta, klobase in kuhana slanina). Ugotovili so koncentracije Fe, Mg in Zn in so bile naslednje (v mg/kg): Fe 1,24 – 63,9; Mg 86,2 – 333 in Zn 7,08 – 64,6. Najvišje vsebnosti elementov v različnih vrstah mesa in mesnih izdelkov so bile (mg/kg): Fe 53,2 v svinjskih ledvicah, Mg 263 v govedini in Zn 51,7 tudi v govedini. Najnižje vrednosti so bile v mg/kg: Fe 6,33 v svinjini, Mg 173 v svinjskih ledvicah in Zn 13,2 v različnih mesnih izdelkih. Med različnimi skupinami živil so pri vseh treh elementih opazili statistično značilne razlike. Ocenjeni povprečni dnevni vnos (EDI) Fe, Mg in Zn v različnih vrstah živil, ki prispevajo k priporočenemu vnosu hrane (RDA) za ženske in moške, je bil v naslednjih razponih (odstotki): Fe 0,07 - 1,33; Mg 0,09 - 0,17 in Zn 0,40 - 1,29. Rezultati, pridobljeni v skupinah živil, testiranih za raven Fe, se ujemajo s podatki iz literature, za Mg in Zn pa so bile ugotovljene razlike.

Ključne besede: Fe; Mg; Zn; meso; mesni izdelki; ICP-OES; Hrvaška

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Original Scientific Article

FERTILITY OF GILTS WITH PROLONGED PREINSEMINATION ANESTRUS AFTER PROGESTAGEN-eCG TREATMENT

Ivan Stančić[1]*, Darko Bošnjak[1], Ivan Radović[1], Dragan Gvozdić[2], Božidar Savić[3], Blagoje Stančić[1]

[1]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, [2]Faculty of Vetrinary Medicine, University of Belgrade, [3]Scientific Veterinary Institute of Serbia, Belgrade, Serbia

*Corresponding author, E-mail: dr.ivan.stancic@gmail.com

Summary: The objective was to determine the effects of Regumate® alone or Regumate®+eCG treatment on the synchronization of estrus and fertility in delayed puberty (n=30+30) and normal cyclic gilts (n=30+30). Gilts were fed daily a complete diet containing 20 mg/gilt Regumate® for 18 days. Twenty-four hours after the last feeding of Regumate®, all gilts received an i.m. injection of 1,000 IU eCG. Gilts were inseminated artificially (AI) 12 and 24 h after first detection of standing estrus. More normal cyclic gilts (84.6%) than delayed puberty gilts (66.7%) expressed estrus by d 4.8 after Regumate® alone treatment ($p < 0.05$). However, similar proportion of normal cyclic (90%) and delayed puberty gilts (93%) were in estrus by d 4.2 after Regumate® + eCG treatment ($p > 0.05$). These findings indicate that about 70% of delayed puberty gilts had established cyclic ovarian activity (i.e. sexually mature, cyclic gilts), while about 30% were truly delayed puberty gilts (sexually immature, prepubertal acyclic gilts), before progestagen treatment. AI of delayed puberty gilts resulted in 80 to 85.7% farrowing rate and 10.50 to 11.04 liveborn piglets per litter. Obtained results demonstrate that progestagen treatment may be an effective tool for increasing gilts' reproductive efficiency and reducing the number of gilts culled from the breeding herd due to prolonged preinsemination anestrus.

Key words: progestagen; eCG; fertility; prolonged anestrus; gilt

Introduction

The most common reason for culling gilts from the breeding herds is prolonged preinsemination anestrus, i.e. when estrus is not detected in gilts older than 8 months of age (1, 2). Estrus may be undetected in gilts that have not established pubertal cyclic ovarian activity and those gilts are defined as truly delayed puberty gilts (3), or in cyclic, sexually mature, but behavioural anestrus gilts (4, 5).

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Recent investigation on large swine breeding farms in Vojvodina (Serbia) demonstrated that 30 to 40% of gilts failed to exhibit behavioural estrus even after 8 months of age, thus increasing economic problems for swine producers. These gilts are culled from the breeding herd as delayed behavioural anestrus gilts, i.e. prolonged pre-insemination anestrous gilts (5, 6, 7). However, previous results of post mortem examination of reproductive organs have shown that many of these gilts actually have cyclic ovarian activity, and that behavioural delayed anestrus may be the result of poor estrus detection technology on farm (8, 9, 10, 11). Based on these findings, we assume

that treatment with progestagen preparation could result in a good estrus response and fertility parameters (farrowing rate and litter size) after artificial insemination of delayed behavioural anestrus gilts. Namely, it has been demonstrated that treatment with progestagen preparation alone is highly effective in synchronizing estrus in randomly cycling gilts, but not in prepubertal (acyclic) individuals (12, 13).

Therefore, the aim of this paper was to compare fertility in prolonged preinsemination anestrus gilts and normal cyclic pubertal gilts, after artificial insemination in estrus synchronized by progestagen treatment.

Materials and methods

General. The experiment was conducted at a swine breeding farm in Vojvodina (Republic of Serbia) with the capacity of 5,500 sows, with average lactation duration of 30 days. Gilts were maintained in groups of twenty to twenty five in a building for replacement gilts. All studied animals were fed and housed in the same way and in the same period of the year. Estrus detection at the farm was conducted once in 24 hours by direct contact with teaser boars.

Experimental animals. Two categories of gilts were used in the experiment (60 gilts in each group). First group: Gilts culled from a breeding herd due to prolonged preinsemination anestrus (estrus not detected until 8 month of age, av. 258 days), i.e. delayed puberty gilts. Second group: Sexually mature (cyclic) gilts with at least one spontaneous estrus cycle, about 210±5 days of age, i.e. normal cyclic gilts. Gilts in this group served as controls.

Hormonal treatment. All gilts were treated with progestogen preparation Regumate® (Allyl trenbolone, Altrenogest, Roussel UCLAF, agro-division Veterinaria, Bernburg, Germany). Gilts were fed 2.5 kg of a complete ration containing 20 mg Regumate® for 18 days. The next day after progestagen treatment, half of the total treated gilts in both groups (n = 30) received an i.m. injection of 1,000 IU eCG - Equine Chorionic Gonadotropin (Folligon®, Intervet-Boxmer, Holland).

Estrus detection. Starting from the second day after hormonal treatment, the behavioural estrus manifestation was tested two times a day, in an interval of about 12 h, by full mature boar contact.

Artificial insemination was performed 12 h and 24 h after detection of standing estrus. Insemination dose, volume 100 ml, contained a minimum of 3 x 10^9 spermatozoa. Disposable SoftGilt Catheter (Minitübe, Germany) was used.

Recorded data: Farrowing rate from insemination in the first estrus after treatment, live born, stillborn and total born piglets were determined for each litter.

Statistical Analyses. Descriptive statistics, t-tests were performed using the 10th edition of the Statistics software package. Descriptive statistics was performed on characteristic intervals: end of treatment to estrus, average piglets born per litter (liveborn, stillborn and total). T-test was used to determine differences between means. Differences in the characteristics on Intervals: end of treatment to estrus (days), average piglets born per litter (liveborn, stillborn and total) were made by a t-test (t-test for Dependent Samples). The difference between number of gilts in estrus and gilts farrowed (from treated) per treatment were tested by t-test (t-test for Dependent Samples). The statistical significance was set at $p < 0.05$. The difference between number of gilts farrowed (form AI) per treatment were tested by t-test (t-test for Independent Samples). The statistical significance was set at $p < 0.05$.

Results

The percentage of delayed puberty and normal cyclic gilts expressing estrus and interval from end of treatment to estrus, after Regumate or Regumate + eCG treatment, are shown in Table 1.

Significantly fewer ($p < 0.05$) delayed puberty gilts were in estrus after treatment with Regumate alone, compared with all other groups. More delayed puberty gilts and normal cyclic gilts were in estrus after Regumate+eCG treatment, compared to Regumate alone treatment ($p < 0.05$). The average end of treatment-to-estrus interval was 4.8 days in Regumate alone group and 4.2 days in Regumate + eCG group, in both delayed puberty and normal cyclic gilts. The difference in duration of these intervals was statistically significant ($p < 0.05$).

Farrowing rate and litter size of delayed puberty and normal cyclic gilts, inseminated in estrus after Regumate or Regumate + eCG treatment, are shown in Table 2.

Farrowing rate was very similar ($p > 0.05$) in normal cyclic gilts treated with Regumate alone (84.6%) or Regumate + eCG (85.2%), as well as in delayed puberty gilts treated with Regumate + eCG (85.7%). Significantly lower farrowing rate (80.0%) was recorded in delayed puberty gilts, treated with Regumate alone ($p < 0.05$). Total litter size, liveborn and stillborn piglets per litter were greater ($p < 0.05$) in normal cyclic and dealyed puberty gilts treated with Regumate + eCG, compared with normal cyclic and delayed puberty gilts in Regumate alone group. These values were not significantly different between normal cyclic and dealyed puberty gilts within the same treatment groups ($p > 0.05$).

Table 1: Estrus manifestation after hormonal treatment

		Hormonal treatment			
		Regumate®		Regumate® + eCG	
		Delayed puberty	Normal cyclic	Delayed puberty	Normal cyclic
No. gilts treated		30	30	30	30
Gilts in estrus	n	20	26	28	27
	%	66.7[a]	86.7[ab]	93.3[b]	90.0[b]
Interval: end of treatment to estrus (days, mean ± SD)		4.8±0.70[a]	4.8±0.81[a]	4.2±0.86[b]	4.2±0.85[b]

ab Values within rows with different superscripts differ ($p < 0.05$).

Table 2: Farrowing rate and litter size

		Hormonal treatment			
		Regumate®		Regumate® + eCG	
		Delayed puberty	Normal cyclic	Delayed puberty	Normal cyclic
No. gilts treated		30	30	30	30
Gilts farrowed (n)		16	22	24	23
Farrowing rate (%)		80.0[a] (16/20)	84.6[b] (22/26)	85.7[b] (24/28)	85.2[b] (23/27)
Average piglets born per litter (mean ± SD)	Liveborn	10.50±1.033[a]	10.41±1.008[a]	11.04±0.999[b]	10.96 ±0.976[b]
	Stillborn	0.69±0.602[a]	0.68±0.716[a]	0.75±0.737[b]	0.95 ±0.926[b]
	Total	11.19±1.047[a]	11.09±1.192[a]	11.79±1.141[b]	11.91 ±1.379[b]

In parentheses: farrowed/inseminated.

ab Values within rows with different superscripts differ ($p < 0.05$).

Discussion

It has been shown that Allyl trenbolone (altrenogest, Regumate®), an orally active progestagen substance, is effective in suppressing follicular development and estrus expressing in gilts. Namely, this progestagen does not prevent normal luteolysis, but continues to block the onset of estrus after luteolysis occurs (14). Therefore, feeding of altrenogest, in a dose of 15 to 20 mg/day/gilt over a period of 14 to 18 days, results in succesfull estrus synchronization in sexually mature gilts (15, 12, 16). Equine CG (eCG) has been frequently used after alternogest (Regumate®) tretament in order to stimulate follicular development and to achieve better synchronization of estrus and ovulation in gilts (14, 17).

