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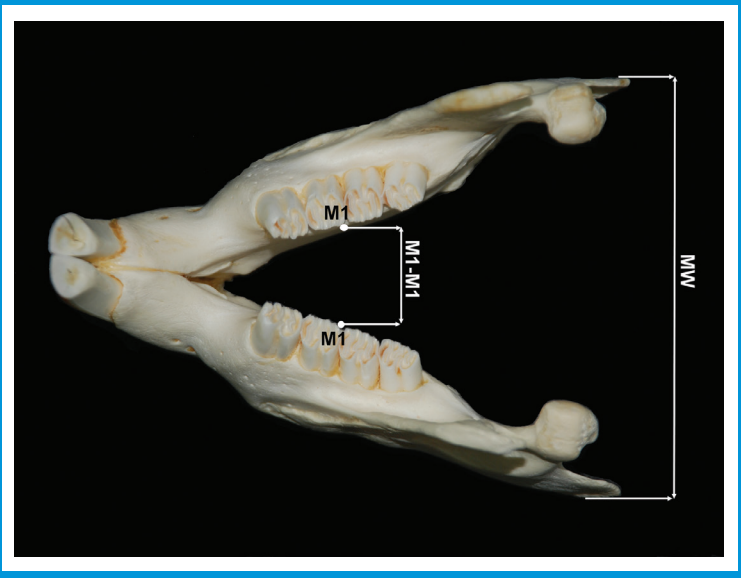

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Original Research Article

CRANIOMETRIC FEATURES OF THE POPULATION OF EUROPEAN BEAVER (*Castor fiber* L.,1758)

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Abstract: The studies of the craniometric features in European beaver (*Castor fiber* L.,1758) were performed using the material of 25 skulls, including 12 females and 13 males from the Pomorze and Kujawy region. For each skull 30 measurements were taken to provide the grounds for the variation in craniometric features and the significance of differences between sexes. In two cases there were observed significant and in three – highly significant differences between males and females of that species. Interestingly, in the shaft of the occipital bone there was found a profound and vast fossa of the skull base.

Key words: craniometry; skull; mandible; beaver

Introduction

Both in terms of functionality and due to the anatomy, the skull is one of the most complicated osseous structures. The sizes of single bones differ for each animal species. The differences result from the order, species and breed the animal represents, which is reflected in the animal classification.

The values of the measurements taken reflect specific features of the skull as well as the effect of the brain size on its shape. In general, the craniometric studies also consider odontometric elements due to their morphological interdependence, despite the fact that tooth must be considered as a part of the digestive system and thus it should not be treated as a component of skeleton. However, upon the teeth development, staring an independent food uptake makes it possible to observe the consequences of the operation of the masticatory apparatus in the skull (1).

The applicable literature offers mostly reports on the skull description; publications on a direct craniometric analysis are much less frequent. Some authors present skull transformations during skull growth.

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The most common material for the study of metrical features involved mostly the skulls of rodents. The pioneer of such studies in Poland was Dehnel (2, 3) who, monitoring the development cycle of skulls *Mikromammalia* observed a phenomenon of a periodical reduction in the cranial capacity in genus *Neomyx* and *Sorex*. Wasilewski (4) formulated similar conclusions in bank vole *(Clethrionomysglareolus)*. Ruprecht (5), on the other hand, performed studies on the effect of the place of origin and gender dimorphism on the skull sizes in muskrat.

The reports by Empel (6) on the skull in wild rabbit demonstrated the effect of the age on some parameters of the cerebral and facial part.

The literature seems to offer some reports on the craniometric measurements of beaver skull. Comparisons of the beaver skulls from the Neolithic times with the contemporary skulls from the Wielkopolska region in Poland were made by Komosa et al. (7). The studies show that the animal age affects respective skull measurements. With age, usually the external sagittal crest and the palate length usually get larger. Rybczyński (8) showed differences in the anatomy of contemporary Canadian beavers and fossil skulls of that species.

The aim of this paper is to provide morphometric characteristics of the European beaver skull in the area of kujawsko-pomorskie province and determining the proportions in its anatomy.

Materials and methods

The observations of the craniometric features in European beaver were performed on 25 skulls, including 12 females and 13 males.

The material was received as a result of reduction cull in the area of the kujawsko-pomorskie province. The animal-use studies were prospectively approved by an animal care and use committee at the UTP – University of Science and Technology. The animals varied in age. Adult individuals were investigated.

The heads were separated from the carcass and exposed to thermal treatment. Having removed soft tissues, the skulls were bleached in 10% perhydrol solution.

On each skull there were marked craniometric points and 30 measurements were taken according to the method proposed by Driesch (9). A detailed description of the methods and how the measurements were taken are given below. For each skull the following measurements were taken: *(A-P)*- total length, *(Cb-P)* - condylobazal length, *(B-P)* – basal length, *(Ect-A)* - the upper neurocranium length, *(A-N)*- cranial length, *(Zl-P)* – viscerocranium length; *(Zy-Zy)*- zygomatic breadth, *(St-P)*- viscerocranial base length, *(Rhi-N)*- the greatest length of nasal bones, *(Po-St)*- the palatine horizontal lamina length, *(ab)*- the greatest diameter of auditory bulla, *(ec)*- breadth dorsal to the external auditory meatus, *(Pml)* –premolar tooth length, *(M1-M3)* – molar teeth row length, *(Ect-Ect)* - frontal breadth, *(Eu-Eu)* – the greatest neurocranium breadth, *(N-B)* - angular neurocranium length, *(A-B)*- skull height, *(FMH)* -foramen magnum height, *(FMW)*- foramen magnum width, *(OCW)*- the greatest occipital condyles breadth, *(JPW)*- the greatest jugular processes base breadth, *(P1m)* the mandible height before P1, *(Id-goc)*- the mandible length, (M1m)- height of mandible before M1, *(Gov-Cr)*- the mandible ramus height, *(M1-M1)*- the intermandibular width for the first molars, *(MW)*- the intermandibular width, *(Et-B)* – cranial cavity depth.

Measurement of cranial capacity was made using a shot no. 6 with a diameter of 2.5 mm. The Duerst method was applied to weigh the amount of shot in the cranial cavity and compare that with the weight of 100 cm^3 of the same type of shot.

The measurement-taking methods are presented in Figs 1-6. The results have been presented in Tables 1 and 2. The following were calculated: the arithmetic mean, standard deviation, coefficient of variation. With the method of the analysis of variance with T-test, there were identified the differences in the values of respective measurements between females and males.

Statistica PL program was used for statistical analysis.

Results

The beaver skull demonstrates a low, elongated, flattened-from-the-top profile. At the same time it represents the type of sciuromorphic skull in rodents. Characteristic features are wide zygomatic bones with large arcs and very massive mandible bones. The orbit is always open. The back-of-neck wall is formed by the occipital bone, ensuring the communication between the skull

and the spinal column. In the centre one will find a vast foramen magnum connecting the cranial cavity with the vertebral canal.

Above the foramen magnum you will find the squamous part of the occipital bone the upper border of which creates a clear crest. The lateral parts in a form of jugular processes go down askew and neighbour with mastoid processes of the temporal bone. At the place where the jugular processes communicate with the squamous part of the occipital bone there occur two endosteal foramens. On the basilar part of the occipital bone there is a quite deep and vast fossa (Fig. 1 - F). The fornix of the fossa is made by a very narrow osseous plate. The sphenoid bone together with basioccipital part form the skull base. The sphenoid bone in beaver has been formed from a few osseous units which maintain some independence. The axial part of the sphenoid bone is formed by the shaft built from the rostral part from the presphenoid bone and basisphenoid bones. On both sides of the presphenoid bones there occur two bones in a form of vertical laminas, forming orbital wings. Caudally to them, on the sides of the basisphenoid bone there occur small temporal wings. Between the pterygoid bone and the vertical lamina of the palatine bone there is a vast opening. Internally to the temporal wings there are found uncinate pterygoid bones limiting the vast opening.

The skull fornix in European beaver is made up by vast parietal bones, and clearly visible, and quite a large interparietal bone. On the skull fornix there is clearly visible sagittal crest which splits rostrally running symmetrically to zygomatic processes of the frontal bones. Symmetrical temporal bones make lateral surfaces of neurocranium; what is characteristic is a small squamous part which in its initial fragment gives off the zygomatic process running lateral. Around the acoustic opening, there occurs a surrounded prominent skeleton of auditory canals. From the lower part of auditory canals there run osseous crests towards the lower surface of tympanic bulla. The mastoid part laterally creates a narrow osseous surface and from the side of the back-of-neck surface it supplements the osseous space between the squamous part and jugular processes of the occipital bone.

The frontal bones in beaver supplement the fornix of the skull and their surface is relatively inconsiderable. The frontal lamina neighbours with the nasal, incisive and zygomatic bones. The orbital plate fills the upper part of the orbit. On the frontal bone there occur poorly marked zygomatic processes. The ethmoid bone separates the cranial cavity from the nasal cavity. It is built from small cribriform plates separated with a relatively wide crista galli. Ethmoturbinates fill the caudal part of the nasal cavity and form the ethmoid labyrinth there.

The fornix of the nasal cavity is made up by nasal bones, in the rostral part – quite wide, they get narrowed caudally and, wedge-like, get squeezed between frontal bones. Caudal borders of the nasal bones reach towards the back, far beyond the caudal end of the incisive bones. The incisive bones in beaver are relatively large, their dorsal parts gets squeezed between the nasal bones and frontal bones. The lateral surfaces are vast, with a clearly visible, running across, swelling. In those bones there are imbedded extremely large and well-developed incisor teeth. The maxillae are a continuation of the lateral surface of viscerocranium; there in deep alveoli, there are imbedded premolar tooth and molar teeth. The deep and vast infraorbital fossa of jawbones makes up the attachment for a part of the rumen. The lower part of fossa which is defined by the osseous crest makes the infraorbital foramen visible. The zygomatic bone makes up the main component of the zygomatic arch, communicating with the maxilla which, in a form of a think osseous lamella makes the anterior border of the zygomatic arch. The upper fragment of the zygomatic bone forms the anterioventral border of the orbit. A small frontal process does not limit the orbit clearly. In its lower part the zygomatic bone gives off temporal process, which anastomoses with the zygomatic process of temporal process. The lacrimal bone, small in size on the skull fornix, in a form of a small osseous lamella goes then onto the anterior surface of the orbit. The bone includes a vast lacrimal foramen. The palatine bones form together with palatine lamina of the maxilla of hard palate. On the border of the connection of those bones, visible double palatine foramens which are usually located asymmetrical. At the bottom of the nasal cavity there runs the nasal vomer which ends up at the 2/3rd of the length of the nasal cavity.

Mandible in beaver relatively large, supplementing the ventral part of the skull makes it assume the shape of a very heavily obtuse wedge.