In our study, more normal cyclic gilts (84.6%) than delayed puberty gilts (66.7%) expressed estrus by d 4.8 after Regumate® alone treatment ($p < 0.05$). However, similar proportion of normal cyclic (90%) and delayed puberty gilts (93%) were in estrus by d 4.2 after Regumate® + eCG treatment ($p > 0.05$). These findings indicate that about 70% of delayed puberty gilts, had established cyclic ovarian activity (i.e. sexually mature, cyclic gilts), while about 30% were truly delayed puberty gilts (sexually immature, prepubertal acyclic gilts), before progestagen treatment. Previous researches demonstrated that daily feeding of Regumate® alone, at levels of 15 to 20 mg/d for 14 to 18 d synchronized estrus in a large proportion of treated randomly cycling gilts, but not in prepubertal (acyclic) gilts. On the other hand, eCG stimulates the onset of estrus in prepubertal gilts but does not synchronize estrus in cycling females (11 - 14, 16 - 20). Thus, a combination of Regumate® and eCG treatments could be an effective way of synchronizing estrus in a group of gilts for which the cycling status is unknown (16).

Satisfactory farrowing rate (80% to 85.7%) and litter size (10.4 to 11.0 liveborn piglets) were obtained in treated group of gilts, after artificial insemination in estrus synchronized by Regumate®. Total litter size, liveborn and stillborn piglets per litter, tended to be greater ($p < 0.05$) in gilts treated with Regumate® + eCG combination than in gilts treated with Regumate® alone. It has been recently demonstrated that sexually mature (cycling) gilts given eCG 24 h after withdrawal of Regumate® had a higher ovulation rate than did gilts given Regumate® alone (11, 14, 17, 18, 21).

The results obtained in this study show that treatment with Regumate® + eCG combination results in good estrus synchronization and high fertility (farrowing rate and litter size) in delayed behavioural anestrus gilts (i.e. gilts with unknown cyclic status). Therefore, progestagen treatment may be an effective tool for enhancing reproductive efficiency in herds of replacement gilts. This can significantly reduce the number of gilts culled from the breeding herd due to prolonged preinsemination anestrus.

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PLODNOST PRI MLADICAH SVINJ S PODALJŠANIM PREDOSEMENITVENIM ANESTRUSOM PO ZDRAVLJENJU Z PROGESTAGENOM eCG

I. Stančić, D. Bošnjak, I. Radović, D. Gvozdić, B. Savić, B. Stančić

Povzetek: Cilj raziskave je bil določitev učinka zdravljenja z Regumate® ali z Regumate® v kombinaciji z eCG na sinhronizacijo estrusa in plodnost pri mladicah svinj v podaljšani puberteti (n = 30+30) in pri normalnih mladicah v pojatvenem ciklusu (n = 30+30). Mladice so bile dnevno 18 dni hranjene s popolno krmo, ki je vsebovala 20 mg Regumate®/svinjo. Štiriindvajset ur po zadnjem krmljenju mladic z mešanico, ki je vsebovala Regumate®, so vse mladice prejele i/m injekcijo 1000 IU eCG. Mladice so bile nato umetno osemenjene 12 in 24 ur po prvem odkritju estrusa. Več mladic z običajnim ciklusom (84,6 odstotka) kot mladic v zapozneli puberteti (66,7 odstotka) je imelo izražen estrus 4,8 dni po zdravljenju samo z Regumate® ($p < 0,05$). Po zdravljenju z Regumate® v kombinaciji z eCG pa je bil ugotovljen podoben delež mladic z običajnim ciklusom (90 odstotkov) in mladic v zapozneli puberteti (93 odstotkov) v estrusu 4,2 dni po zdravljenju ($p > 0.05$). Te ugotovitve kažejo, da je imelo približno 70 odstotkov mladic v zapozneli puberteti urejeno ciklično dejavnost jajčnikov (tj. spolno zrele, ciklične mladice), medtem ko je bilo približno 30 odstotkov mladic pred zdravljenjem s progestagenom resnično zapoznelih (spolno nedozorele, predpubertetne aciklične mladice). Umetna osemenitev je pri mladicah v zapozneli puberteti povzročila 80 - 85,7 odstotno stopnjo prasitev in 10,5 - 11,04 živorojenih pujskov na gnezdo. Dobljeni rezultati kažejo, da je lahko zdravljenje s progestagenom učinkovito orodje za povečanje reproduktivne učinkovitosti mladic in zmanjšanje števila mladic, izločenih iz plemenske črede zaradi daljših predosemenitvenih anestrusov.

Ključne besede: progestagen; eCG; rodnost; podaljšan anestrus; mladica

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Original Scientific Article

EPIZOOTIOLOGICAL, CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF SHEEP FLOCKS INFECTED WITH *BRUCELLA OVIS* IN THE REPUBLIC OF SERBIA

Miloš Petrović[1], Silvio Špičić[2], Aleksandar Potkonjak[3], Branislav Lako[3], Miloš Kostov[4], Željko Cvetnić[2]

[1]Veterinary Specialist Institute Niš, Serbia; [2]Croatian Veterinary Institute Zagreb, Croatia; [3]Faculty of Agriculture, University of Novi Sad, Department for Veterinary Medicine, Novi Sad, Serbia; [4]Military Hospital, Pathology Department, Niš, Serbia

*Corresponding author, E-mail: cvetnic@veinst.hr

Summary: This paper describes a study on *Brucella ovis* infection of sheep flocks in southern Serbia. Using serological testing, positive reactions were confirmed in 67 (29.8%) and suspicion in 31 (13.8%) out of the total 225 tested sheep sera samples. Rams originated from 113 flocks with 4751 sheep from 28 settlements in the Pirot region of southern Serbia. Pathological changes indicative of *Brucella ovis* infection were confirmed by macroscopic examination in the testes of 7 (58.3%) out of 12 examined rams. In 7 (58.7%) animals, unilateral epididymitis with pronounced hypertrophy of the tail, body and head of epididymis was confirmed. Using bacteriological and molecular techniques, the presence of *B. ovis* was confirmed in the samples (testes, epididymis, lymph nodes) in 11 (91.7%) out of 12 rams. The clinical manifestation of ovine epididymitis arose following the procurement of rams and mating season. The disease was more pronounced in flocks in which it occurred for the first time. On the basis of the results presented here, it can be concluded that *B. ovis* infection causes substantial economic losses in sheep production, and is manifested in reduced conception (24.85%), miscarriages (5.38%), reduced number of lambs (0.74 lambs per ewe) and increased perinatal mortality (7.78%).

Key words: *Brucella ovis*; epizootiology; clinical changes; pathological changes; Serbia

Introduction

Brucella (B.) ovis causes chronic disease in sheep which manifests in characteristic testes and epididymis changes in rams and placentitis in ewes. It is estimated that this disease causes substantial economic losses in flocks where there is no disease control (7). Losses are seen in reduced fertility of rams, miscarriages in ewes, early deaths of avital lambs, removal of infected animals from the flock and bans on trade. *B. ovis* is considered to be the most important causative agent of infectious reproductive disorders in sheep worldwide (1, 11, 12, 28).

The main symptoms of the disease in rams are epididymitis and, less commonly, orchitis. Infected rams have lower sperm quality and reduced fertility. Infection can result in sterility. Changes are usually unilateral, rarely bilateral. Pathological changes are most often found in the tail, less often in the body and the head of the epididymis. The percentage of infected rams in the country is very high, and ranges from 20–60%, and infected flocks from 45–75%. In countries with advanced control programmes, the prevalence is significantly lower,

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though complete eradication is difficult to achieve. It strongly depends on situation in country/region (6, 7, 8, 11, 25, 27, 39).

Infection with *Brucella ovis* has been confirmed in virtually all countries with relevant sheep production (5, 9, 15, 33). It has been proven in neighbouring countries, i.e. Romania (21) and Croatia (18, 42), and has been described in Slovenia (30), Austria (40), Italy (23), Switzerland (26), Spain (35) and Ukraine (19).

The objective of this study was to confirm the epizootiological, clinical and pathomorphological characteristics of *Brucella ovis* infection in sheep flocks in a sheep-raising region in Serbia.

Material and methods

Epizootiological survey and clinical examination

On the basis of results of serological testing (indirect enzyme linked immunosorbent assay, ELISA; and complement fixation test, CFT), an epizootiological survey and clinical examination of seropositive rams were conducted. Serological testing was conducted on 225 ram sera samples originating from 113 flocks with 4751 sheep, from 28 settlements in the Pirot region of southern Serbia. The epizootiological survey and clinical examination of seropositive rams was conducted in 7 (30.4%) out of 23 seropositive flocks of sheep, in 6 (42.9%) out of 14 settlements in which seropositive animals were confirmed. The surveyed flocks were labelled with codes S36, S46, S57, S58, S63, S64 and S74.

Pathomorphological and bacteriological testing

Biological materials were sampled from seropositive rams for pathomorphological and bacteriological testing. The testes and epididymis were sampled after the castration of three rams, and the testes, epididymis and lymph nodes (*lnn. inguinalis, lnn. ilicimediales* and *lnn. lumbalesaortici*) were sampled following the slaughter of nine rams. The observed macroscopic changes are described below in detail.

During the pathoanatomical examination, several tissue sections were taken from the macroscopically altered parts of the organs, and from the direct surroundings of the seemingly unaltered tissue. The size of tissue sections was typically 1.5 cm x 1 cm x 0.5 cm.

Tissue sections were fixed in 10% buffered neutral formalin, and prepared according to standard procedures (37). Tissue preparations were stained using the standard hematoxylin and eosin (HE) method and were microscopically analysed on a *Leica DM 1000* microscope with a digital *Leica EC3* camera (32). In the staining process, hematoxylin (*Mayer's hematoxylin, Bio Optica*, Italy) and eosine (1% aqueous solution, BioGnost, Croatia) solutions were used.

Bacteriological processing and identification of isolates

Bacteriological testing was conducted on 21 samples of epididymis, testes and lymph node tissues originating from 12 rams from 7 flocks, from 6 settlements. Several grams of delivered materials (testes and lymph node tissue) were examined and about 1 ml of homogenate was inoculated onto a selective nutritional substrate, i.e. blood agar, Brucella agar and modified semi-selective nutritional substrate according to Thayer-Martin. Plates with inoculated material were incubated at 37°C in the presence of 10% CO_2, and colony growth was observed in daily intervals. Isolates were identified on the basis of colony morphology, growth in the presence of an atmosphere with 5–10% CO_2, production of H_2S, growth on substrates with the addition of 20μg/ml thionine and basic fucsin and agglutination of antiserum R (4, 17, 34). In order to prove that the isolates belong to the genus *Brucella,* a PCR method based on the replication of the part of the genome that codes the synthesis of protein BCSP-31 characteristic for genus *Brucella* was used. The expected size of the replication product is approximately 440 bp (41). For identification of *Brucella* species, a multiplex PCR (Bruce-ladder, Ingenasa, Spain) method was used (22).

Results

Results of the serological testing

Using indirect enzyme immunoassay testing, a positive reaction was found in 67 (29.8%) and suspicion in 31 (13.8%) out of 225 examined ram

serum samples. Seropositive rams originated from 16 settlements (57.1% of the investigated settlements in the Pirot region) and 34 flocks (30.1% of investigated flocks in the Pirot region). The complement fixation test confirmed a positive reaction in 41 (18.2%) out of 225 examined rams. Seropositive rams originated from 14 settlements (50% of investigated settlements in the Pirot region) and 23 flocks (20.4% of investigated flocks in the Pirot region).