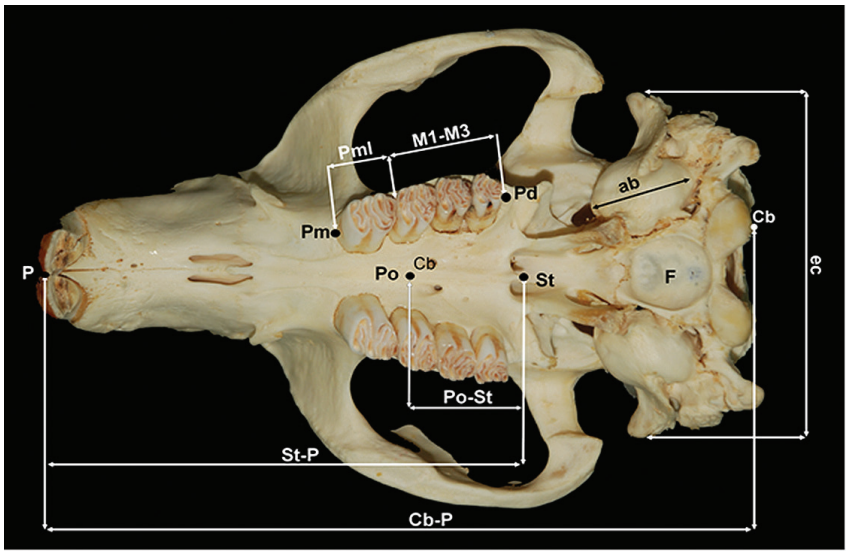

Figure 1: The beaver skull – ventral view

(Cb-P)- condylobazal length, (St-P)- viscerocranial base length, (Po-St)- palate length, (ab)-the greatest diameter of auditory bulla, (ec)- breadth dorsal to the external auditory meatus, (M1-M3) – molar teeth row length, (Pml) –premolar tooth length, F - shaft of the occipital bone

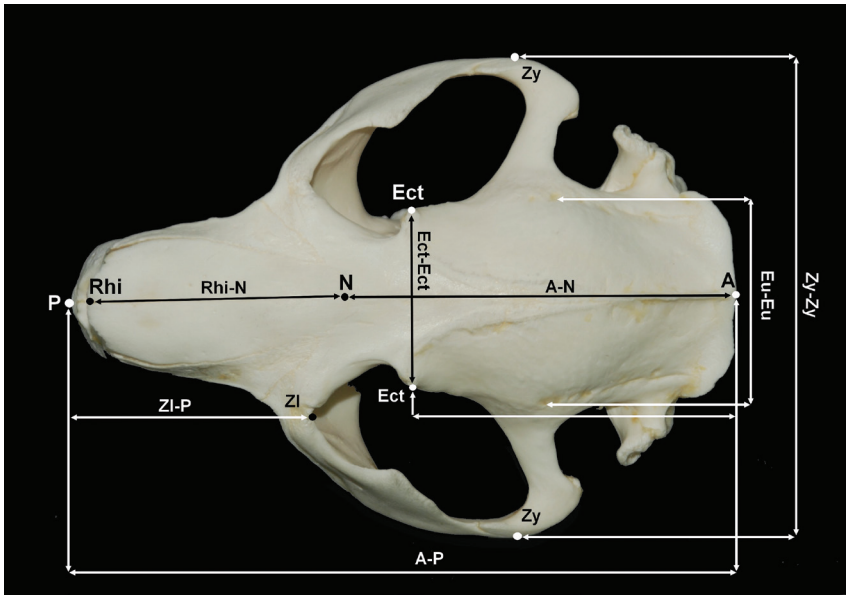

Figure 2: The beaver skull – dorsal view

(A-P)- total length, (A-N) - cranial length, (Zy-Zy)- zygomatic breadth, (Rhi-N)- the greatest length of nasal bones, (Ect-Ect) - frontal breadth, (eu-eu) – the greatest neurocranium breadth, (Zl-P) – viscerocranium length

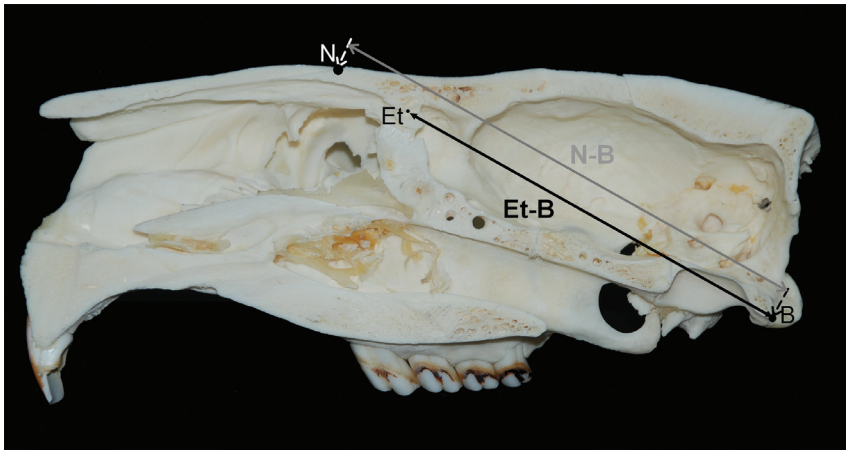

Figure 3: Cranial cross-section in beaver

(N-B)- angular neurocranium length, (Et-B) – cranial cavity depth

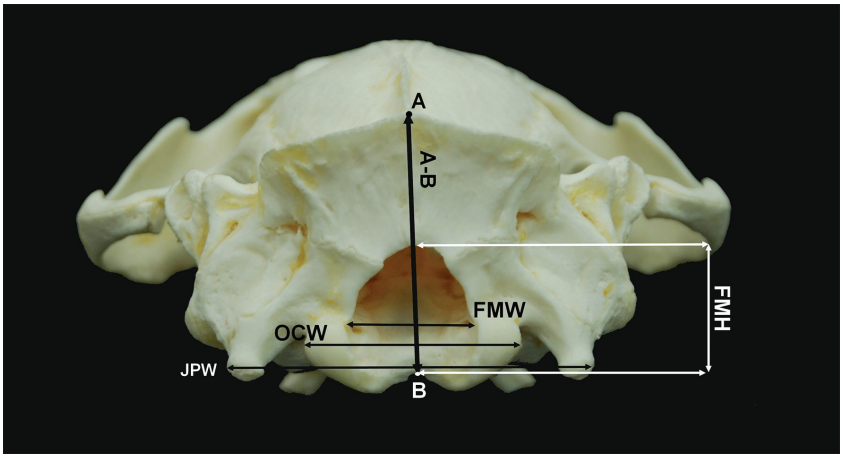

Figure 4: The beaver skull – planum nuchale view

(A-B)- skull height, (FMH) -foramen magnum height, (FMW)- foramen magnum width, (OCW)- the greatest occipital condyles breadth, (JPW)- the greatest jugular processes base breadth

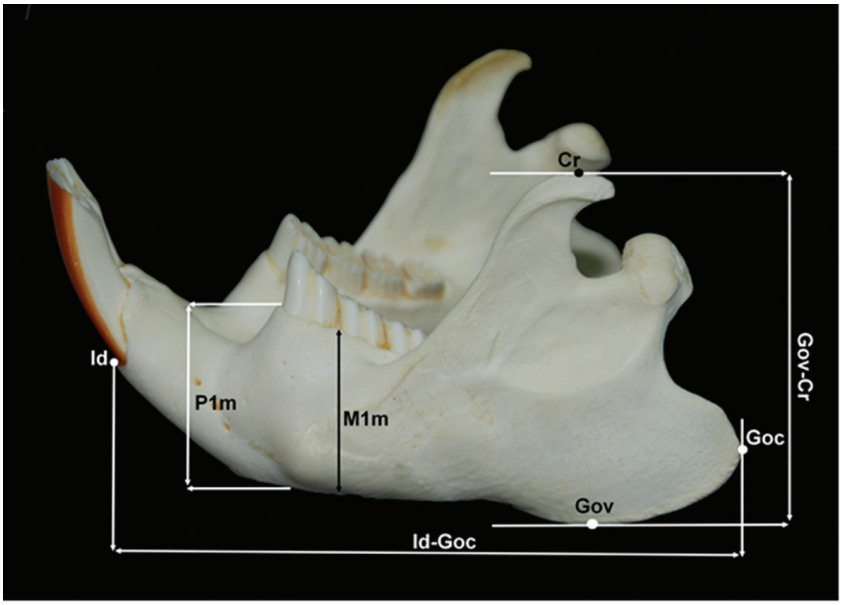

Figure 5: The mandible of beaver - side view

(P1m) - the mandible height before P1, (Id-goc)- the mandible length, (M1m)- height of mandible before M1, (Gov-Cr)- the mandible ramus height

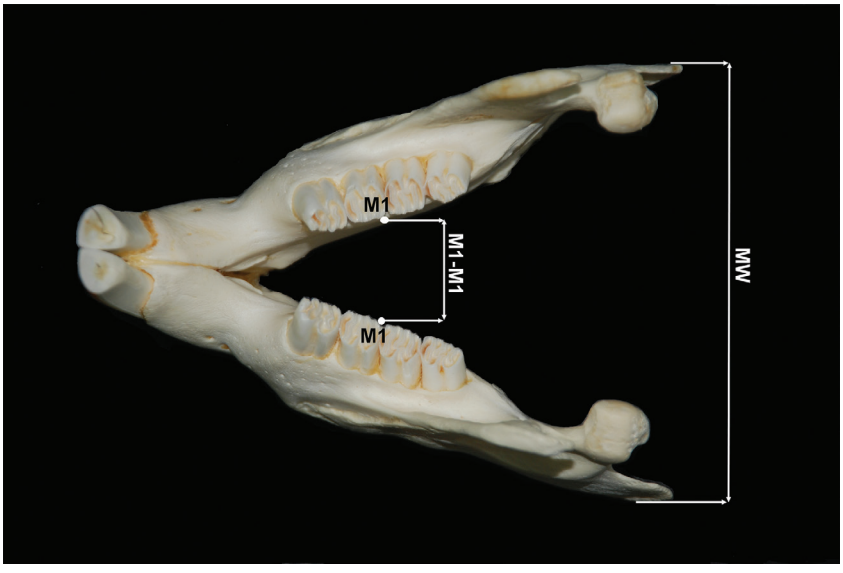

Figure 6: The mandible of beaver – dorsal view

(M1-M1) - the intermandibular width for the first molars, (MW)- the intermandibular width

The inferior part of the border of the mandible together with the border of the fornix determine the angle of 30°. The mandible is a double bone; both mandibles, the right one and the left one, communicate with each other in intermandible synchondrosis. The connection in beaver is quite permanent. The mandible body relatively high in the anterior part includes a vast dental alveolus for lower incisors. In the caudal part it ends up with a vast angular process. The branch of the mandible includes two processes: the coronoid process and condyloid process. Between the condyloid process and the angular process there occurs quite a deep cut.

In the maxilla and mandible there occur a total of 20 teeth: 2 incisors each, 2 premolar teeth each, and 6 molar teeth each. Between the incisors and premolar teeth there is a vast free space – diastema. Incisors are strongly arc-bent, from the front covered with brown-orange enamel, chisel-like-finished. The premolar teeth and molar teeth are similar to each other, their masticatory surface forms transverse enamel folds, it allows for crushing even the finest food. All the teeth are rootless, continuously attrited with wear and tear they stick out throughout their life from alveoli. According to the craniometric examinations, the largest total length (*A-P*) was 14.77 cm in males. This parameter in the female group was characterized by a higher coefficient of variation of 4.21%, the difference between the two groups was 0.95 cm and was not statistically significant.

The highest condylobazal length (*Cb-P*) in males was 14.04 cm, while in females it was 13.67 cm. The coefficient of variation was 2.53% and 7.53%, respectively. The difference between these groups was statistically insignificant.

The upper neurocranium length (*Ect-A*) was 8.42 cm in males and 7.13 cm in females. The difference between the groups was statistically significant. We also observe a fairly high coefficient of variation in females, which is 7.99% compared to males, where it was only 1.49%.

In the case of cranial length (*A-N*) the measurement was 8.92 cm in males and 9.12 cm in females. Coefficient of variation was 12.22% in females and only 3.08% in males.

Very high statistically significant differences between males and females were recorded for the angular neurocranium length (*N-B*) and the difference was 0.3 cm.