Results of the epizootiological survey

The results of the epizootiological survey indicate that the clinical manifestations of epididymitis in sheep in all flocks appeared after introduction of new rams or ewes and during the mating season. Manifestation of the disease was more pronounced in flocks in which the disease appeared for the first time. Based on results of the epizootiological survey in this study, it can be concluded that infection with *B. ovis* causes substantial economic losses in sheep production, seen in consequential reduction of conception (24.85%), miscarriages (5.38%), reduced number of lambs (0.74 lambs per ewe) and perinatal mortality (7.78%) (Table 1).

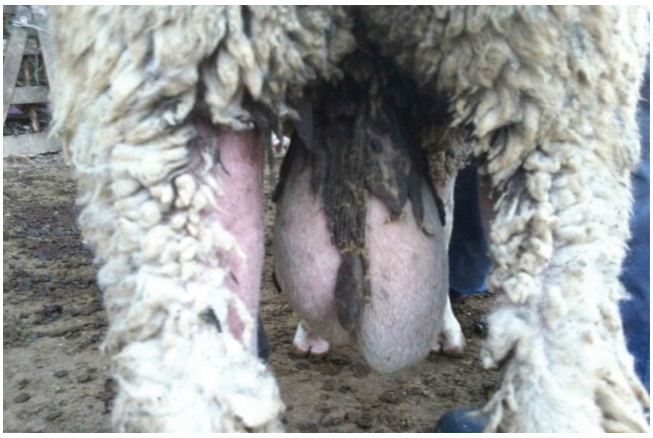

Figure 1: Scrotum asymmetry

Results of clinical examination

Clinical examination by adspection and palpation of epididymis and testes was conducted on 12 rams from 7 seropositive flocks, and in 5 (41.7%) seropositive rams, asymmetry of scrotum and unilateral enlargement of the epididymis tail were established. The enlargement of the epididymis was up to the size of chicken's egg. Increased sensitivity and pain during palpation was exhibited by 80% of rams with changes to epididymis (Table 2 and Figure 1).

Table 1: Manifestation of sheep epididymitis in seropositive flocks based on the epizootiological survey

Flock code	No. of rams	No. of ewes	Duration of disease (years)	Asymmetry of testes			Sheep		Lambs	
				Enlargement of epididymis and testes			Did not conceive	Miscarried	Births per ewe	Perinatal mortality
				Unilateral	Bilateral	Total				
S 36	3	55	4	(0/3)	(0/3)	(0/3)	(5/55)	(0/50)	(52/55)	(0/52)
				0%	0%	0%	9.09%	0%	0.95	0%
S 46	4	87	1	(1/4)	(0/4)	(1/4)	(34/87)	(3/53)	(55/87)	(16/55)
				25%	0%	25%	39.08%	5.66%	0.63	29.09%
S 57	1	7	1	(0/1)	(0/1)	(0/1)	(1/7)	(0/6)	(6/7)	(0/6)
				0%	0%	0%	14.28%	0%	0.86	0%
S 58	2	47	1	(2/2)	(0/2)	(2/2)	(14/47)	(0/33)	(34/47)	(6/34)
				100%	0%	100%	29.79%	0%	0.72	17.65%
S 63	11	310	4	(3/11)	(1/11)	(4/11)	(34/310)	(16/276)	(271/310)	(15/271)
				27.27%	9.09%	36.36%	10.96%	5.80%	0.87	5.54%
S 64	1	11	3	(1/1)	(0/1)	(1/1)	(2/11)	(0/9)	(9/11)	(0/9)
				100%	0%	100%	18.18%	0%	0.82	0%
S 74	8	175	1	(1/8)	(0/8)	(1/8)	(82/175)	(9/93)	(87/175)	(3/87)
				12.50%	0%	12.50%	46.86%	9.68%	0.5	3.45%
TOTAL:	30	692		(8/30)	(1/30)	(9/30)	(172/692)	(28/520)	(514/692)	(40/514)
				26.67%	3.33%	30%	24.85%	5.38%	0.74	7.78%

Table 2: Results of the clinical examination of epididymis and testes of seropositive rams by adspection and palpation

Settlement	Flock code	Ram ID	Ram age	Serological results		Asymmetry of scrotum Enlargement of epididymis		
				ELISA	RVK	Unilateral	Bilateral	Total
Lukanjske pojate	S 36	6150254	2	+	+	0	0	(0/1) 0%
Nišor	S 46	6629594	3	+	+	0	0	(1/2) 50%
		4337349	3	+	+	1	0	
Ponor	S 57	4412845	2.5	+	+	0	0	(0/1) 0%
	S 58	4412829	3.5	+	+	1	0	(2/2) 100%
		2452058	4.5	+	+	1	0	
Rosomač	S 63	1448438	2.5	+	+	1	0	(1/2) 50%
		9413681	2.5	+	+	0	0	
Rsovci	S 64	6414294	5	+	+	1	0	(1/1) 100%
Slavinja	S 74	9332931	2.5	+	-	0	0	(0/3) 0%
		5412026	2.5	+	+	0	0	
		5625496	2.5	+	+	0	0	
Total:				12	11	(5/12) 41.67%	(0/12) 0%	(5/12) 41.67%

Table 3: Percent of established pathoanatomical changes in seropositive rams

Flock code	Ram ID	Hypertrophy of the epididymis				Spermato-celes	Atrophy of the testes	Granulomas in epididymis and testes	Total rams with confirmed changes
		total	tail	body	head				
S 36	615024	0	0	0	0	0	0	0	(0/1) 0%
S 46	662954	0	0	0	0	0	0	0	(1/2) 50%
	433739	1	1	1	0	0	0	0	
S 57	441285	0	0	0	0	0	0	0	(0/1) 0%
S 58	441289	1	1	0	0	0	0	0	(2/2) 100%
	245208	1	1	1	1	1	1	1	
S 63	144848	1	1	1	1	1	0	1	(1/2) 50%
	941361	0	0	0	0	0	0	0	
S 64	641424	1	1	1	0	0	1	1	(1/1) 100%
S 74	933291	0	0	0	0	0	0	0	(2/3) 66,67%
	541206	1	1	0	0	0	0	0	
	562546	1	1	0	0	0	0	0	
Total:		(7/12) 58,33%	(7/12) 58,33%	(4/12) 33.33%	(2/12) 16.67%	(2/12) 16.67%	(2/12) 16.67%	(3/12) 25%	(7/12) 58,33%

Results of pathomorphological testing

The macroscopic examination of epididymis and testes of 12 rams confirmed changes indicating *B. ovis* infection in 7 (58.33%) rams. The examination revealed lesions of varying degrees characteristics for acute and chronic phases of the disease. In acute phase, changes were necrotic, while in chronic phase granulomas, fibrosis and atrophy of testes and epididymis were observed (Table 3, Figures 2 and 3).

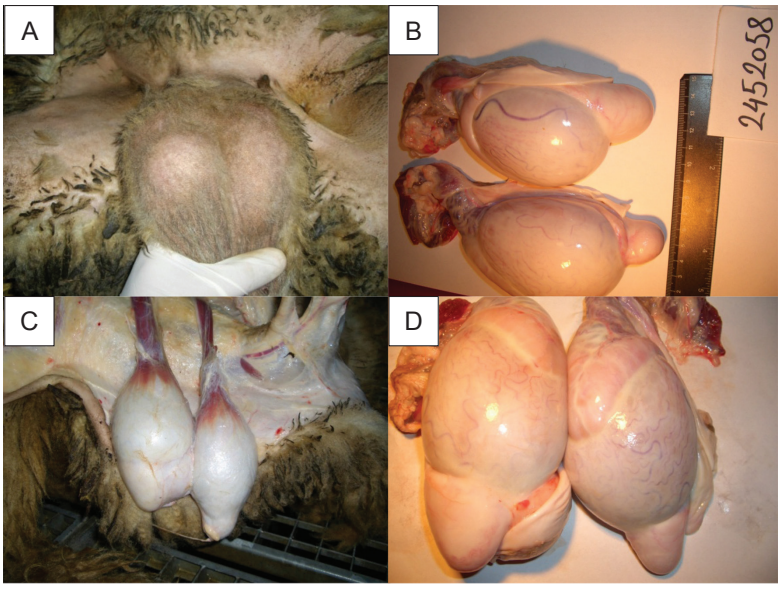

Figure 2: A) Increased epididymis tail. B) The unilateral enlargement of the whole epididymis, partly with lagging, accrued *tunica vaginalis*. C) "*In situ*" position of both testicles: abnormal, enlarged right testis and epididymis tail with spermatocele. D) Spermatocele of the epididymis tail, attached to the tunic vaginitis, which does not separate easily.

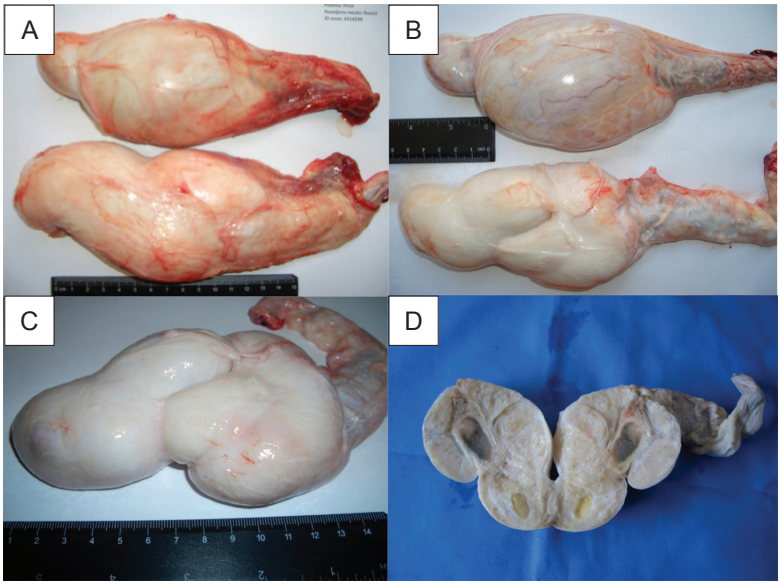

Figure 3: A) Both testes with accompanying envelopes. B) The testes without sheath. Upper testicle has distinctive appendages; lower atrophic testis with enlarged epididymis presents nodular changes, atypical anatomy, and is sporadically covered with stripe adhesions. C) Right testis with nodular changes, epididymis tail with cystic, slightly wave like wall. D) Sagittal section of the right testis and epididymis. Enlarged, deformed atrophic testis with gnarls and epididymis with granulomatous areas.