Yet another parameter measured which demonstrates a high significance of the difference between the groups was the length of the viscerocranium (*Zl-P*). In females one can see a high value of the coefficient of variation (10.06%) which in the male group accounts for 2.89% only. This measurement is also characterized by the high significance of the difference between the studied groups.

The greatest length of nasal bones (*Rhi-N*) as one of the few parameters was characterized by the high significance of the differences, while the minimum and maximum values were at a similar level.

The length of the palate (*Po-St*) was characterized by a high coefficient of variation of 10.49% in the female group, which was significantly lower in comparison with males.

In the case of the greatest diameter of auditory bulla (*ab*), also in females the coefficient of variation was twice as high, but the difference between means was not statistically significant.

One of the measured parameters is the breadth dorsal to the external auditory meatus *(ec)*, which is distinguished only by a very high coefficient of variation in females - 9.77%.

One shall consider the length of the row of molar teeth noteworthy; with its outstanding high coefficient of variation accounting for 11.60% in the male group. In females, on the other hand, it was 5.15%. The measurement showed significant differences across the means. The frontal width of the skull is the parameter that is the only one characterized by a similar and high coefficient of variation in both the group of females and males. Accordingly, it is 10.52% and 11.38%.

The frontal breadth *(Ect-Ect)* is the only parameter that has similar and a high coefficient of variation in both males and females, 10.52% and 11.38% respectively.

The largest internal eye height reached the highest variability coefficient among all tested males and was 12.10%.

The next measured parameters were: skull height, cranial length and occipital cap height, values not statistically different between groups.

The measurement of the height of the foramen magnum showed the highest value of the coefficient of variation in females and it accounted for 19.54%. It was the highest value of all the 74 measurements in total. Besides, the difference between the sexes was highly significant. The other measurements of the occipital bone (foramen

Table 1: Statistical characteristics of the caniometric measurements in European beaver

No	Measurement	Sex	n	x	RANGE	S_x	V_x	Difference
1	A-P	♂	21	14,00	11,58 – 14,77	0,40	2,86	0,95
		♀	19	13,05	12,50 - 14,70	0,55	4,21	
2	Cb-P	♂	21	13,46	9,99 – 14,04	0,39	2,13	0,96
		♀	19	12,49	11,59 – 13,67	0,94	7,53	
3	B-P	♂	21	13,98	13,84-14,14	0,91	0,65	0,87**
		♀	19	13,11	12,82-13,39	1,98	1,51	
4	Ect-A	♂	21	6,85	6,37 – 8,40	0,10	1,46	0,05*
		♀	19	6,80	5,99 - 7,29	0,54	7,94	
5	A-N	♂	21	8,69	5,19 – 8,89	0,27	3,10	0,83
		♀	19	7,85	7,09 - 9,16	0,96	12,20	
6	Zl-P	♂	21	6,04	5,36 – 7,90	0,17	2,81	-0,10**
		♀	19	6,15	5,72 - 6,52	0,62	10,08	
7	Zy-Zy	♂	21	10,39	8,06 – 11,91	0,54	5,20	0,77
		♀	19	9,62	8,79 - 11,98	0,53	5,51	
8	St-P	♂	21	9,06	7,96 – 9,21	0,24	2,65	0,57
		♀	19	8,49	7,73 - 9,67	0,54	6,36	
9	Rhi-N	♂	21	2,95	2,59 – 3,49	0,25	8,47	-0,07**
		♀	19	3,02	2,21 - 3,21	0,22	7,28	
10	Po-St	♂	21	1,23	0,99 – 1,42	0,05	4,06	0,06
		♀	19	1,17	1,10 - 1,31	0,12	10,26	
11	ab	♂	21	1,73	1,38 – 1,90	0,06	3,47	0,13
		♀	19	1,59	1,25 – 1,91	0,11	6,92	
12	ec	♂	21	1,75	1,32 – 1,91	0,09	5,14	0,11
		♀	19	1,63	1,54 – 2,01	0,16	9,81	
13	Pml	♂	21	1,03	0,92 – 1,07	0,12	11,65	0,03*
		♀	19	1,00	0,77 – 1,19	0,05	5,00	
14	M1-M3	♂	21	2,21	1,79 – 2,34	0,09	4,07	0,13
		♀	19	2,08	2,02 – 2,48	0,13	6,25	
15	Ect-Ect	♂	21	3,20	2,31 – 3,41	0,36	11,25	0,30
		♀	19	2,89	2,69 – 3,79	0,30	10,38	
16	Eu-Eu	♂	21	3,71	3,02 – 3,94	0,11	2,96	0,16
		♀	19	3,54	2,99 – 3,97	0,20	5,65	
17	N-B	♂	21	8,99	8,91-9,13	0,59	6,56	3,02**
		♀	19	8,69	8,60-8,78	0,72	8,28	
18	A-B	♂	21	3,89	2,99 – 3,98	0,05	1,28	0,22
		♀	19	3,68	2,91 – 4,02	0,26	7,06	
19	FMH	♂	21	1,49	1,17 – 2,31	0,15	10,07	-0,08**
		♀	19	1,57	1,25 – 1,77	0,31	19,74	
20	FMW	♂	21	1,73	1,36 – 1,94	0,04	2,31	0,11
		♀	19	1,62	1,57 – 1,82	0,07	4,32	
21	OCW	♂	21	3,13	2,79 – 3,32	0,05	1,60	0,14
		♀	19	2,99	2,89 – 3,19	0,08	2,67	
22	JPW	♂	21	5,53	4,69 – 5,89	0,17	3,07	0,30
		♀	19	5,24	5,18 – 5,63	0,22	4,20	
23	Et-B	♂	21	7,77	73,39-79,96	1,63	2,90	0,98*
		♀	19	7,67	75,47-73,39	0,84	1,09	
24	Capacity [ml]	♂	21	41,54	39-43	1,39	3,35	0,04*
		♀	19	41,50	40-43	1,00	2,41	0,45

Table 2: Statistical characteristics of craniometric measurements of mandible in European beaver

No	Measurement	Sex	n	x	RANGE	S_x	V_x	Difference
25	P1m	♂	21	3,41	3,01 – 3,49	0,16	4,69	0,17
		♀	19	3,24	3,19 – 3,75	0,16	4,94	
26	Id-goc	♂	21	10,76	8,88 – 10,81	0,18	1,67	0,59
		♀	19	10,17	10,49 – 11,19	0,47	4,62	
27	M1m	♂	21	3,02	2,19 – 3,12	0,08	2,65	0,23
		♀	19	2,79	2,88 - 3,14	0,26	9,32	
28	Gov-Cr	♂	21	6,18	5,65 – 6,29	0,09	1,46	0,15
		♀	19	6,03	5,89 – 6,32	0,16	2,65	
29	M1-M1	♂	21	1,64	1,33 – 1,99	0,07	4,27	0,02*
		♀	19	1,62	1,51 – 1,77	0,18	11,11	
30	MW	♂	21	7,86	6,62 – 8,30	0,28	3,56	0,45
		♀	19	7,41	6,44 – 8,26	0,64	8,64	

magnum width and occipital condyles as well as the greatest width of base of jugular processes) were non-significant.

Interestingly, the highest width of jugular processes demonstrated the lowest value of the coefficient of variation among the two groups and it accounted for 2.21% in females and 0.92% in males.

The largest mandibular width (M1-M1) reached a high value of variation coefficient in females (11.39%) and the difference was statistically significant.

For other mandibular measurements, i.e. *(P1m)* the mandible height before P1, *(M1m)*- height of mandible before M1, *(MW)* - the intermandibular width, *(Gov-Cr)*- the mandible ramus height, *(Id-goc)*- the mandible length no statistically significant differences between the sexes were noticed.

The study of cranial cavity capacity and its length (*Et-B*) showed statistically significant differences between sexes.

In conclusion, among the 30 measurements received, only in five cases differences were statistically highly significant and in five cases it was statistically significant.

Discussion

The studies of the metric variation in the European beaver skull have facilitated determining characteristic features of the species. The skull of the beaver, the biggest rodents living in Poland, is an extremely massive structure, slightly flattened from the top. Among the mammal order it represents the type of sciuromorphic structure. The characteristic feature of the beaver skull, both extinct and contemporary forms, are wide zygomatic bones with large arches and very massive mandible bones with wide shafts (8).

A specific feature if the beaver skull is, unusual in other mammal species, a vast and deep fossa of the skull base covered with mucous membrane. In literature, you cannot find any information about the role of this structure in the life of beavers. The applicable literature seems not to offer information on the role it plays in the life of that species.

The beaver skull shows a slight effect of sexual dimorphism on the variation in the craniometric features. Of all the parameters analysed only 3 measurements differed highly significantly and they concerned the following parameters: the length of the viscerocranium, the greatest length of nasal bones, cranial base length, the height of foramen magnum and angular height of the neurocranium. A similarly inconsiderable variation resulting from sexual dimorphism was noted in muskrat by Ruprecht (5). Assuming the standard deviation as the measure of population variations, one can assume that the Kujawy-Pomorze beaver population shows a slight variation in craniometric features and the only exception is the total length of the skull and the condylobazal length where the standard deviation reached higher values.

The studies of the craniometric features facilitated providing characteristics of the Kujawy –Pomorze European beaver population. The measurements show that the average total length of the beaver skull in females was 13.05 cm and in males – 14.00 cm. Yet another parameter usually described when providing skull characteristics is the maximum skull width, measured in zygomatic arches the mean of which was also higher in the male group and it accounted for 10.39 cm, while in females – for 9.62 cm. Considering at least one parameter connected with the mandible, it is noteworthy mentioning the maximum width of diaphyseal processes since the difference across the means in both groups was one of the few significant ones.

Considering the coefficient of variation, it assumed the highest value among the females and it accounted for 19.74% for (FMH) -foramen magnum height. The lowest value of the parameter was 0.65%, for the basal length (B-P) which refers to males. Both values are a threshold at the same time for the group of females and males studied.

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KRANIOMETRIČNE ZNAČILNOSTI V POPULACIJI EVROPSKEGA BOBRA (*Castor fiber* L., 1758)

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Povzetek: Študija kraniometričnih značilnosti evropskega bobra (*Castor fiber* L., 1758) je bila izvedena na 25 lobanjah 12 samic in 13 samcev evropskega bobra s področij Pomorze in Kujawy na Poljskem. Na vsaki lobanji je bilo opravljenih 30 meritev, ki so bile podlaga za ugotavljanje raznolikosti v kraniometričnih značilnostih in njihovem pomenu pri razlikah med spoloma. V dveh primerih so opazili značilne in v treh primerih zelo značilne razlike med samci in samicami. Zanimivo je, da je v zatilnici najdena globoka in obsežna jama na bazi lobanje.

Ključne besede: kraniometrija; lobanja; mandibula; bober

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Original Research Article

MYCOBACTERIA IN AQUARIUM FISH: ARE FISH HANDLERS AWARE OF THEIR ZOONOTIC POTENTIAL?

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Abstract: Mycobacteria potentially pathogenic for humans are commonly present in aquarium fish sold in Slovenian pet shops. *Mycobacterium marinum* is the most important causative agent of skin mycobacteriosis in humans. The purpose of the present study was to evaluate, by means of a questionnaire, aquarium hobbyists' and pet shop salespersons' awareness of the health risks related to aquarium fish handling.

A total of 198 participants took part in the study, 76.3% of these were aquarium hobbyists, and 23.7% were pet shop salespersons with up to 15 years of fish handling experience. About one third (35.8%) of all participants recognized that fish may be a source of infection for humans and that fish may contract tuberculosis. Fewer (24.4%) were aware of the fact that fish tuberculosis is a zoonotic disease. The vast majority of respondents were unfamiliar with the clinical manifestations of either fish tuberculosis or cutaneous mycobacteriosis in humans. Despite the generally acknowledged belief that aquarium water may pose a risk to human health, the respondents' aquarium handling practices were surprising as the majority never used waterproof gloves.