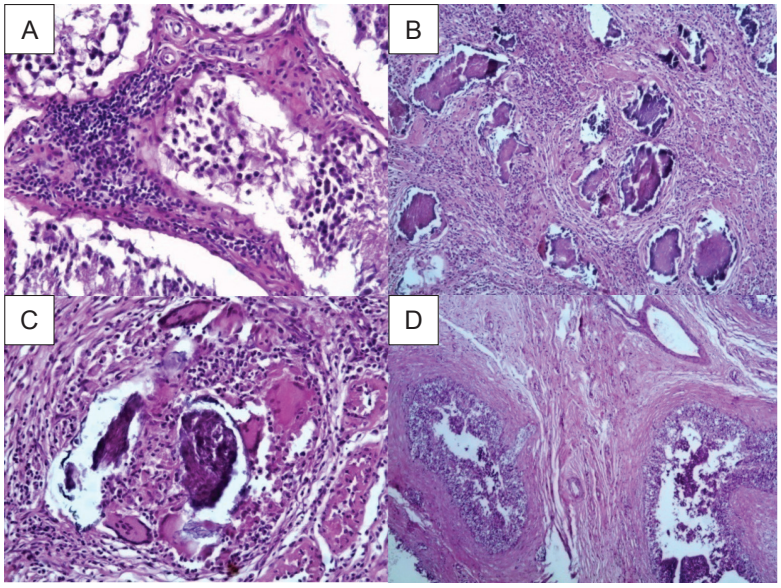

Figure 4: A) Pronounced interstitial mononuclear inflammatory infiltrate and seminiferous tubules with reduced sperm production and desquamated germinative epithelium (H & E, x200). B) The testicular atrophy with numerous microcalcifications, indicated by diffuse interstitial fibrosis and accumulation of inflammatory cells (H & E, x100). C) Spermatic granuloma with central accumulation of spermatozoa, surrounded by chronic inflammatory mononuclear and multinucleated giant cells in the testis (H & E, x200). D) Papillary hyperplasia with necrosis of epithelial cells, and interstitial fibrosis (H & E, x100)

Microscopic examination of epididymis confirmed vacuolar degeneration, papillary hyperplasia and necrosis of duct epithelial cells, and oedema and fibrosis of the interstitial area. In individual ducts, the formation of microabscesses and intraluminal necrotic tissue detritus were observed. In the interstitial area, mixed cell inflammatory infiltrate composed of plasma cells, lymphocytes and histiocytes was observed. Testes significantly reduced production of spermatozoids, with desquamated germinative epithelium in places. Individual preparations showed spermatic granulomas with a central accumulation of spermatozoids surrounded by chronic mononuclear inflammatory and multinucleated giant cells (Figure 4).

Results of bacteriological testing

Bacteriological testing was conducted on 21 epididymis, testes and lymph node tissue samples originating from 12 rams. A total of 20 isolates were obtained from the material of 11 (91.7%) rams.

Results of molecular testing

All 20 isolates and the standard referential strains of *B. abortus 544, B. melitensis 16M, B. ovis 63/290* and *B. suis 1330* were identified as *Brucella* using PCR. They were identified on the basis of the replication product of around 440 base pairs.

Species identification was done using multiplex PCR method (*Bruce-ladder, Ingenasa, Spain*). All 20 brucella isolates were identified as *Brucella ovis*. The PCR profile of isolates overlapped with the profile of referential strain *B. ovis 63/290*. The expected sizes of PCR products for *Brucella ovis* were 1071, 794, 587, 450 and 152 base pairs.

Discussion

In recent decades, serologically positive animals on *B. ovis* infection have been confirmed in the Republic of Serbia, but the causative agent has never been isolated and identified (29, 31, 36).

In this study, indirect enzyme immunosorbent assay tests confirmed positive reactions in 67 (29.8%) and suspicion in 31 (13.8%) of the total 225 tested ram blood sera samples. Seropositive rams originated from 16 settlements (57.1% investigated settlements in the Pirot region) and 34 flocks (30.1% of investigated flocks in the Pirot region).

Complement fixation test confirmed a positive reaction in 41 (18.2%) of the 225 investigated rams. Seropositive rams originated from 14 settlements (50% of investigated settlements in the Pirot region) and 23 flocks (20.4% of the investigated settlements in the Pirot region).

All seropositive flocks showed clinical manifestations of sheep epididymitis that appeared following the purchase of infected rams, less commonly ewes, and during the mating season. In flocks where the disease occurred for the first time after mating, flock owners reported a high incidence of the disease, and in flocks where sheep epididymitis was present for years the symptoms intensity was lower. Literature states that *B. ovis* infections in sheep caused lambing reduction by 30% in newly infected flocks, and 15–20% in flocks where the disease is endemic (9). Following infection, the number of live births can be reduced by a quarter, 16% of lambs die within the first 6 weeks of life, and 20% of lambs remain infertile (30). The results of the epizootiological survey presented here closely corroborate these reports.

Earlier studies showed that about 50% of *B. ovis* infected rams exhibit epididymitis, though clinical symptoms often withdraw (8). After test inoculation of nine rams intraprepucially and conjunctively with *B. ovis,* the following was seen: eight of nine rams were serologically positive (AGID and CFT). Thirty days after infection, six (66.7%) out of nine rams developed clinical changes in epididymis tail (increased volume and change in consistency) which regressed in 50% of those rams. Five (83.3%) out of six rams had unilateral changes, while four (66.6%) showed sensitivity to palpation (13). Numerous pathogens other then *B. ovis* are often responsible for macroscopic testicular alterations in rams. This restricts the significance of clinical examination of epididymis and testes of rams in diagnosis of sheep epididymitis (7, 8, 43, 44). Various bacterial species *(Actinobacillus seminis, Histophilus somni, Salmonella enterica subsp. diarizonae, Brucella melitensis, Escherichia coli)* can also cause clinical epididymitis (3, 14, 16, 20, 24). Also, it has been shown that in many cases of ram epididymitis, sterile granulomas are caused by trauma (44).

The clinical examination of 12 seropositive rams by epididymis and testes adspection and

palpation showed an increased percentage of rams with changes to epididymis and testes in comparison to epizootiological data. Asymmetry of scrotum and unilateral enlargement of epididymis tail was confirmed in 5 (41.7%) rams. Pathomorphological examination of epididymis and testes of 12 rams confirmed characteristic macroscopic findings of unilateral epididymitis with pronounced hypertrophy of epididymis tail in 7 (58.3%) rams, changes in epididymis body were found in 4 (33.3%) rams and in epididymis head in 2 (16.7%) rams. Microscopic examination of epididymis confirmed vacuolar degeneration, papillary hyperplasia and necrosis of duct epithelial cells, oedema and fibrosis of interstitial areas. In individual tubules, formation of microabscesses and intraluminal necrotic tissue detritus was observed. The interstitial area contains mixed cell inflammatory infiltrate composed of plasma cells, lymphocytes and histiocytes. Individual testes showed pronounced atrophy and fibrosis of sperm canals, presence of numerous microcalcifications with surrounding giant cell reactions and marked interstitial fibrosis with diffuse accumulation of chronic inflammatory cells.

The present study corresponds to research of other authors. Study conducted on 267 serologically positive rams, palpation confirmed enlarged testes in 125 (46.8%) rams. Following pathohistological examination, the number of animals exhibiting enlarged testes and epididymis was increased (7). Pathoanatomical changes were found in 68 (43.6%) out of 156 rams. An enlarged epididymis was confirmed in 38 (24.4%) rams, testicular atrophy in 14 (8.9%) and granulomas in testes and epididymis tissue in 16 (10.3%) tested rams. The pathohistological examination of epididymis in rams infected with *B. ovis* revealed interstitial oedema, fibrosis and perivascular infiltrates of lymphocytes and plasma cells. The same author confirmed epithelial hyperplasia with intraepithelial cysts containing neutrophils mixed with mononuclear cells (42). Granulomas surrounded by lymphocytes, epithelial and giant cells were a common find. A proliferation of intertubular connective tissue, small extratubular spermatozoic granulomas, necrosis and calcification were observed in individual preparations (38).

Epizootiological analysis of obtained results, clinical and pathomorphological examination in the present study indicate a higher percentage of rams with changes to epididymis and testes in relation to results of cited authors. This is a direct consequence of the lack of diagnostic testing for sheep epididymitis, long-term presence of the infection and lack of disease eradication programmes.

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EPIZOOTIOLOŠKE, KLINIČNE IN PATOLOŠKE ZNAČILNOSTI ČRED OVC, OKUŽENIH Z *BRUCELLO OVIS* V REPUBLIKI SRBIJI

M. Petrović, S. Špičić, A. Potkonjak, B. Lako, M. Kostov, Ž. Cvetnić

Povzetek: Članek opisuje raziskavo okužbe čred ovc z *Brucello ovis* na jugu Srbije. Pri serološkem testiranju so bile potrjene pozitivne reakcije v 67 (29,8 odstotka) in sumljive reakcije v 31 (13,8 odstotka) od skupno 225 testiranih seroloških vzorcih ovc. Ovni so izvirali iz 113 čred z 4751 ovcami iz 28 naselij v pokrajini Pirot v južni Srbiji. Patološke spremembe, ki kažejo na okužbo z *Brucello ovis*, so potrdili z makroskopskim pregledom mod pri 7 (58,3 odstotka) od 12 preučevanih ovnov. Pri 7 živalih (58,7 odstotka) so potrdili enostransko vnetje nadmodka z izrazito hipertrofijo repa, telesa in glave nadmodka. Z uporabo bakterioloških in molekularnih metod so potrdili prisotnost *B. ovis* v vzorcih (moda, nadmodek, bezgavke) pri 11 od 12 ovnov (91,7 odstotka). Klinična izraženost vnetja nadmodka ovnov je nastala po nabavi ovnov in po parjenju. Bolezen je bila izrazitejša v čredah, v katerih je prišlo prvič do pojava bolezni. Na podlagi predstavljenih rezultatov je mogoče sklepati, da okužba z *B. ovis* povzroča velike gospodarske izgube v proizvodnji ovc, ki se kaže v zmanjšani zmožnosti obrejitve (24,85 odstotka), s splavi (5,38 odstotka), z zmanjšanim številom jagnjet (0,74 jagnjeta na ovco) in povečano umrljivostjo jagnjet ob rojstvu (7,78 odstotka).

Ključne besede: *Brucella ovis*; epizootiologija; klinične spremembe; patološke spremembe; Srbija

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Review Article

ANIMAL MODELS OF HUMAN PATHOLOGY - OUR EXPERIENCE

Martina Perše, Anton Cerar*

Institute of Pathology, Medical Experimental Centre, Faculty of Medicine, University of Ljubljana, Slovenia

*Corresponding author, E-mail: anton.cerar@mf.uni-lj.si

Summary: Animal models are often criticized as not reflecting human pathology in all aspects of a disease. However, how closely an animal model resembles the human pathology or how an animal model is produced is not a matter of debate or judgment of a good animal model. It is important to recognize that any animal model has its own advantages and limitations that need to be taken into account. The choice of an animal model should be based on the scope and aims of a particular study and the characteristics and limitations of a particular model.
In our experience, work with animal models is a special branch of laboratory animal science that requires specific knowledge and attention. The aim of the present paper is thus to highlight the knowledge and experience we have obtained through work with chemically induced animal models and to draw attention to various factors that need to be taken into account when working with animal models. The characteristics of some chemically induced animal models that have been adopted and used at the Medical Experimental Centre (animal models of gastric, colorectal and mammary carcinogenesis, colitis and acute nephrotoxicity) are briefly introduced as examples and their similarities to the corresponding human disease discussed. The main factors that may seriously affect the validity of the results when using a particular animal model are also highlighted. Some experience-based recommendations when using animal models are mentioned at the end of the paper.

Key words: animal models; human pathology; carcinogenesis; animal welfare; ethics

Introduction

Animal models are often criticized as not reflecting human pathology in all aspects of a disease. However, how closely an animal model resembles the human pathology or how an animal model is produced is not a matter of debate or judgment of a good animal model. By definition, an animal model is "a living organism in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal"(1). It is important to recognize that any animal model has its own advantages and limitations that need to be taken into account. The choice of an animal model should be based on the scope and aims of a particular study and the characteristics and limitations of a particular model (1,2).

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Animal models

Animal models of human pathology can be broadly divided on the basis of the mode of production into spontaneous, induced and transgenic (1,2).