Several differences were revealed between the two groups of respondents. Pet shop salespersons were better educated on fish handling, and more were aware of the health risks linked to aquarium fish than aquarium hobbyists. The latter often perceived pet shop salespersons as relevant sources of information. However, overall awareness of the zoonotic potential of fish mycobacteria proved to be fairly low. Therefore, more effort should be made to increase the awareness of the role of mycobacteria in infections associated with exposure to aquarium fish.

Key words: fish tank granuloma; fish tuberculosis; mycobacteria; questionnaire; zoonosis

Introduction

Fish are the third most popular pet globally, owned by 12% of more than 27,000 respondents in an international pet ownership survey (1). With increasing fish popularity, owners should be sensitized to public health issues related to handling aquarium fish. Fish tank water hosts highly diverse microbial communities, including pathogens which pose a potential risk to the pet industry, fish in trade, and humans (2). Several pathogens associated with aquarium fish are zoonotic and may be transmitted to humans through cuts or abrasions of the skin and through direct contact via bites, stings, and spine or pincer injuries. The epidemiology of aquarium fish related infections is complex as fish may often act as asymptomatic carriers transmitting the pathogens to humans, commensal microbes may become zoonotic pathogens, and clinical manifestation of the disease in fish may have little relevance to clinical signs in affected humans (3). Groups at risk include individuals frequently exposed to fish, their products, or their environment and persons with impaired immune systems.

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Bacteria are the primary causative agents of zoonotic infections related to aquarium fish, and mycobacteria are probably the best known zoonotic fish-borne pathogens. Three mycobacterial species most commonly associated with fish disease are *Mycobacterium* (*M.*) *marinum*, *M. fortuitum*, and *M. chelonae*. However, a variety of other nontuberculous mycobacteria (also called mycobacteria other than tuberculosis, atypical, opportunistic, or environmental mycobacteria) reported in humans have also been associated with fish mycobacteriosis (4). The disease is usually systemic, characterised by granulomas in multiple organs or tissues. The affected fish show no specific clinical signs until the appearance of overt symptoms such as scale loss, pigment changes, emaciation, ulceration, and behavioural changes. In addition to fish, mycobacteria can infect a number of other aquatic organisms and can replicate in various protozoan hosts (5).

In humans, fish-borne or water-borne mycobacterial infection leads to superficial granulomatous inflammation, most commonly seen on the extremities; however, deeper tissues may also be involved. The localized form of the disease presents with nodular or ulcerated lesions while the sporotrichoid form is associated with lymphatic spread (6). Although rare, disseminated infections have mostly been described in immunocompromised patients (7) but have also been reported in immunocompetent persons (8). Diagnosis of skin mycobacteriosis (also called fish tank/swimming pool granuloma or fisherman's/fish-fancier's finger) is difficult due to its nonspecific and insidious presentation; therefore, a delay in diagnosis is commonly observed (9, 10). Once diagnosed, the infection can be effectively treated with antibiotics although surgical intervention may sometimes be required (6).

Surveys on the presence of mycobacteria in aquarium fish from pet shops in Slovenia indicated a high level of contamination with mycobacteria, potentially pathogenic for humans. *M. marinum* was detected in 20.7% and 10.6% of mycobacteria-positive fish, respectively (11, 12). Fish tank granuloma is rarely detected in humans in Slovenia; only 12 cases have been reported from 2000 through 2018 in a population of approximately two million (M. Žolnir-Dovč, unpub. data). Nevertheless, the disease is very likely underdiagnosed.

The purpose of the present study was to evaluate the awareness of aquarium hobbyists and pet shop salespersons of the health risks associated with aquarium fish handling.

Material and methods

To evaluate the awareness of aquarium hobbyists and pet shop salespersons of the health risks associated with aquarium fish, participants were invited to complete an anonymous questionnaire developed by the authors. The questionnaire was comprised of 20 questions on fish-related training, fish, and aquarium water-handling practices, knowledge of fish tuberculosis and fish tank granuloma, as well as possible personal experiences with the infections. The questionnaire was first tested on a pilot group of 10 people with fish handling and/or questionnaire development experience to determine question clarity, identify additional response options, and to assess the length of time necessary to provide answers. The questionnaire was revised accordingly. The data was evaluated and analysed using the Statistic Program for the Social Sciences (SPSS, Version 24.0).

Results

A total of 198 respondents participated in the study (28.3% men, 71.1% women, mean age 26 years, age range 12 to 69 years). Of these, 76.3% were aquarium hobbyists (AH) and 23.7% were pet shop salespersons (PSS) with up to 15 years of fish handling experience. The two groups of respondents differed in several factors.

The majority of the respondents (84.8%) had no special fish-related education, i.e., attendance of specialised courses or lectures. However, PSS were better trained (23.4%) than AH (2.7%). When asked about self-education, only 35.5% of the respondents reported reading literature with website publications being the most common source of information, followed by books and advice from PSS. There was a statistically significant difference ($p<0.001$) between AH and PSS; 25.3% of the former and 68.1% of the latter reported reading literature for self-educational purposes.

Regarding knowledge of the zoonotic nature of fish diseases, only 35.8% of the respondents were positive that some fish diseases could be transmitted to humans, while 53.4% of the respondents were unsure of this possibility.

Table 1: Summary of answers related to the respondents' knowledge of fish tuberculosis and fish tank granuloma

Answer	Yes		No		I do not know		Fisher's exact test
Statement/query	PSS† (%)	AH‡ (%)	PSS (%)	AH (%)	PSS (%)	AH (%)	p value
Some aquarium fish diseases can be transmitted to humans	69.6	52.6	5.0	16.0	24.5	4.1	<0.001
Aquarium fish may contract tuberculosis	73.9	24.5	6.5	4.8	19.6	70.7	<0.001
Fish tuberculosis-causing bacteria may be dangerous to human health	56.5	14.3	6.5	8.8	37.0	76.9	<0.001
Aquarium water may be a source of harmful microbes causing human infection	84.8	71.4	2.2	0.7	13.0	27.9	0.059
Are you familiar with clinical signs of fish tuberculosis?	32.6	4.1	67.4	95.9	/	/	<0.001
Are you familiar with clinical signs of fish tank granuloma?	2.2	3.4	97.8	96.6	/	/	1.000

†PSS, pet shop salespersons; ‡AH, aquarium hobbyists

Table 2: Recommended precautions to prevent the transmission of fish tuberculosis (17, 18)

Recommendation	Description
Buy healthy fish from trusted suppliers	Healthy fish have smooth, sleek, shiny scales, and intact fins. Fish bodies should not have any bumps or lesions.
Monitor the health status of fish daily	Symptoms of illness may include abnormal swimming, scale loss, coloration changes, lack of appetite, loss of body weight, skin ulcers, eroded fins, belly swelling. If a fish looks sick, place it in a tank by itself to prevent other fish in the main aquarium from getting sick. Immediately remove any dead fish from the aquarium to reduce the risk of spreading the disease to other fish. Consult a veterinarian specialized in fish diseases.
Do not release unwanted fish into nature	Never release aquarium fish into any waterway. Do not dispose of water or sick fish into storm-water or street drains. By following these recommendations, you will help to preserve the health of free-range and cultured aquatic animals.
Dispose of dead fish safely	Put dead fish into a plastic bag and place them in the waste that is not intended for recycling.
Dispose of aquarium tank water safely	Pour the aquarium waste water down a household sink. Do not use kitchen or bathtub sinks. Do not use the waste water to water house or garden plants.
Follow good hygiene practice	Wash hands before and after feeding the fish. Help children feeding the fish to wash their hands thoroughly. Do not allow children younger than five to come into contact with aquarium water and fish. Do not allow people with impaired immune system to clean the aquarium. When cleaning the aquarium, always wear waterproof gloves which should completely cover the parts of the arms immersed in water. Use utensils intended for aquarium cleaning only.
Pay attention to your health	If you notice skin lesions characteristic of skin mycobacteriosis, consult your physician and make sure to mention that you have a pet fish.

Similarly, 36.3% of the respondents confirmed that fish may contract tuberculosis, with a notable difference ($p<0.001$) between PSS and AH (73.9% vs. 24.5%). However, only 24.4% of the respondents agreed that the bacteria causing fish tuberculosis might endanger human health. Again, a higher awareness of this fact was notable among PSS (56.5% agreed) compared to AH (14.3% agreed) (Table 1).

Furthermore, 10.8% of the respondents claimed that they could recognise clinical signs of fish tuberculosis (among them, PSS showed a statistically significant predominance over AH: 32.6% vs. 4.1%), while only 3.1% had ever heard of fish tank granuloma (Table 1). The majority of the respondents also denied ever having had cutaneous lesions resembling the lesion presented in a photograph on the questionnaire.

Considering the general belief expressed by 74.6% of the respondents, that aquarium water may contain microbes harmful to humans, their aquarium handling practices were surprising, as the majority (74.6%) never used waterproof gloves when cleaning the aquarium or handling fish.

Discussion

To the best of our knowledge, the present study is one of a very few published surveys on the awareness of zoonotic potential of fish mycobacteria. The findings presented herein support previously reported observations that fish handlers' awareness of aquarium fish related health risk is poor. A French nationwide study, including 40 pet shop salespersons, revealed that 75% of the respondents knew little or nothing about skin mycobacteriosis in humans and 52.5% knew little or nothing about the disease in fish (13). On the other hand, when the knowledge and experience in terms of fish tuberculosis were assessed among the members of three tropical fish clubs in the United Kingdom (14), 60% of the respondents claimed that they could probably (or definitely) recognize fish tuberculosis. However, only 30% were aware of the risk of skin infection after contact with fish.

We have demonstrated the high level of contamination of aquarium fish with mycobacteria, which are potentially pathogenic to humans, in our previous studies (11, 12). Ornamental fish sold in pet shops in Slovenia mostly originate from fish farms in southeast Asia and are imported either directly from the country of origin or through distribution centres in Europe. Therefore, we believe that the presence of mycobacteria in aquarium fish in Slovenia reflects a global state in the ornamental fish industry. Other studies have also reported high proportions of mycobacteria positive aquarium fish (15, 16). Considering the rising number of people with weakened immune system, raising the awareness among fish handlers is essential to prevent mycobacterial infections, particularly since fish handling practices have been found to be risky as the majority of the respondents never used waterproof gloves when cleaning aquariums or handling fish. These findings correlate with previous reports. Of the 40 respondents included in a study by Gray et al. (14), 85% immersed their hands at least once a week in the fish tanks, and only two (5%) ever wore gloves. Similarly, Schmoor et al. (13) reported that 95% of the aquarium fish salespersons cleaned the aquariums without wearing gloves and 65% handled dead fish with bare hands. A total of 90% of salespersons immersed their unprotected hands into the aquariums on a daily basis.

There is another aspect to consider. The present study revealed that the respondents to the questionnaire most often disposed of aquarium waste water by pouring it into the sink/toilet (83%, data not shown). Authorities recommend disposing aquarium tank water into household sinks and not into street or storm-water drains in order to prevent the transmission of pathogens into the environment (17, 18). It should, however, be noted that not all household sinks are appropriate for waste water disposal. Some respondents reported using the kitchen or bath sinks, which present risky practices. Cases of mycobacterial infections in young children have been described where fish tanks were cleaned in bathtubs in which children were also bathed (19, 20). In order to follow good hygiene practices at home, it is also inappropriate to dispose of aquarium waste water in areas devoted to food preparation. The use of separate supplies and tools, intended for aquarium cleaning alone, is highly recommended.