Spontaneous animal models originate from naturally occurring mutations, identified and maintained in a particular strain. The best known spontaneous model is probably the nude mouse. Nowadays, there are several hundred strains with inherited disorders resembling similar conditions in humans (see http://www.jax.org). Some of them have been employed at the Faculty of Medicine. For example, Goto-Kakizaki (GK) rats, a spontaneous polygenic model for lean diabetes type II (Prof. Dr. Gorazd Drevenšek) and spontaneously hypertensive rats (SHR), a model for studies in hypertension and cardiovascular disorders (Prof. Dr. Ruda Zorc).

Transgenic animal models are developed with the use of genetic engineering technology, which, since the 1980s, has enabled the production of more than 10,000 transgenic animal models all over the world. The first transgenic mouse created in Slovenia, termed *Cyp51* (lanosterol-14α-demethylase) knockout mouse, was the work of Prof. Dr. Simon Horvat (3). According to the rules for strain nomenclature, (4) the correct designations of *Cyp 51* knock-out mice are B6.129SV-Cyp51<tm1Bfro>, B6.129SV-Cyp51<tm1.1Bfro>, B6.129SV-Cyp51<tm1.1Bfro>&Cyp51<tm1.1Bfro>Tg(Alb/cre)21Mgn etc.

Transgenic models are generally used to elucidate the role of a particular gene in an organism or in the pathogenesis of a specific disorder. For example, *Crem* (cAMP response element modulator) and *Cyp 51* knock-out mice are used to study cholesterol metabolism (Prof. Dr. Damjana Rozman) (5-8).

Induced animal models are usually healthy animals in which a certain pathological process, behavior or other condition is induced experimentally, either surgically or by the administration of biologically active substances, usually termed chemical agents (1,2).

In our experience, work with animal models is a special branch of laboratory animal science that requires specific knowledge and attention. The aim of the present paper is thus to highlight the knowledge and experience we have obtained at the Medical Experimental Centre (MEC) through work with animal models and to draw attention to various factors that need to be taken into account when working with animal models. Some experience-based recommendations when using animal models are mentioned at the end of the paper.

Chemically induced animal models of human pathology at MEC

Chemically induced animal models are widely used models because they are relatively easy to induce, are reproducible and are widely available. Treatment can begin before exposure to the agents, during or after the induction period, or through all phases. Such protocols are usually used to assess the promotional or protective effects of the tested factor and, when followed closely, provide data that are fairly reproducible. Chemically-induced pathologies are also nowadays frequently used in genetically engineered mice and rats to study various basic mechanisms of induced diseases and to elucidate the role of a particular deleted or inserted gene in the pathogenesis of a particular disease.

In the following section, we briefly introduce the characteristics of some chemically induced animal models that have been adopted and used at MEC. Their similarities to the corresponding human disease and the main factors that may seriously affect the validity of the results when using a particular animal model are also highlighted in order to emphasize the enormous number of factors that need to be controlled when using animal models.

MNNG animal model of gastric carcinogenesis

MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) is an efficient direct carcinogen for the induction of gastric carcinoma in rodents (9). The optimal conditions for tumor induction are related to the concentration of MNNG in the drinking water and the duration of administration. A standard mode of application of MNNG is 100 μg/mL in drinking water for 6 months.

MNNG induces erosion, regeneration and benign hyperplastic adenomatous changes that precede the development of malignant adenocarcinomas, which infiltrate through the muscle layers and

involve the serosa (10). Carcinomas are most often found in the antrum of the glandular stomach and rarely in the corpus (Fig. 1a) (11). We have also found rare cases of squamous cell papillomas (Fig. 1b) in squamous gastric mucosa and malignant mesenchymal tumors (12).

The histological structure of induced adenocarcinomas in rat is similar to human gastric adenocarcinomas. Metastases of adenocarcinomas to the liver or the lymph nodes have only occasionally been noted in rats. Differentiated tumors (Fig. 1c) occur more frequently in rats, while human tumors show higher proliferation and metastases formation (10).

Since MNNG is degradable by light, the solution must be protected from light. The strain, sex and age of rats affect the incidence of gastric carcinoma. The use of inbred strains in carcinogenesis studies is recommended due to the many advantages they offer (the tumors are more readily transplantable, immunologic and molecular tests in genetically uniform animals are easier to perform etc.) (10,11,13).

DMH animal model of colorectal carcinogenesis

DMH (1,2 dimethylhidrazine) and its metabolite AOM (azoxymethane) are highly specific carcinogens that require metabolic activation for the induction of colorectal tumors. In short-term studies, colon carcinogenesis is usually induced by two s/c applications of DMH (150 mg/kg) or AOM (15 mg/kg) given one week apart and animals are scored for aberrant crypt foci (ACF) 8-12 weeks after the application. Tumor outcome depends on the total amount of carcinogen administered and the latency period. In long-term studies, DMH is administered weekly for 15 weeks in a relatively low concentration (20 mg/kg) and animals are scored for the number of colonic lesions 20 weeks later (Fig. 2a,b,c) (14-17).

Animals develop aberrant crypt foci (Fig. 2b), adenomas and various types of carcinomas, usually well differentiated adenocarcinomas (Fig. 2c) and, less frequently, mucinous, signet-ring cell type or undifferentiated carcinomas. Undifferentiated, mucinous and signet-ring cell carcinomas appear mostly in the proximal part of the colon and are usually surrounded by lymphoid tissue aggregates of intestinal mucosa. Tumors that are capable of metastasis are almost exclusively mucinous and signet ring cells carcinomas of the proximal colon. Adenocarcinomas of the distal colon have not been shown to metastasise (18). We have also observed a few cases of anal squamous cell carcinoma in CBA mice.

DMH/AOM colon carcinogenesis is a multistep process with morphological, histological and molecular features similar to those seen in human sporadic colon carcinogenesis, including similarities in response to some promotive and preventive agents (explained in detail in (18)).

In contrast to humans, metastases to the liver and lung are very uncommon in the DMH/AOM rat model. However, various rat and mouse strains differ in susceptibility to these carcinogens (19,20). The susceptibility for DMH/AOM-induced colorectal carcinogenesis is also sex (21) and age dependent (22,23). Since DMH is an indirect carcinogen, particular attention needs to be paid to the potential interference of a preventive compound with the metabolic pathway of DMH (24).

DSS animal model of colitis

Colitis is induced by DSS (dextran sulphate sodium) dissolved in the drinking water. Acute colitis is usually induced by continuous administration of 2-5% DSS for a short period (4-9 days). Chronic colitis can be induced by continuous treatment of low concentrations of DSS or cyclical administration of DSS; for instance, 4 cycles of DSS treatment for 7 days followed by 10 days of water (25,26).

The clinical manifestation of DSS colitis in the acute phase may include weight loss, diarrhea, occult blood in stools, piloerection, anemia, while clinical manifestations in the chronic phase of colitis do not usually reflect the severity of inflammation or histologic features found in colons. Macroscopic features include a shortened edematous colon. Typical histological changes of acute DSS-colitis are mucin depletion, epithelial degeneration and necrosis leading to the disappearance of epithelial cells (Fig. 3a). The latter is accompanied by neutrophil infiltration of the lamina propria and submucosa, cryptitis, crypt abscesses and phlegmonous inflammation in the mucosa (Fig. 3b) and submucosa. Shallow erosions also usually appear. Chronic changes consist of mononuclear leukocyte infiltration, crypt architectural

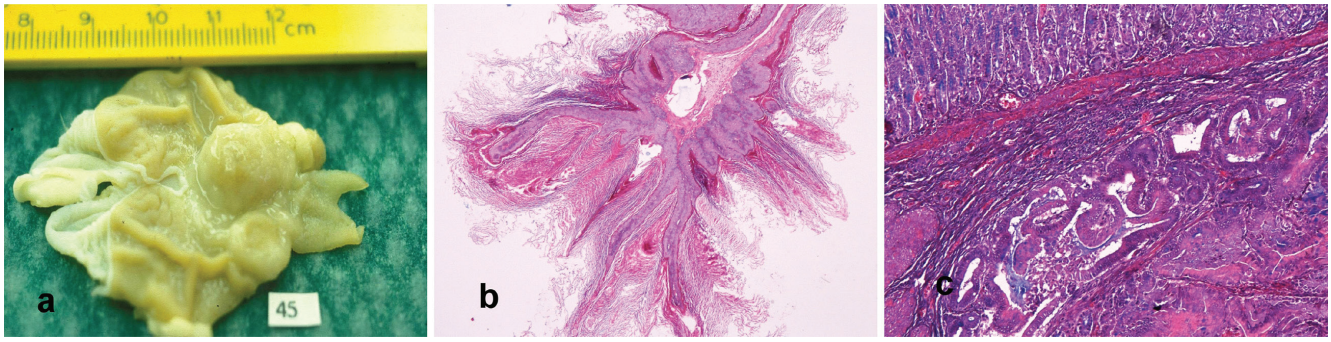

Figure 1: MNNG induced stomach carcinoma in Wistar rat that had been given MNNG solution for 6 months

a) On the right is a short segment of duodenum. A sessile (wide-based) tumor is situated in the antral region. On the opposite edge, there is a white area of squamous mucosa. b) Papillary squamous papilloma of the stomach. There is no sign of epithelial dysplasia. The tumor has a typical arborescent configuration. c) Well-differentiated adenocarcinoma of the stomach. The tumor is infiltrating the submucosa under the normal mucosa (upper edge) (Kreyberg stain).

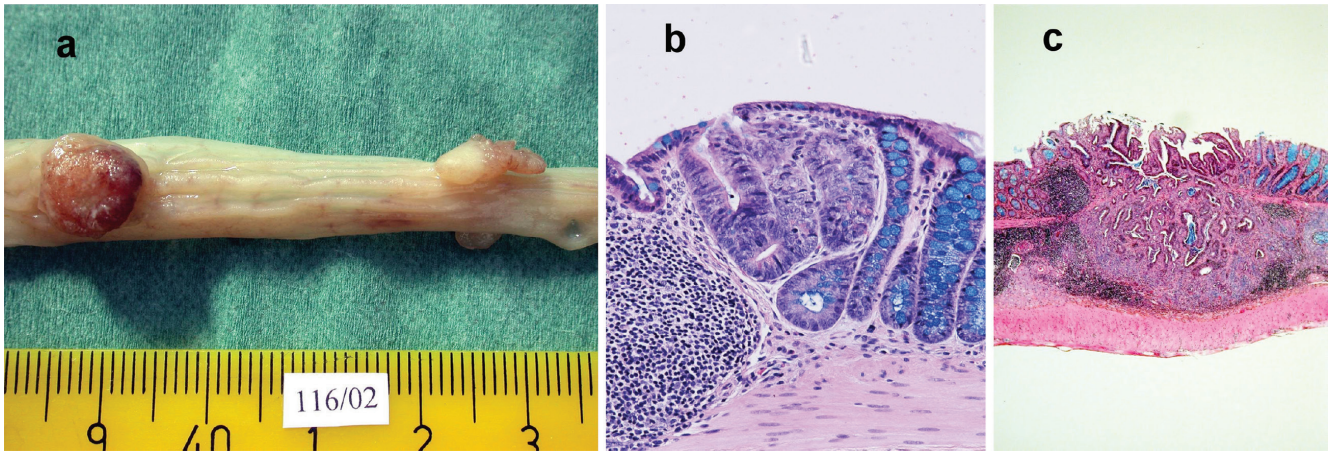

Figure 2: DMH induced colorectal tumors in Wistar rat. DMH has been injected s/c weekly for 15 weeks

a) The right tumor is a papillary adenoma while the left tumor is a sessile and ulcerated carcinoma. b) Dysplastic aberrant crypt focus situated in the vicinity of a lymphoid follicle. There is moderate epithelial dysplasia (Kreyberg stain). c) Well-differentiated colorectal adenocarcinoma infiltrating submucosa. Carcinoma invasion is accompanied by fibroplasia. The carcinoma is situated in the vicinity of a lymphoid follicle (Kreyberg stain).

disarray, a widening of the gaps between crypt bases and muscularis musosae, deep mucosal lymphocytosis, and transmural inflammation with lymphoid follicles (Fig. 3d). Re-epithelisation of rectal and distal colonic erosions by the squamous epithelium and moderate epithelial regeneratory atypia simulating dysplasia at the edge of chronic erosions are also found (Fig. 3c) (27).