In conclusion, PSS demonstrated a higher level of knowledge and more appropriate fish handling practices. They were also perceived as a relevant source of information for AH and tended to be more aware of fish tuberculosis and its zoonotic potential. Nevertheless, poor general knowledge

of the clinical manifestations of fish tuberculosis and fish tank granuloma was demonstrated. Efforts should, therefore, be made to increase awareness of the role of mycobacteria in infections associated with exposure to aquarium fish. A step in this direction was taken by publishing a leaflet describing the disease in fish and humans along with the precautions needed to prevent infection. The leaflet was distributed to the public on several occasions and is available (in Slovenian only) at https://www.vf.uni-lj.si/si or from the corresponding author upon request. In addition, an excerpt from the leaflet, related to the recommended precautions, is presented in Table 2 to give an overview of the preventive measures that aquarium fish handlers may introduce into their daily practices.

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MIKOBAKTERIJE PRI AKVARIJSKIH RIBAH: ALI SE AKVARISTI ZAVEDAJO NJIHOVEGA ZOONOTSKGA POTENCIALA?

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Povzetek: Za ljudi potencialno patogene vrste mikobakterij so v Sloveniji splošno prisotne pri akvarijskih ribah, ki so naprodaj v trgovinah z domačimi ljubljenčki, med drugimi tudi vrsta *Mycobacterium marinum*, ki je najpomembnejša povzročiteljica kožne mikobakterioze pri ljudeh. Namen raziskave je bil, s pomočjo vprašalnika, oceniti zavedanje ljubiteljskih akvaristov in prodajalcev v trgovinah z akvarističnim programom o zdravstvenih tveganjih, povezanih z rokovanjem z ribami.

V raziskavi je sodelovalo 198 oseb, 76,3 % ljubiteljskih akvaristov in 23,7 % prodajalcev v trgovinah, ki so v povprečju imeli do 15 let izkušenj pri rokovanju z akvarijskimi ribami. Približno tretjina (35,8 %) sodelujočih se je strinjala s trditvijo, da so ribe lahko vir okužbe za ljudi in da ribe lahko zbolijo za tuberkulozo. V manjšem deležu (24,4 %) so se sodelujoči strinjali s trditvijo, da je ribja tuberkuloza zoonoza. Velika večina anketirancev ni poznala kliničnih znakov ribje tuberkuloze in kožne mikobakterioze pri ljudeh. Kljub splošno izraženemu prepričanju, da lahko voda iz akvarija predstavlja tveganje za zdravje ljudi, je bila splošna praksa rokovanja z ribami presenetljiva, saj velika večina sodelujočih pri rokovanju z ribami nikoli ne uporablja vodoodpornih rokavic.

Med obema skupinama anketirancev smo odkrili več razlik. Prodajalci v trgovinah so bolj poučeni o rokovanju z ribami in bolj ozaveščeni o zdravstvenih tveganjih povezanih z akvarijskimi ribami, kot ljubiteljski akvaristi. Slednji prodajalce dojemajo kot zaupanja vreden vir informacij. V splošnem je ozaveščenost o zoonotskem potencialu mikobakterij pri akvarijskih ribah precej slaba, zato bi morali nameniti več pozornosti ozaveščanju o vlogi mikobakterij pri okužbah, povezanih z akvarijskimi ribami.

Ključne besede: kožna mikobakterioza; ribja tuberkuloza; mikobakterije; vprašalnik; zoonoza

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Original Research Article

PSEUROTIN A FROM *Aspergillus fumigatus* Fr. AUMC 8002 EXHIBITS ANTICANCER ACTIVITY AGAINST HEPATOCELLULAR CARCINOMA *IN VITRO* AND *IN VIVO*

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Abstract: This study investigated the *in vitro* and *in vivo* anti-hepatocellular carcinoma activity of pseurotin A isolated from the n-butanol extract of *Aspergillus fumigatus* Fr. AUMC 8002 (nBE-AF).The *in vitro* anticancer activity of nBE-AF was measured against a human hepatocellular carcinoma cell line (HepG2) using a sulforhodamine-B (SRB) assay. The intraperitoneal median lethal dose (LD_{50}) of nBE-AF was determined in rats. Hepatocellular carcinoma was induced in rats by a single intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg b.wt.) followed by subcutaneous injections of carbon tetrachloride (CCl_4) (3 ml/kgb.wt.) weekly for 6 weeks. After administration of these carcinogens, 1/10 and 1/20 LD_{50} doses of nBE-AF were administered intraperitoneally daily. NBE-AF exhibited significant cytotoxic activity against HepG2 cells. Administration of DEN and CCl_4 significantly elevated the serum levels of liver function and tumour markers and significantly downregulated tumour necrosis factor-α gene expression. Moreover, DEN and CCl4 decreased immunohistochemical Bax expression and increased Bcl-2 expression in the liver. Co-treatment with nBE-AF mitigated the DEN+CCl_4-induced alterations in a dose-dependent manner. Histopathological evaluation of the liver substantiated the above biochemical results. These results confirmed that nBE-AF, via its major isolated secondary metabolite, pseurotin A, exerted an anti-hepatocarcinogenic effect and could be used as a chemopreventive agent for hepatocellular carcinoma..

Key words: *Aspergillus fumigatus* extract; pseurotin A; hepatocellular carcinoma; anticancer; cytotoxic

Introduction

Cancer is a leading cause of death worldwide. Approximately 7.6 million deaths occur from cancer annually worldwide, and this number is expected to increase to 13.1 million in 2030 (1). Hepatocellular carcinoma (HCC) is the fifth most common tumour and the second most common cause of cancer mortality worldwide (2). The major risk factors for HCC are hepatitis B or C virus infection, food additives, toxic industrial chemicals, alcohol consumption, and exposure to environmental carcinogens such as aflatoxins and nitrosamines (3). Diethylnitrosamine (DEN) is one of the most potent hepatocarcinogenic nitrosamines found in agricultural chemicals, cosmetics and alcoholic beverages (4). DEN has been commonly used as a cancer initiator for the induction of HCC in experimental animals. Metabolized DEN in the liver generates reactive oxygen species (ROS) and induces oxidative stress and liver cell injury (5, 6).Furthermore, DEN is a genotoxic compound that forms alkyl DNA adducts and causes several nuclear aberrations in the rat liver that ultimately lead to the development of HCC (7).Carbon

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tetrachloride (CCl_4) is a powerful environmental toxicant that produces severe hepatic damage via the generation of reactive free radicals. Currently, there is no established effective chemotherapy for liver cancer, and chemotherapy is considered of limited value (8).One current approach to control hepatic cancer is chemoprevention, a programme of cancer management in which the occurrence of tumourigenesis could be completely prevented, slowed, or reversed by the use of natural or synthetic products (9).Indeed, the complementary and alternative use of natural products in the treatment of liver diseases has greatly increased worldwide. Currently, the identification of bioactive ingredients from plants, marine organisms and microorganisms to inhibit tumourigenesis in different types of animal models of carcinogenesis is attracting substantial attention (10). Fungi are important sources of bioactive secondary metabolites containing novel compounds with unique structural characteristics. These organisms have proven to be a rich and promising source of novel anticancer, antibacterial, antitumour and anti-inflammatory agents (11). *Aspergillus fumigatus* is a saprotrophic fungus that is widespread throughout nature. *A. Fumigatus* is found in soil and decaying organic matter and plays a vital role in carbon and nitrogen recycling. Numerous bioactive compounds, such as alkaloids, dibenzofurans, dioxopiperazine and indolediketopiperazine, have been isolated from *A. fumigatus* (12, 13). In the present study, subsequent culture and fractionation of the n-butanol extract of *A. fumigatus* (nBE-AF) resulted in the isolation of pseurotin A as a major secondary metabolite. Pseurotin A (an alkaloid) is a major secondary metabolite isolated from *Pseudeurotium ovalis* (14) and *A. Fumigatus* (15).To our knowledge, detailed studies about the anticancer effects of pseurotin A have not yet been reported. Therefore, the aim of this study was to evaluate the chemopreventive effect of nBE-AF via its major isolated secondary metabolite, pseurotin A, against HCC in rats.

Materials and methods

Chemicals

DEN was purchased from Sigma Chemicals Co. (St. Louis, MO, USA), whereas CCl_4 was obtained from El-Gomhoria Chemicals Co. (Zagazig, Egypt).

Fungal strain and growth conditions

The *Aspergillus fumigatus* Fr. AUMC 8002 strain was previously isolated and characterized from Roquefort cheese from the Sharkia and Assiut Governorates, Egypt. This fungal strain was maintained on Czapek yeast extract agar medium and incubated at 30ºC for 7 days, followed by monthly subculture.

N-butanol extract of A. fumigatus Fr. AUMC 8002

An Erlenmeyer conical flask(250 ml capacity) containing 50 ml of Czapek yeast extract broth medium was inoculated with 1 ml of *A. fumigatus* Fr. AUMC 8002 fungal spore suspension, and the culture flasks were incubated at 30°C for 7 days. After incubation, the fermented substrate was extracted using 1:1(v/v) n-butanol. The n-butanol extracts were washed with equal volumes of distilled water and dried over anhydrous sodium sulfate, filtered and concentrated by air-drying. The obtained crystals were preserved in dark bottles in a refrigerator at 4ºC and subjected to further analysis.

In vitro anticancer activity

The *in vitro* anticancer activity of nBE-AF against HepG2 cells was measured using a SRB assay (16). Briefly, cells were placed in a 96-multiwell plate (104cells/well) for 24 h before treatment with the compounds to allow attachment of the cells to the surface of the plate. nBE-AF was dissolved in dimethylsulfoxide (DMSO), and different concentrations (5, 12.5, 25 and 50 mM) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. The cell monolayer was incubated with nBE-AF for 48 h at 37°C in an atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was then removed by four washes with 1% acetic acid, and the bound stain was recovered with Tris-EDTA buffer. The colour intensity was measured in an enzyme-linked immunosorbent assay (ELISA) reader (microplate reader; Sunosik; SPR-960B; China). The relationship between the surviving fraction and the nBE-AF concentration was plotted to obtain the survival curve for the

HCC cell line after the specified time, and the IC_{50} (the concentration required for 50% inhibition of cell viability) was calculated.

Isolation and chemical identification of the major compound isolated from nBE-AF

The extract was subjected to silica gel open column chromatography [Merck silica gel 60H (70–203 mesh)] and elution with a stepwise gradient of 100% hexane to 100% EtOAc to (1:1) EtOAc-MeOH to yield 4 major fractions (F1-F4).

Fraction (3), eluted with hexane–EtOAc (7:3), yielded a pale yellow solid precipitate, which was purified by recrystallization from MeOH to yield pseurotin A. Thin-layer chromatography (TLC) was carried out using Merck Kiesel gel 60 pf254 plates. Preparative TLC was used for purification of the compound using chloroform and methanol (4:1) as the mobile phase, yielding a white crystalline solid. The chemical structure of the isolated compound was elucidated according to elemental analysis of C, H, N and halogen in a Perkin Elmer CHN 2400 and spectroscopic analysis including infrared (IR), nuclear magnetic resonance (^{1}HNMR), carbon-13 nuclear magnetic resonance (^{13}C NMR) and mass spectra. These analyses were carried out at the Micro-analytic Center, Cairo University, Egypt, as follows:

IR spectra

The IR spectra of the isolated compound were recorded from KBr discs using an FTIR Spectrophotometer 460 (4000–400 cm^{-1}).