The histological changes are the features of inflammatory bowel disease (IBD) in man, some of them of ulcerative colitis (regular rectal localization) and some of Crohn's disease (transmural inflammation with disseminated lymphoid follicles, focal lesions) (28,29).

Many factors can influence the susceptibility, onset, severity and responsiveness to DSS induced colitis, such as DSS (concentration, molecular weight, duration of DSS exposure), genetic (strain, substrain and gender) and microbiological (microbiological status and intestinal flora) factors of the animal, which is discussed in detail elsewhere (27).

MNU animal model of mammary carcinogenesis

MNU (N-methylnitrosourea) is a highly specific carcinogen for the mammary gland. Induction is usually i/p in a dose of 50 mg/kg (30-32). Animals develop mammary tumors (Fig. 4a) classified as adenomas and carcinomas. Carcinomas are invasive and non-invasive, and are termed *in*

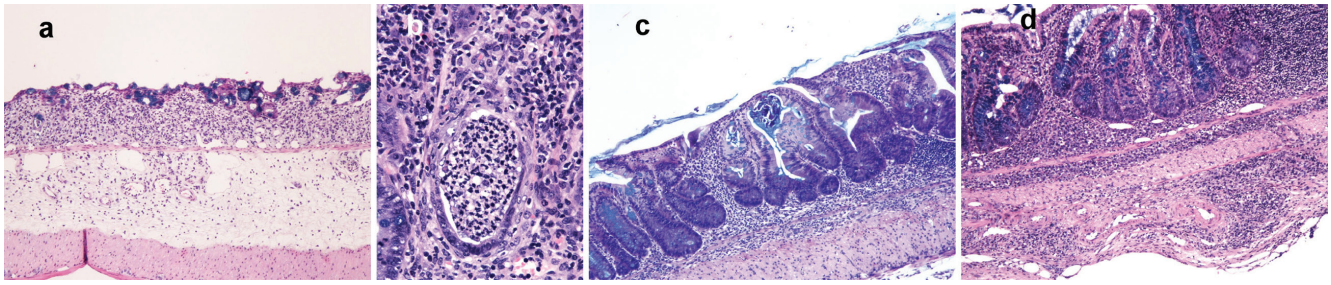

Figure 3: DSS lesions of colorectal mucosa in C57BL/6JOlaHsd mice. The mice have been given a 3% DSS solution for 5 days

a) Early DSS lesion. There is severe depletion of the epithelial cells and mucinous depletion of the colorectal mucosa. Mild mononuclear and neutrophil inflammatory cell infiltration is present in the submucosa (Kreyberg stain). b) Crypt abscess as an early DSS lesion of the colorectal mucosa. There is mucinous depletion in the epithelial cells and dense mixed inflammatory infiltration of the lamina propria (Kreyberg stain). c) In addition to severe inflammatory changes, there are moderate epithelial atypia (nuclear stratification, numerous mitoses, budding) indistinguishable from dysplasia, characteristic of neoplasia (Kreyberg stain). d) Transmural inflammatory infiltration in a chronic phase of DSS colitis. There is mucinous depletion, deep lymphocytosis, architectural crypt anomalies (Kreyberg stain).

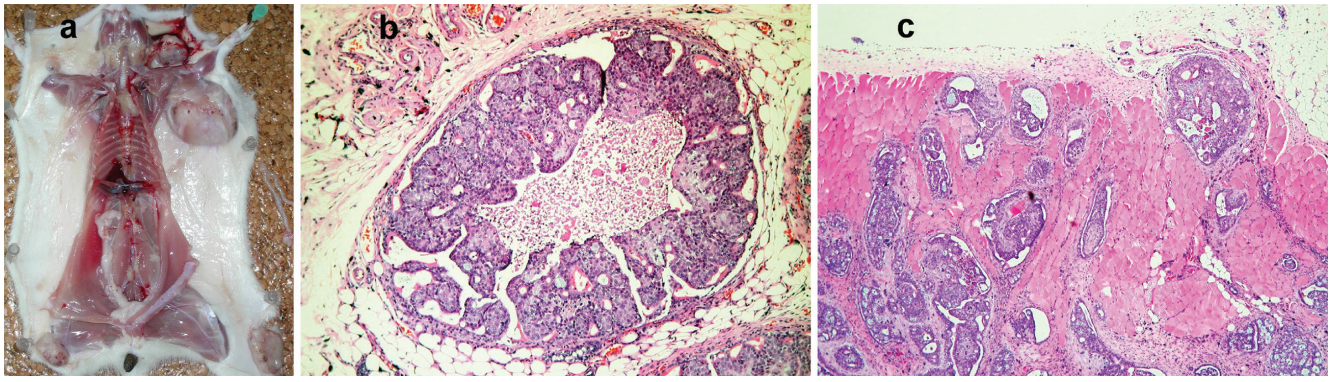

Figure 4: MNU induced mammary tumors in Sprague Dawley female rats. The first palpable tumors were observed 9 weeks after two i/p applications of MNU (50mg/kg)

a) Tumors are mainly localized in axillary and inguinal regions. b) Mammary in situ carcinoma. The tumor is surrounded by intact ductal wall. c) Mammary invasive carcinoma. Moderately differentiated carcinoma infiltrates throughout the skeletal muscle wall.

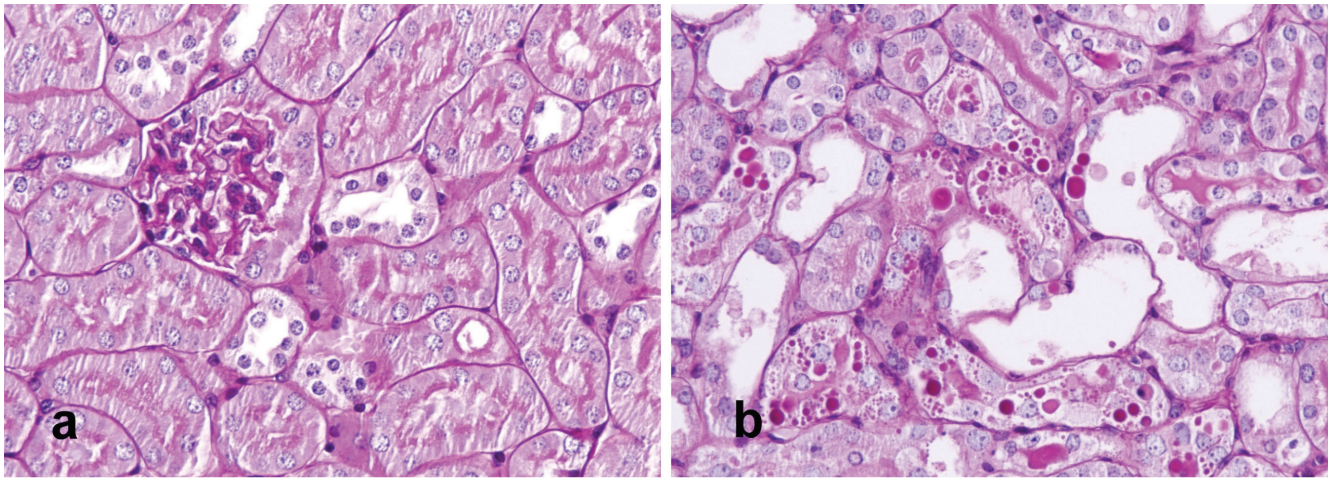

Figure 5: Early cisplatin induced kidney cortical lesions in Balb/c male mouse (5th day after i/p cisplatin application - 18 mg/kg)

a) Normal kidney cortex in Balb/c male mouse. Microvilli of proximal tubuli and basal membranes are intensively stained (PAS stain).b) There is severe necrosis and apoptosis of ductal epithelial cells, with dilatation of ducts and disappearance of cells (PAS stain).

situ ductal carcinomas (Fig. 4b). Histologically, carcinomas are composed of papillary, cystic, cribriform, solid or rarely tubular patterns. A massive stromal response, demonstrated by inflammatory infiltration and fibrosis, has frequently been observed. Invasive carcinomas (Fig. 4c) may ulcerate the skin, contain large areas of necrosis and hemorrhage and invade the local neighboring tissues, such as muscle, salivary gland or lymph nodes. Metastases in a local lymph node and in the liver or spleen are rare (33).

There is substantial evidence suggesting that the MNU animal model mimics human breast cancer in terms of the rat tumor's histopathology, mammary ductal epithelial cells origin, dependence on ovarian hormones for tumor development, and altered expression of TGFB, erbB2 and cyclin D1 (34). The MNU induced breast cancer model has therefore been used extensively to evaluate preventive and therapeutic agents for human breast cancer (35-38) and to study malignant progression (39).

It has been shown that the tumor incidence, latency period and tumor multiplicity are strain (40), dose and age (40-42) dependent, irrespective of the route of administration (42). Mammary carcinogenesis can be influenced by strain, diet, age, dose, time of day and year of carcinogen administration, and immune and endocrine system status, as well as other still unknown factors that may cause changes in tumor induction in rodents under identical conditions (43).

Cisplatin animal model of acute nephrotoxicity

Cisplatin is an effective antitumor drug with nephrotoxic activity, which is a serious problem in human medicine. Depending on the dosage of cisplatin, animals may develop various clinical and pathohistologic features of acute kidney injury. After cisplatin administration, animals often show dehydration and weight loss, a decreased number of white blood cells and hemoglobin content and bone marrow cellularity, significantly increased levels of serum creatinine and blood urea. Treatment with high doses of cisplatin usually elicits severe acute toxicity, followed by death within 3-5 days after administration (44).

Histologically, cisplatin nephropathy in rodents is characterized by early degenerative changes in the proximal tubule and consists of hydropic degeneration, cytoplasmic vacuolization and tubular dilatation. However, frequent histological features also include apoptotic and necrotic cells found in the tubules of the corticomedullary area, predominantly in the pars recta of the proximal tubule (Fig. 5) (45).

The onset, severity and mortality rate of cisplatin induced nephrotoxicity depend on the vehicle, concentration and duration of cisplatin exposure, application route, genetic background of animals (strain and substrain, gender), age and other conditions (such as food and water access).

Limitations and modifying factors of animal models

As demonstrated in the previous section, there are many factors that can influence the course of a disease in a particular chemically induced animal model and thus affect the characteristics of the model and the validity of the results. In chemically induced animal models most common modifying factors are the route of application and the dose of chemical agent used. Rarely mentioned but very important are also the preparation of the agent (pH, light, shelf life) and the application of the prepared solution, which should be fresh, sterile and properly protected. If the compound is sensitive to light and is not properly protected, then the concentration of the solution is not under control and, consequently, the outcome of the study may be significantly affected, i.e., unreproducible. When transgenic models are used, it is important to be aware that they were created in a laboratory and may therefore contain unknown mutations in other parts of the genome. In transgenic or spontaneous models, the genetic background of animals plays an important role in the characteristics of the model. The breeding and naming of a transgenic or spontaneous model is thus a factor that may significantly affect the characteristics of a model (4,46,47). To avoid misinterpretation of the results, one should closely follow the rules for strain nomenclature of the International Committee on Standardized Genetic Nomenclature for Mice and Rats (4,47).