Mass spectra

The mass spectra (direct inlet, FAB ionization) of the isolated compound were recorded using a mass spectrophotometer (MS-5988). The product was subjected to a stream of high-energy electrons at elevated temperatures of up to 100°C, yielding cleavage fragments, which were characterized by the mass/charge (m/e) values from the mass spectral data.

^{1}HNMR

Spectra were recorded on a Varian Mercury VX-300 NMR spectrometer using DMSO-d6 as a solvent and tetramethylsilane (TMS) as the internal chemical shift reference. Shifts (in ppm relative to TMS, with coupling constants in Hz) were obtained with the Varian Mercury VX-300 NMR spectrophotometer (500 MHz for ^{1}H and 125 MHz for ^{13}C).

Animals

Healthy adult male Sprague-Dawley rats weighing 150–170 g were provided by the Animal Research Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt. Animals were acclimated to laboratory conditions for 2 weeks prior to starting the experiment. Rats were kept in metal cages during the experimental period, maintained on a 12 h light-dark cycle in a temperature range of 21–24 °C and a relative humidity of 50–60%, and supplied a standard diet. The protocols were approved by the Ethics of Animal Use in Research Committee of Cairo University.

Determination of the intraperitoneal median lethal dose (LD_{50})

An acute toxicity screen was performed for nBE-AF according to the Spearman-Karber arithmetic method (17). Eighty-four adult male Sprague-Dawley rats were divided into 14 groups (six per group). The animals were fasted overnight with only water provided. The control group received no treatment. Groups 2 through 14 received intraperitoneal injections of fungal extract dissolved in water at different concentrations ranging from 500 to 6500 mg/kg body weight (b.wt.). The mortality of the animals was observed after 24 h.

$LD_{50} = LD100 - \sum (a \times b)/n$

n = total number of rats in a group.

a = difference between two consecutive doses of injected fungal extract.

b = average number of dead animals between two succeeding doses.

LD_{100} = lethal dose leading to 100% death of all test animals.

Induction of HCC

HCC was induced by a single intraperitoneal injection of DEN at a dose of 200 mg/kg b.wt.

dissolved in normal saline, as previously described by Sarkar et al. (18). After two weeks, animals received subcutaneous injections of CCl_4 in corn oil (50% (v/v), 3 ml/kg b.wt.) weekly for 6 weeks to promote the carcinogenic effect of DEN (6).

Experimental design

Sixty rats were divided into six groups (10/group) for an experimental period of 32 weeks. Group I served as the normal control group and received vehicle alone. Group II was the HCC (DEN+CCl_4) control rat group. Groups III (DEN+CCl_4+ 1/10 LD_{50}) and IV (DEN+CCl_4+ 1/20 LD_{50}) were injected intraperitoneally with fungal extract dissolved in distilled water at doses of 508 and 254 mg/kg b.wt., respectively, daily. Group V (1/10 LD_{50}) was intraperitoneally injected with 508mg/kg b.wt. nBE-AF alone daily. Group VI (1/20 LD_{50}) received intraperitoneal injection of nBE-AF alone (254 mg/kg b.wt.) daily. At the end of the experiment, blood samples were collected from the medial canthus of the rats and were centrifuged at 3000 rpm for 10 min to separate serum. Serum samples were preserved at -20°C until use for biochemical analysis. Then, rats were anaesthetized using diethyl ether and sacrificed by cervical dislocation, and liver tissue was rapidly removed and perfused with ice-cold saline. Liver specimens were immediately frozen in liquid nitrogen and stored at -80°C for analysis of gene expression. Other liver specimens from all groups were preserved at -20°C for the assessment of antioxidant status or in 10% neutral buffered formalin for histopathological and immunohistochemical investigations.

Biochemical analysis

Assessment of liver function

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated according to the method described by Bergmeyer et al. (19). Serum alkaline phosphatase (ALP) activity was measured by the method of King and Armstrong (20).Serum levels of albumin, total protein and total bilirubin (TBL) were determined according to the methods described by Grant etal. (21) and Tietz (22, 23), as appropriate.

Quantitative determination of serum tumour markers

Alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) levels were measured using ELISA kits provided from Bio-Check, Inc. (Foster City, CA, USA).

Assessment of antioxidant activity

Liver specimens were homogenized (10% (w/v)) in a potassium phosphate buffer solution (pH 7.4) using a tissue homogenizer. Samples were then centrifuged at 3000 rpm for 15 min. The supernatant was used to estimate superoxide dismutase (SOD) activity (24), catalase (CAT) activity (25), glutathione peroxidase (GPx) activity (26), reduced glutathione (GSH) levels (27) and malondialdehyde (MDA) concentrations (28).

Estimation of hepatic tumour necrosis factor-α (TNF-α) expression

Total RNA was extracted from 30 mg of liver tissue using an RNeasy Mini Kit (Qiagen, Heidelberg, Germany) according to the manufacturer's instructions. The purity of the total RNA was determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Total RNA (0.5 µg) was reverse transcribed into cDNA using a Qiagen LongRange2 Step RT-PCR Kit following the manufacturer's instructions. One microliter of total cDNA was mixed with 12.5 µl of 2× SYBR® Green PCR Mix with ROX from BioRad, 5.5 µl of autoclaved water, and 0.5 µl (10 pmol/µl) of each forward and reverse primer for the target genes. The expression values were normalized to an internal housekeeping control (*β*-actin) gene. The expression of the TNF-*α* gene was assessed using real-time PCR. The primer sequences are shown in Table (1). PCR reactions were conducted in a Rotor-Gene Q cycler (Qiagen, Heidelberg, Germany). The real-time qPCR program included an enzyme activation step at 94°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. Detection of the fluorescent product was carried out at the end of the 72 °C extension period. The amplification data were collected by a sequence detector and analysed with sequence detection software. For each assay, a standard curve was generated using increasing amounts of cDNA, and the slopes of all curves indicated adequate PCR conditions (slopes of 3.3-3.6). The RNA concentration in each sample was determined from the threshold cycle (Ct) values and calculated with the sequence detection software supplied by the manufacturer. The quantitative fold changes

in mRNA expression were determined relative to the ß-actin mRNA levels in each corresponding group and calculated using the $2^{-\Delta\Delta Ct}$ method.

Histopathological examination

Liver samples were preserved in 10% neutral buffered formalin and were then processed and stained with haematoxylin and eosin (H&E) for histopathological examination using light microscopy (29).

Immunohistochemical investigation

Liver sections were prepared for immunohistochemical detection of Bax and Bcl-2 using primary rabbit polyclonal anti-rat Bax and Bcl-2 antibodies (Thermo Fisher Scientific Inc., Fremont, CA, USA), respectively. This technique was carried out via the avidin–biotin complex method (30). Negative control sections were incubated in phosphate-buffered saline instead of the primary antibody, and all tissue sections were examined under a light microscope.

Statistical analysis

The data are presented as the means ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan's test for comparison between different experimental groups. Analyses were performed using IBM SPSS statistics computer software (version 22). P-values of < 0.05 were considered statistically significant.

Results

In vitro anticancer potency

To explore the *in vitro* anticancer potency of nBE-AF against HepG2 cells, cell proliferation was determined by an SRB assay. The cytotoxic effects of nBE-AF on HepG2 cells were determined by treating cells with different concentrations of nBE-AF. Upon treatment with increasing concentrations of nBE-AF, treated cells showed a significant decrease in viability. nBE-AF was active against HepG2 cells, with an IC_{50} of 22.2 µg/ml (Figure 1).

Preliminary characterization of nBE-AF

nBE-AF is a faint yellow-green colour and dissolves only in DMSO with heating and shaking and in hot water. nBE-AF produces one yellow spot on TLC with an R_f value of 0.3 using methanol solvent and melts at 203°C. Elemental analysis indicated that the ratio of the main elements (C/H/N) was 2.73/2.77/0.33 for compound 32. The extracted natural compound was found to be a polymer of the furanose ring with n=3 units. The data obtained by spectral analysis, IR spectrophotometry and mass spectrometry indicated that the M^+ parent ions were a polymer of the furanose ring. The mass spectrometry data showed that the M^+ parent ion (430) indicated n= 3 fragmentation, and the IR spectra showed the presence of bands at 1116 and 1039 cm-1, corresponding to the ether linkage (-O-) and (C=C), respectively, in the furanose moiety (Helal et al., 2018 unpublished data).

Chemical identification of the major compound isolated from nBE-AF

Preparative chromatographic purification of nBE-AF yielded the pseurotin A compound as the major compound isolated from nBE-AF. The molecular formula of this compound was determined using spectroscopic tools (IR, ^1H and ^{13}C NMR, elemental analysis and mass spectrometry) as illustrated in Figure 2. The structure of pseurotin contains a unique and highly oxidized azaspirocyclic framework in the form of a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione ring system. Numerous analogues were found that differed in the substitution at positions C2 and C8 (Figure 2).

IR (KBr, cm^{-1})

The IR spectra showed absorption bands for hydroxyls and NH groups: 3399 cm^{-1} (br, 3OH and NH), 3080 cm^{-1}(C-H, aromatic), 2936 cm^{-1} (C-H aliphatic), 1656 cm^{-1} (br, 3C=O, α, β-unsaturated and amidic), and 1246 cm^{-1} (-O-, ether).

^1H NMR (DMSO-d6)

The ^{1}HNMR data indicated shifts with δ= 2.52, 2.72, 2.88 and 2.52 ppm (t, J=3H, CH3, CH2); 2.72 and 2.88 (2s, J=6H, 2CH3); 3.05 (q, 2H, CH2CH3);

3.40, 3.53, and 3.79 (3t, 2CHOH); 3.87(s, 1H, NH, exchange with D2O); 4.26, 4.88, and 5.17 (3t, 3OH, exchange with D2O); 6.56 (d,1H, J= 10.2 Hz olefinic); 6.96 (d, 1H, J = 16.9 Hz, olefinic); and 7.89(s,5H, Ar-H).

13C NMR (DMSO-d6)

The ^{13}C NMR results indicated shifts with δ = 29.81, 31.29, 35.20, and 36.33 (3CH3 and CH2); 63.48, 70.07, 70.32 and 75.25 (4CHOH); 81.37, 92.17, 92.62, 97.26, 98.49, 102.3, 104.4, and 104.6 (2C=C and 4Ar-C); and 162.9, 174.2 and 179.8 (3C=O).

Mass spectrometry

The fast atom bombardment mass spectrometry (FABMS) data contained a peak (m/z= 432 [M+H]+), identified as C22H25NO8, that indicated the parent ion at M+1 [m/z= 431.45 (M + H+, 19.54%)] with fragment ions at m/z values of 54.95 (67.56%), 57.10 (72.88%), 69.10 (99.75%), 70.05 (61.02%), 71.10 (69.86%), 81.05 (67.17%), 83.05 (92.59%), 85.05 (73.24%), 97.10 (100%) as the base peak, 111.0 (63.06%), and 430.35 (47.96%).

The parent ion indicates that the molecular formula of this compound is $C_{22}H_{25}O_8N$; the compound was soluble in ethyl acetate, acetone, ethanol and isopropanol.

Median lethal dose of nBE-AF

The intraperitoneal LD_{50} of nBE-AF in rats was 5083 mg/kg b.wt., suggesting that nBE-AF was highly safe. The therapeutic doses were (1/10 of the LD_{50}) 508.3 mg/kg b.wt. and (1/20 of the LD_{50})254.2 mg/kg b.wt. (Table 2).

Effect of nBE-AF on liver function and serum tumour markers

DEN+ CCl_4-treated rats showed significantly ($p < 0.05$) increased serum ALT, AST and ALP activity and TBL levels compared with those of the control group. However, the groups co-treated with DEN and nBE-AF (III and IV) exhibited significant dose-dependent reduction in the levels of these markers compared with those in the DEN+CCl_4-treated group. In addition, the serum albumin and total protein levels were significantly decreased in rats treated with DEN and CCl_4 relative to those in control group. Compared with DEN+CCl_4 treatment alone, co-administration of nBE-AF with DEN significantly increased serum albumin and total protein (Table 3).The serum levels of the tumour markers AFP and CEA were significantly increased in the DEN+CCl_4-treated group compared with those in the control group but were significantly decreased by co-treatment with 1/10 and 1/20 LD_{50} doses of nBE-AF (Figure 3).

Effect of nBE-AF on hepatic antioxidant status

The activities of the hepatic antioxidant enzymes GR, GPx and SOD were significantly decreased in DEN-treated group compared with those in control animals (Table 4). Compared to the DEN-treated group, the groups treated with nBE-AF showed significantly increased hepatic GR, GPx and SOD activities (Table 4). A significant increase in the MDA level, which is a marker of lipid peroxidation, was observed in DEN-treated rats relative to that in control rats (Table 4). Treatment with nBE-AF for 24 weeks showed a significant protective effect against DEN-induced lipid peroxidation (Table 4). Therapeutic treatment with nBE-AF for 24 weeks resulted in a significant decrease in MDA levels compared to those in DEN-treated rats (Table 4). DEN administration led to a significant reduction in hepatic GSH content compared with that in control rats (Table 4), but treatment with nBE-AF significantly improved hepatic GSH levels compared to those in DEN-treated group (Table 4).

Estimation of hepatic TNF-a expression

Expression of the TNF-*a* gene was significantly ($p < 0.05$) upregulated in the livers of DEN+CCl_4-treated rats relative to that in control group rats. Additionally, this expression level was significantly downregulated in a dose-dependent manner in the nBE-AF-co-treated groups (III and IV) relative to that in theDEN+CCl_4-treated group (Figure 4).

Histopathological changes

Light microscopy of H&E-stained liver sections from rats in the control and nBE-AF-treated groups

showed normal polygonal hepatocytes with round nuclei arranged in strands, along with a normal central vein, Kupffer cells and blood sinusoids (Figure 5A). The livers of rats in the DEN+CCl_4-treated group revealed highly demarcated HCC near the central vein and some hepatocytes with a vacuolated cytoplasm and deeply stained pyknotic nuclei, in addition to dilated blood vessels (Figure 5B). Severely congested blood vessels and hepatic portal vein, along with portal vein dilation, were also observed (Figure 5C). The lesions in the group treated with DEN+CCl_4+1/10 LD_{50} nBE-AF were distinctly reduced relative to that in the DEN-treated group. The livers of rats treated with DEN+CCl_4+1/10 LD_{50} nBE-AF showed a significant improvement in hepatocyte morphology, as represented by the normal central vein (Figure 5D). Moreover, Kupffer cells were noticed around the portal tract, in addition to some congested blood vessels and a reduction in the size of the hepatic portal vein to a normal shape (Figure 5E). The livers of rats in the DEN+CCl_4+1/20LD_{50} nBE-AF group revealed moderate improvements in hepatocyte morphology, reductions in inflammation and degenerative changes, and reactive hepatocytes with condensed chromatin and small nuclei (Figure 5F).

Table 1: Oligonucleotide sequences used for real-time PCR analysis of the TNF-α and β-actin genes

Gene	Primer sequences	GenBank accession number
TNF-α	F: 5'-CCAGGAGAAAGTCAGCCTCCT -3' R: 5'-TCATACCAGGGCTTGAGCTCA -3'	NM_012675
ß-actin	F:5'-AAGTCCCTCACCCTCCCAAAAG -3' R: 5'-AAGCAATGCTGTCACCTTCCC -3'	V01217

Table 2: Determination of the LD_{50} of nBE-A

Group	Difference between consecutive doses (a)	No. of dead animals	Mean mortality between two consecutive doses (b)	a × b
Control	0	0	0	0
500 mg/kg b.wt.	500	0	0	0
1000 mg/kg b.wt.	500	0	0	0
1500 mg/kg b.wt.	500	0	0	0
2000 mg/kg b.wt.	500	0	0	0
2500 mg/kg b.wt.	500	0	0	0
3000 mg/kg b.wt.	500	0	0	0
3500 mg/kg b.wt.	500	0	0	0
4000 mg/kg b.wt.	500	0	0	0
4500 mg/kg b.wt.	500	2	1	500
5000 mg/kg b.wt.	500	3	2.5	1250
5500 mg/kg b.wt.	500	4	3.5	1750
6000 mg/kg b.wt.	500	5	4.5	2250
6500 mg/kg b.wt.	500	6	5.5	2750
Sum				8500

LD_{50} = LD - Σ (a × b)/N

LD is the lowest lethal dose among all animals in each group; N is the number of animals in each group; ∑ = the sum of (a × b).

LD_{50} =6500-8500/6= 6500-1416.6= 5083 mg/kg b.wt.

Table 3: Effect of nBE-AF on the serum levels of liver function markers in DEN-treated rats (Mean ± SE, n = 10)

Groups	ALT (U/L)	AST(U/L)	ALP(U/L)	Albumin(mg/dL)	Total protein(mg/dL)	TBL (mg/dL)
Control	67.74 ± 3.13[a]	169.42 ± 2.24[a]	131.60 ± 2.37[a]	3.98 ± 0.08[a]	7.56 ± 0.07[a]	0.70 ± 0.03[d]
DEN+CCl_4	118.36 ±2.97[d]	279.38±2.93[d]	197.00 ± 2.02[d]	2.24 ± 0.079[d]	4.25 ± 0.16[d]	1.19 ± 0.024[a]
DEN+CCl_4+1/10 LD_{50} nBE-AF	86.34 ± 1.08[b]	188.40±4.34[b]	156.40 ± 3.55[b]	3.38 ± 0.09[b]	6.6 ± 0.27[b]	0.76 ± 0.026[c]
DEN+CCl_4+1/20 LD_{50} nBE-AF	97.76 ± 2.20[c]	226.48±4.76[c]	180.00 ± 3.22[c]	2.98±0.05[c]	5.75 ± 0.07[c]	0.88 ± 0.019[b]
1/10 LD_{50} nBE-AF	70.26 ± 2.10[a]	174.38±0.82[a]	128.80 ± 2.63[a]	3.82 ± 0.14[a]	7.43± 0.15[a]	0.65 ± 0.02[d]
1/20 LD_{50} nBE-AF	69.32 ± 4.27[a]	174.40 ± 1.25[a]	130.20 ± 2.59[a]	3.92 ± 0.07[a]	7.13 ± 0.05[a]	0.67 ± 0.02[d]

Means within the same column carrying different superscripts are significantly different ($p < 0.05$).

Table 4: Effect of nBE-AF on oxidative stress markers in the liver of DEN-treated rats (Mean ± SE, n = 10)

Groups	SOD (U/g tissue)	CAT(U/g tissue)	GPx (ng/g tissue)	GSH(mmol/g tissue)	MDA(nmol/g tissue)
Control	11.00 ± 0.55[a]	66.00 ± 3.09[a]	0.74 ± 0.03[a]	1.19 ± 0.04[a]	82.53 ±1.53[d]
DEN+CCl_4	4.81± 0.71[d]	31.52 ± 2.06[d]	0.1 ± 0.014[d]	0.42 ± 0.03[d]	178.85 ± 1.68[a]
DEN+CCl_4+1/10 LD_{50} nBE-AF	8.73 ± 0.31[b]	54.00 ± 2.03[b]	0.62 ± 0.01[b]	0.92 ± 0.03[b]	98.19 ± 1.15[c]
DEN+CCl_4+1/20 LD_{50} nBE-AF	7.17 ± 0.09[a]	41.36 ±1.77[c]	0.44 ± 0.01[c]	0.75 ± o.02[c]	127.35 ± 1.49[b]
1/10 LD_{50} nBE-AF	11.25 ± 0.148[a]	66.40 ±2.86[a]	0.79 ± 0.01[a]	1.05 ± o.05[a]	82.96 ± 0.75[d]
1/20 LD_{50} nBE-AF	11.70 ± 0.51[a]	67.04 ± 1.77[a]	0.79 ± 0.01[a]	1.14 ± o.04[a]	81.16 ± 0.67[d]

Means within the same column carrying different superscripts are significantly different ($p < 0.05$).

Immunohistochemical findings

Immunohistochemical staining with the pro-apoptotic Bax antibody in rat liver tissue revealed weak expression in control and nBE-AF-treated rats (Figure 6A-C). The livers of rats in the DEN+CCl_4-treated group showed preneoplastic nodules with a marked reduction in the expression of Bax (Figure 6D). However, the livers of rats in the DEN+CCl_4+1/10 LD_{50} nBE-AF and DEN+CCl_4+1/20 LD_{50} nBE-AF groups showed increased nuclear and cytoplasmic expression of Bax (Figure 6E and F).

Immunohistochemical staining with the anti-apoptotic Bcl-2 protein in rat liver showed weak expression in control and nBE-AF-treated rats (Figure 7A-C). The livers of rats in the DEN+CCl_4-treated group exhibited preneoplastic nodules with marked expression of Bcl-2 (Figure7D), while the livers of rats in the groups treated with DEN+CCl_4+1/10 LD_{50} nBE-AF and DEN+CCl_4+1/20 LD_{50} nBE-AF showed moderate expression of Bcl-2 (Figure 7E and F).

nBE-AF Cytotoxicity

Conc µg/ml	HepG2 cells - 32 A
0.000	1.000000
5.000	0.737376
12.500	0.726812
25.000	0.308240
50.000	0.313170

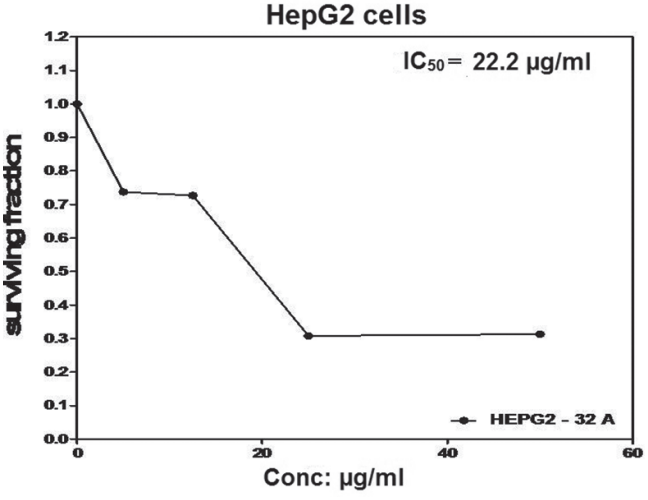

Figure 1: Cytotoxicity of nBE-AF against HepG2 cells

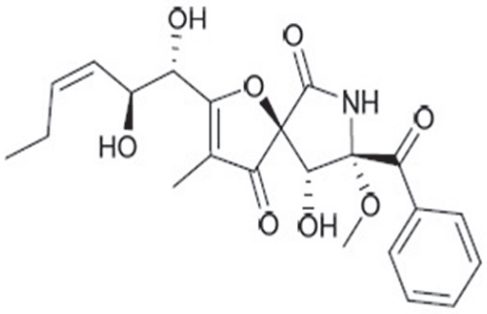

Figure 2: Chemical structure of pseurotin A isolated from nBE-AF

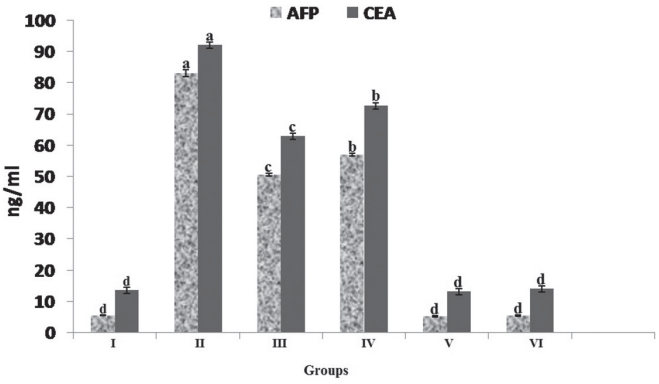

Figure 3: Effect of nBE-AF on the serum AFP and CEA levels in DEN-treated rats. The bars with differing letters indicate significant differences ($p<0.05$) (mean ±SE, n=10)