Anyone who uses animal models should be aware that environmental conditions, microbiological factors, such as microbiota and the micobiological state of the animal (i.e., germ free, SPF, conventional) and genetic factors, such

as species, strain, gender and very often also the age of the animals, are important factors that can influence the characteristics of an animal model and affect the variability and the validity of the results.

Advantages of animal models

Although all animal models have embedded limitations, they are valuable tools for understanding various aspects of a disease, including the pathogenesis, pathophysiology of the disease and for discovering novel therapeutic targets and drugs. Research on laboratory animal models also has advantages.

In contrast to clinical or epidemiological studies, research on laboratory animals is carried out in standardized conditions. Environmental conditions (i.e., light/dark period, temperature, humidity, diet, water, bedding, housing conditions, equipment etc.) and microbiological conditions are controlled and known. Laboratory animals are kept in highly controlled environments with limited exposure to pathogens (48). Their microbiological status is regularly monitored. The genetic characteristics of animals are usually known, particularly when using inbred or other isogenic strains. The use of genetically identical animals enables highly reproducible experiments to be performed and the role of genetic factors in the pathogenesis of a particular disorder to be elucidated (49-51).

Experience based recommendations for the responsible use of animal models

Many questions about human disorders have been solved thanks to animal models and many advances have been incorporated into human health care. However, although animal models are indispensable tools in biomedical research, their use in research is not an automatic right. Scientists have a moral, ethical and legal obligation to conduct experiments on animals responsibly. This means that scientists should be well-trained and well-informed before performing an experiment on animals. There are many protocols in the literature for establishing a particular animal model. Searching for literature about a particular model is therefore an essential task. Most animal models were developed several decades ago. This means that a lot of information about both the characteristics and the limitations or other modifying factors of a particular model can be found before its use. Anyone wishing to establish a particular model in a laboratory and without any experience with animal models, is recommended to consult experts with adequate experience of animal models. Those who already have experience with animal models know that establishing an animal model in a laboratory for the first time requires experience, a thorough examination of the literature and preliminary experiment. As indicated above, there are many factors that can influence the characteristics of a particular animal model. The aim of preliminary experiment is to establish a reliable and reproducible animal model with the same characteristics, regardless of the laboratory in which the model is used. Preliminary experiment should be performed on a sufficient number of animals to obtain statistically significant results and ascertain the characteristics of the chosen model and should closely follow the protocol used. Sometimes even small discrepancies, such as age, sex, sub-strain of the animals, infection with potential pathogenic microorganism or merely application at an inappropriate time of the day, may result in failure to establish a reliable model. It is important to bear in mind that studies meet ethic and scientific criteria only when they are performed on reliable and reproducible animal models. The establishment of a reliable and reproducible animal model in the laboratory is thus a prerequisite for further research. After the establishment of a reliable and reproducible animal model, responsible research on animal models, which includes careful preparation, observation and monitoring of the animals during the research, can commence.

During experiments, careful observation of the animals is of great importance. This includes regular monitoring of the animals' weight, diet and water consumption (at least on a weekly basis). For example, monitoring DSS consumption is necessary, especially when animals are exposed to various therapeutic strategies, which may lower the consumption of DSS (increased fluid intake or thirst) (52). Changes in body weight and diet and water consumption are important measurable data that can show alterations in an animal's health.

Careful evaluation and recording of the clinical status of animals on a daily basis, including

weekends or holidays, is necessary. It is advisable to create a list of all expected alterations in the clinical status of an animal model before starting the experiment. A list of the scale or grade of observed and monitored changes in animals' health or behavior can serve as a guide in daily monitoring of animals in research. Monitoring and recording of all expected and unexpected occurrences in animal models by an experienced person with special skills and care are advised on a daily basis.

Avoiding any actions that can cause unnecessary stress to the animals is required. Stress can influence many parameters in the body, including the immune system, carcinogenesis, inflammatory diseases etc. and consequently affect the animals' welfare, as well as the scientific results. The creation of instructions about humane endpoints and actions for avoiding unnecessary suffering of animals is recommended. These instructions on humane endpoints should take into consideration all aspects of the research, i.e., the aim of the study, use of the optimal model, conditions under which the experiment is performed and animal welfare. For example, in an animal model of mammary carcinogenesis, the weight or expanse of individual or total tumor mass per animal is important data. It can affect animal behavior or even the animal's health. The influence on an animal's welfare can be even greater in the case of malignant necrotizing tumors that ulcerate. Such animals usually have an increased number of neutrophils, an indication of an inflammatory response. Thus, not only animal welfare or humane endpoints but also the validity of the scientific results can be confounded because of the unsuitable state of an animal or of factors other than those investigated (33,53).

Finally, at the end of an experiment thorough examination of each animal (autopsy) and accurate identification and interpretation of all observed alterations that have occurred in a particular model is required for the responsible conduct of an animal experiment. All expected and unexpected macroscopic lesions should be recorded and taken for further analysis. Particular attention should to be paid to unexpected lesions, i.e., lesions that are not a characteristic of a particular model. Some clinically healthy animals may possess sporadic inherited malformations or even hidden disorders that cannot be seen before careful autopsy. In such cases, identification of a lesion and its cause, as well as evaluation of its potential influence on the results of the study, is needed to avoid misinterpretation of the obtained results (54). It is advisable that, at autopsy, not only the organ/tissue of interest but various organs and tissues are taken for further analysis. Although animal models do not reflect corresponding human disorders in all aspects of the disease, most of the obtained results are needed adequately to characterize the animal model used and to corroborate the validity of the research performed.

In conclusion, although animal models do not represent the complexity of human disease, they are valuable and indispensable tools, which provide a wide range of options for investigating mechanisms and therapeutic options. However, the validity of the obtained results greatly depends on the quality and specificity of the experimental question asked and the responsible use of animals. From the point of view of animal welfare, humane endpoints, ethical considerations and scientific validity, it is very important to have enough information and knowledge about animal models to design the experiment responsibly and to evaluate the results properly.

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ANIMAL MODELS OF HUMAN PATHOLOGY - OUR EXPERIENCE

M. Perše, A. Cerar

Summary: Animal models are often criticized as not reflecting human pathology in all aspects of a disease. However, how closely an animal model resembles the human pathology or how an animal model is produced is not a matter of debate or judgment of a good animal model. It is important to recognize that any animal model has its own advantages and limitations that need to be taken into account. The choice of an animal model should be based on the scope and aims of a particular study and the characteristics and limitations of a particular model.

In our experience, work with animal models is a special branch of laboratory animal science that requires specific knowledge and attention. The aim of the present paper is thus to highlight the knowledge and experience we have obtained through work with chemically induced animal models and to draw attention to various factors that need to be taken into account when working with animal models. The characteristics of some chemically induced animal models that have been adopted and used at the Medical Experimental Centre (animal models of gastric, colorectal and mammary carcinogenesis, colitis and acute nephrotoxicity) are briefly introduced as examples and their similarities to the corresponding human disease discussed. The main factors that may seriously affect the validity of the results when using a particular animal model are also highlighted. Some experience-based recommendations when using animal models are mentioned at the end of the paper.

Key words: animal models; human pathology; carcinogenesis; animal welfare; ethics

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Review Article

DIVERGENT SELECTION EXPERIMENTS IN POULTRY

Dušan Terčič

Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia

E-mail: dusan.tercic@bf.uni-lj.si

Summary: The importance of quantitative genetics is obvious for poultry breeders as most traits of economic value are quantitative. To better understand quantitative traits, much research has been conducted using short- and long-term divergent selection experiments in chickens and quails over the last decades. In the past, divergent selection experiments were conducted for a variety of reasons such as estimating genetic parameters, testing alternate breeding schemes, measuring selection limits and plateaus, testing theoretical basis relating population sizes and selection intensities, searching for correlated responses to selection on a focal trait, ascertaining the symmetry of responses in a particular trait, etc. Genetically correlated traits are known to respond to indirect selection pressures caused by directional selection on other traits. Thus correlations are of great interest to the breeders. Additionally, information on genetic correlations between traits may provide insight into the biological mechanisms involved in generating differences between selection lines. Divergent selection experiments resulted in a wealth of unique populations of chickens/quails that are very useful for subsequent biochemical or physiological studies, and for studying of genotype by environment (GxE) interactions. GxE interactions are of fundamental importance in poultry breeding because their involvement influences breeding procedure. Geneticists have long been concerned with identifying key genes responsible for variation in quantitative traits. Intercrosses between divergently selected chicken/quail lines have led to the identification of several quantitative trait loci affecting for growth, egg production and quality, feed consumption, disease resistance and other traits.

Key words: poultry; divergent selection; correlated responses; quantitative trait loci

Introduction

With the rise of genetics as a field of science in the 1920's and 1930's, artificial selection experiments became a standard tool in quantitative genetic research. They have been and continue to be a powerful tool to yield information on quantitative traits in terms of their underlying genetic variability, the relationships between traits and their effects on performance (fitness). In addition, selection experiments have provided stocks that have been useful for many other topics, from estimating mutation rates to understanding the molecular, biochemical, and physiological foundations of trait variation (1, 2). Several designs of selection experiments can be distinguished: selection may be practiced in a single direction without a control population, or, control can be maintained to remove environmental variation over generations. In an alternative design, such as divergent selection, the selection is practiced in two lines in opposite directions for the same trait and the difference in performance between the lines is recorded. Common environmental effects are again eliminated (3). This paper briefly reviews poultry divergent selection experiments and some of the results obtained to discuss aspects of

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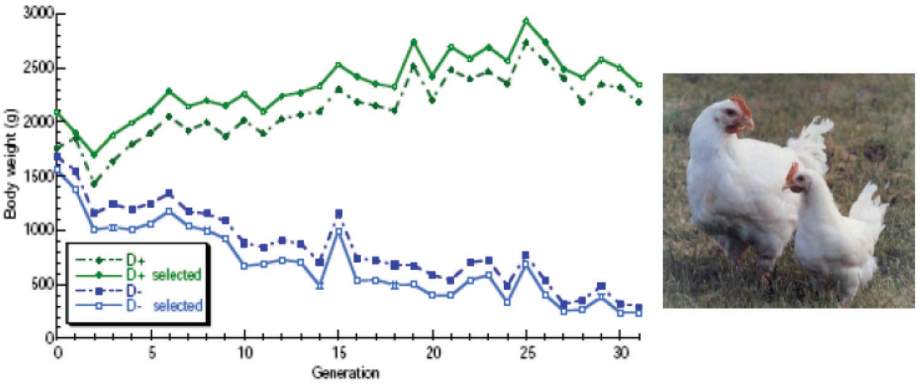

Figure 1: Response to selection for high (D+) and low (D-) 8-wk weight (»selected«=chickens selected to be parents of the next generation). In the photo, both, the low and high weight line birds, are of selection age, where they show almost an eight-fold weight difference (10).

selection experiments that should be considered for the continuation of existing experiments or before future poultry selection experiments are established.

Divergent selection experiments

The motivation to apply divergent selection on quantitative characters was the following (4): a) to demonstrate the principles of quantitative genetics; b) to test for possible departures from predictions; c) to investigate the long-term responses and selection limits; d) to establish differentiated populations for further genetic, physiological or nutritional studies; e) to estimate genetic parameters such as heritabilities; f) to investigate changes in other traits which may be correlated; g) to compare alternative selection schemes. Selection experiments may be viewed in the short- and long-term. Evolutionary biologists, especially quantitative geneticists, are usually interested in the long-term response to selection, which is usefully considered in terms of fixation probabilities of alleles underlying the trait(s) under selection. Over the short term (a few generations), the response to selection will primarily be a function of the alleles segregating in the base population (5). More than 30 generations of selection might be needed to accomplish most of the goals of long-term selection experiments (6). In classical selection experiments, chickens (layers, broilers) and quails can serve as good models for several reasons. They are fairly easy and relatively cheap to maintain in larger numbers, they produce comparatively large numbers of offspring and many interesting characteristics are easy to score (body weight-BW, plumage colour, body composition, metabolic and immunological traits, etc.). This, in addition to a short generation time and high recombination rate, renders chickens and quails suitable as models for genetic studies (7, 8).

Selection traits

The majority of divergent selection experiments in poultry can be grouped according to the selection objectives of either growth and carcass traits, or, various physiological, endocrinological, nutritional, immunological, behavioral, and molecular traits. Several selection experiments are presented in Table 1. Single trait long-term selection experiments with closed populations provide information that cannot be obtained from multitrait selection experiments or from analyses of commercial breeding programs where introgression may occur (9). Because of that, the bulk of studies carried out in poultry have been single-trait selection experiments. Terčič and Holcman (10) (Figure 1) as well as Dunnington and Siegel (11) have summarized the results from an unprecedented and classical long-term selection experiments of 31 and 38 generations, respectively. In both experiments, the common founder populations originated from meat-type crosses. The two lines have been maintained as closed populations selected for either high or low BW at 8 weeks (wk) of age. Various aspects of direct and correlated effects of selection for BW were investigated in depth to understand the mechanisms of selection at organismal, cellular, and molecular levels (10, 11). Different lines (selected, relaxed, dwarf) and line crosses proved to be excellent models for the study of long-term effects of selection for growth (6).

The majority of studies have one selection objective, but few studies have several selection objectives for direct comparison of selection

Table 1: Selection objectives, experimental designs and duration of several selection experiments in chickens

Selection objective	Design[1]	Generations of selection / Reference
8-wk BW	D	16 / (12) ; 31 / (10)
8-wk BW	D+C	38 / (11)
proportion of abdominal fat	D	7 / (13)
residual feed consumption	D	15, 18 / (14)
apparent metabolisable energy corrected for zero nitrogen balance	D	8 / 15
responsiveness to photoperiod	D	4 / (16)
large and small yolk proportions	D	1 / (17)
feather pecking behaviour	D+C	5 / (18)
resistance to Rous sarcoma virus	D	18 / (19)
immune response in chickens	D+C	18 /(20)
ascites incidence	D+C	10 / (21)
serum immunoglobulin levels		3 / (22)
exponential growth rate to 14 or 42 days of age	D	5 / (23)
incidence of tibial dyschondroplasia	D+C	10 / (24)
phytate phosphorus bioavailability	D	3 / (25)

[1]Divergent selection with (D + C) or without (D) a control

strategies. Inclusion of two selection objectives enables measurement of the direct response in trait X, the correlated response in trait Y, and vice versa, in the complementary selection lines, and permits testing of predicted and realised responses to selection (26). For example, divergent selection in generations 10 to 13 of Japanese quail for either 4-week BW or for laying hen yolk precursor (indirectly by increased total plasma phosphorus-HP, decreased total plasma phosphorus-LP) for improvement the shell characteristics, has enabled direct comparison of the selection strategies (27). Selection for several objectives may be more informative than selection for only one objective. To determine correlated responses between growth at different ages and body composition, chickens were divergently selected for exponential growth rate (EGR) to 14 or 42 days of age over five generations (23). During the selection experiment, selection for fast EGR14 or EGR42 increased fat at the age of selection. However, selection for fast EGR42 increased BW and percentage fat at 42 d of age, whereas selection for fast EGR14 increased BW but not fat at 42 d of age (23). Several studies have selected on a correlated trait, rather than on the trait of interest. Divergent selection for serum immunoglobulin M and G levels in chickens changed antibody producing cells as well as other immunocompetent cells that modulate the immune response of the selected lines (22). Divergent selection for total plasma phosphorus in Japanese quail was applied in order to reduce the fearfulness as measured by tonic immobility and consequently to reduce the mortality (28).

Correlated responses

The selection experiment provides an effective resource to estimate genetic covariances with traits in the selection criterion, particularly for traits which are either difficult or expensive to measure (26). For instance, a selection on feed efficiency of laying hens, independent of BW and egg production, requires controlling feed consumption on an individual, or at least on a family basis. This control is costly in time and money. Also, it may be difficult to provide an environment, where genetic improvement is desired. For instance, in selection for resistance to a specific disease because of the cost and risks of exposure (29). In the 1980's, chicken lines were developed that are either high or low in antibody (Ab) production against sheep red blood cells (SRBC). The ultimate goal of developing such lines is to improve some immune parameters, and hence improve resistance to specific diseases (30). Many chickens remain contaminated by

Salmonella for several weeks without showing any symptoms (asymptomatic carriers). Since these healthy carriers are an obstacle to the eradication of Salmonella, selection for increased resistance to Salmonella carrier state could improve both, animal health and food safety. INRA researchers in France have developed two series of divergently selected lines for either low or high carrier state resistance in both, young chicks and adult hens (31). Genetic relationships between carcass composition with meat and eating quality traits are required for the effective inclusion of meat and eating quality traits in breeding programmes and ultimately for the evaluation of alternative selection strategies (26). Many studies showed that inosine-5′-monophosphate (IMP) and intramuscular fat (IMF) contribute to the sensory perception of meat delicacy. From the experiment, where chickens derived from the first two generations of divergent selection for the percentage of IMF and selection for increased IMP content in breast meat were used, it was concluded that both, IMP and IMF contents in chicken meat, have the potential to be increased through genetic selection with little or no positive effect on BW (32). Over the past decade, there has been a highly increased interest in breeding to improve chicken feed efficiency. However, the biological basis of variation in feed efficiency is yet to be fully understood. To examine the biological mechanisms underlying feed efficiency in laying hens, a divergent selection for residual feed intake was started in 1976 (33).

Analysis of genotype-by-environment interaction

The term »genotype-G x environment-E interaction« is most commonly used to describe situations where different genotypes (for instance divergently selected lines) respond differently to different nutritional/climatic environments (34). The importance of GxE interactions to both, the poultry breeder and the poultry producer, appears to be in the choice of performance testing regime in nucleus stocks, to take account of the regime in which commercial animals are reared. Many studies on GxE interactions have been reported for chickens. One cycle of divergent selection for abdominal fat was performed in broilers raised under three different climatic conditions (35). The difference between the high-fat (HF) and low-fat (LF) selection lines had the same magnitude under different environmental conditions that increase or decrease fat deposition. Growth rate (GR) and meat yield depression have been more pronounced in meat-type chicken genotypes (lines) with higher BW and more rapid GR than in those with lower BW and GR (36).

Quantitative trait loci

A powerful way to examine the genetic control of trait variation is to use quantitative trait locus (QTL) analysis, which examines, statistically, the phenotypic effects associated with genetic regions that are delimited by molecular markers (5). Divergently selected lines of chickens for BW, abdominal fatness, immune response, and other traits are frequently used to create marker populations with high amounts of genetic variation for QTL analysis. Calenge et al. (31) used commercial laying hen lines divergently selected for resistance to Salmonella carrier state at two different ages to identify several QTL carrier state resistance variations. Interestingly, previously identified QTL for resistance to carrier state in a chicken F2 experimental population were also validated in divergently selected lines. Simulation studies demonstrated the usefulness of rearing animals more resistant to carrier state in the prevention of Salmonella disease propagation in poultry, in synergy with vaccination (37). The genetics behind complex traits may be studied by intercrossing divergently selected lines with distinct phenotypic differences. One such cross was generated between two lines of chickens divergently selected for BW at eight weeks of age for 25 generations. Intercross facilitated mapping of loci involved in growth and fatness-related traits (38).

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DVOSMERNI SELEKCIJSKI POSKUSI V PERUTNINARSTVU

D. Terčič

Povzetek: Poznavanje zakonitosti kvantitativne genetike je za selekcijske hiše v perutninarstvu zelo pomembno, saj je večina gospodarsko pomembnih lastnosti kvantitativnih. Zaradi boljšega razumevanja omenjenih lastnosti je bilo v zadnjih desetletjih na kokoših in prepelicah izpeljanih veliko kratkotrajnih in dolgotrajnih dvosmernih selekcijskih poskusov. Njihov namen je bil večkraten: ocena genetskih parametrov, testiranje možnih načinov rej živali, merjenje skrajnih selekcijskih meja, testiranje teoretičnih modelov povezanih z velikostmi populacij in intenzivnostmi selekcije, iskanje koreliranih in ugotavljanje asimetričnih učinkov selekcije, itn. Za genetsko korelirane lastnosti je znano, da je mogoče nanje vplivati posredno z neposredno selekcijo na ostale lastnosti. Zato je za selekcioniste poznavanje korelacij velikega pomena. Dodatno lahko poznavanje genetskih korelacij ponudi vpogled v biološke mehanizme, ki povzročajo razlike med selekcijskimi linijami. Dvosmerno selekcionirane linije kokoši/prepelic so dragocen material za naknadno izvajanje biokemijskih in fizioloških raziskav ter za študij interakcij med genotipom in dejavniki okolja. Ker je od interakcij med genotipom in okoljskimi dejavniki odvisna izbira okolja, v katerem bo potekalo testiranje živali, so za selekcioniste v perutninarstvu vitalnega pomena. Genetiki se že vrsto let ukvarjajo z identifikacijo ključnih genov oziroma regij v genomu, ki vplivajo na kvantitativne lastnosti. S križanji med dvosmerno selekcioniranimi linijami kokoši je bilo identificiranih več kvantitativnih lokusov, ki vplivajo na rast, prirejo in kakovost jajc, zauživanje krme, odpornost na bolezni ter na druge lastnosti.

Ključne besede: perutnina; dvosmerna selekcija; korelirane lastnosti; kvantitativni lokusi

Namen ustanovitve in delovanja podjetja MD svetovanje d.o.o. je pomagati podjetjem pri poslovanju z nudenjem produktov in storitev, ki ne spadajo v osnovno dejavnost podjetja. To dosegamo s celovito ponudbo predstavljenih produktov in storitev.

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Vse to nam bo uspelo s trdim delom in kakovostno izvedbo storitev in zaupanih nam nalog, predvsem če bomo sledili naslednjim načelom:

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- vedno delo v dobro stranke,
- strokoven razvoj,
- organizacijsko izpopolnjevanje,
- zagotavljanje visoke stopnje kakovosti storitev z upoštevanjem predlogov naših strank,
- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
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- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
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MD svetovanje, poizvedbe in storitve d.o.o.
Dunajska cesta 421,
1231 Ljubljana – Črnuče

PE Ljubljana-Vič
Cesta dveh cesarjev 403,
1102 Ljubljana

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mdinfo@mdsvetovanje.eu www.mdsvetovanje.eu

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