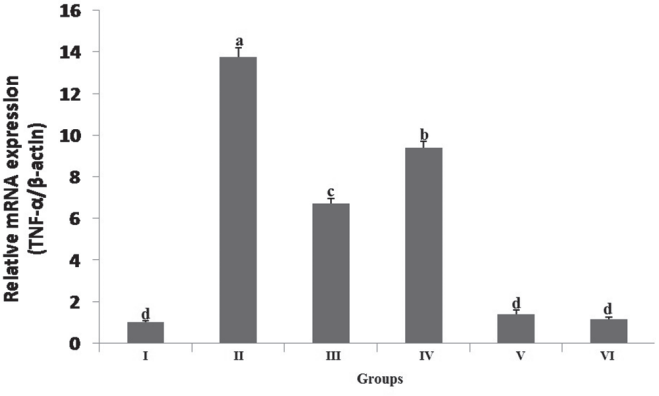

Figure 4: Relative expression levels of the TNF-α gene in the livers of control and experimental rats. The bars with differing letters indicate significant differences ($p < 0.05$) (mean ± SE, n=10)

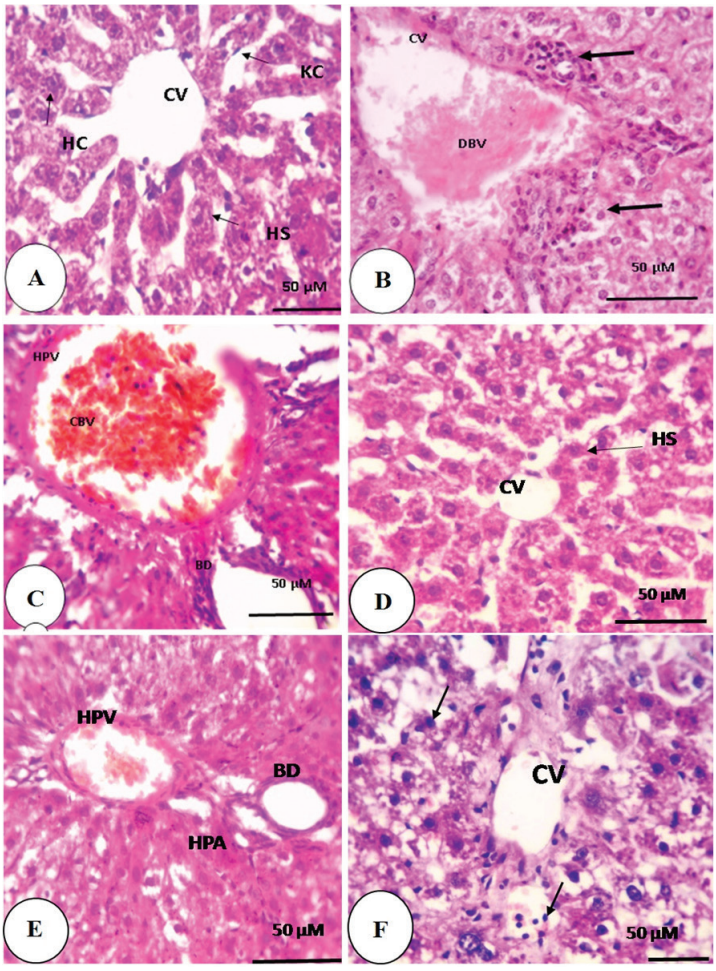

Figure 5: Photomicrographs of H&E-stained liver sections from rats in the control and experimental groups under light microscopy. A; The control group and the groups treated with nBE-AF alone at 1/10 and 1/20 LD_{50} showing normal architecture, indicating the nontoxic nature of nBE-AF. B, C; DEN+CCl_4-treated group showing the following abnormalities: B, Highly demarcated HCC near the central vein (CV) (thick arrow); some hepatocytes have vacuolated cytoplasm with deeply stained pyknotic nuclei. Dilated blood vessels (DBV) can also be seen; and C, Severe congested blood vessels (CBV) and hepatic portal vein (HPV), with portal vein dilation. D, E; DEN+CCl_4+1/10 LD_{50} nBE-AF-treated group showing considerable improvements in hepatocyte morphology. D; Normal central vein. E; The size of the hepatic portal vein was reduced to a normal shape, and Kupffer cells (KC) were visible around the portal tract, along with some congested blood vessels. F; DEN+CCl_4+1/20 LD_{50} nBE-AF-treated group showing moderate improvements in hepatocyte morphology, reductions in inflammation and degenerative changes, and reactive hepatocytes with condensed chromatin (arrowhead) and small nuclei. (HC: Hepatic cells, HS: Hepatic cells arranged in strands, HPA: Hepatic portal artery, BD: Bile duct)

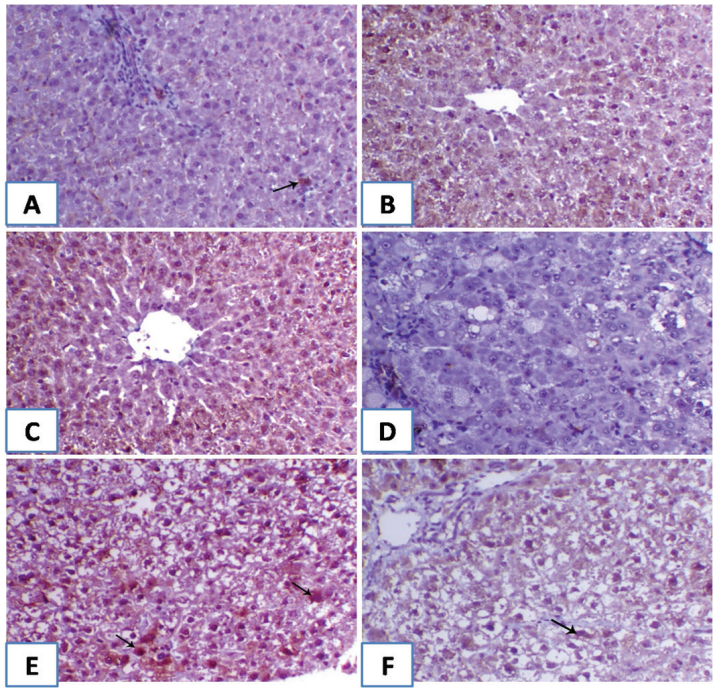

Figure 6: Immunohistochemical staining with Bax in liver tissue from rats in the control (A), 1/10 LD_{50} nBE-AF alone (B), 1/20 LD_{50} nBE-AF alone (C), DEN+CCl_4-treated (D), DEN+CCl_4+1/10 LD_{50} nBE-AF-treated (E) and DEN+CCl_4+1/20 LD_{50} nBE-AF-treated (F) groups. A-C; Weak expression of Bax (arrows). D; Preneoplastic nodule with a marked reduction in Bax expression. E, F; Increased nuclear and cytoplasmic expression of Bax (arrows) (200×)

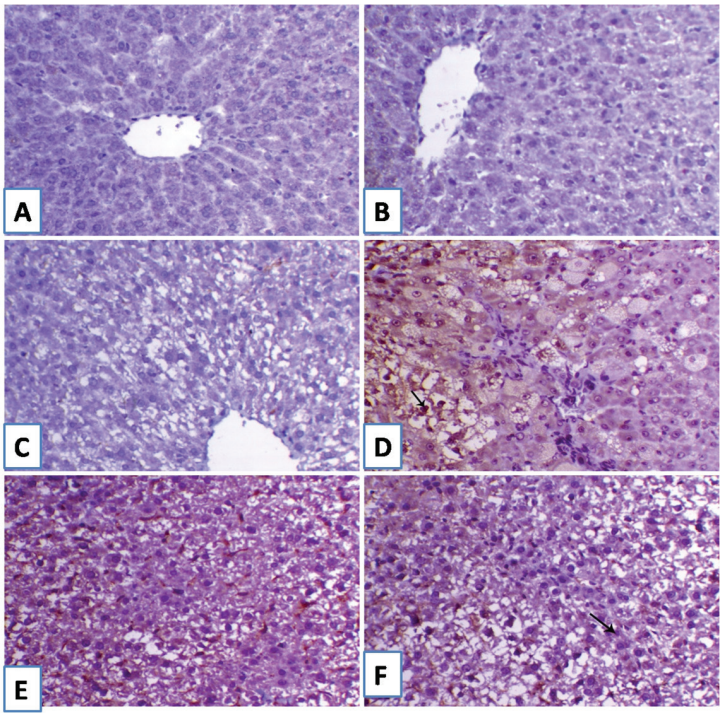

Figure 7: Immunohistochemical staining with Bcl-2 in liver tissue from rats in the control (A), 1/10 LD_{50} nBE-AF alone (B), 1/20 LD_{50} nBE-AF alone (C), DEN+CCl_4-treated (D), DEN+CCl_4+1/10 LD_{50} nBE-AF-treated (E) and DEN+CCl_4+1/20 LD_{50} nBE-AF-treated (F) groups. A-C; Weak expression of Bcl-2. D; Preneoplastic nodule with marked expression of Bcl-2(arrows). E, F; Moderate expression of Bcl-2 (arrows) (200×)

Discussion

In recent years, interest in the use of bioactive metabolites of microorganisms such as actinomycetes and fungi as possible chemopreventive and chemotherapeutic agents against HCC has been increasing worldwide. These derived compounds inhibited the growth and proliferation of tumour cells (31).This study investigated the *in vitro* anticancer activity of nBE-AF on HepG2 cells as well as its chemopreventive potential against DEN+CCl_4-induced HCC in rats. Our results showed that nBE-AF has significant cytotoxicity towards HepG2 cells, with an IC_{50} of 22.2 µg/ml. Therefore, we suggest that nBE-AF has strong cytotoxic activity according to the guidelines of The American National Cancer Institute, which describe an extract with strong cytotoxic activity as an anticancer agent if its IC_{50} value is less than 30 mg/ml (32). In the present study, the major compound isolated from nBE-AF was identified as pseurotin A. Four pseurotin analogues were reported to exhibit potent cytotoxicity against the human breast cancer cell line MCF-7, with the more active pseurotin D inhibiting MCF-7 cell growth with an IC_{50} value of 15.6 µM (33).

DEN and CCl_4 induced liver damage in rats, as indicated by significant increases ($p < 0.05$) in the serum transaminase (ALT and AST), ALP and TBL levels relative to those in control group. Elevations in transaminase levels are considered the most sensitive markers for diagnosing hepatocellular damage (34).The elevated serum ALT and AST levels in DEN + CCl_4-treated rats may be attributed to DEN-induced hepatic damage associated with the loss of the functional integrity of the cell membrane and the leakage of cellular enzymes from neoplastic cells into the circulation (35). Moreover, the elevated ALP activity reflected pathological alterations in biliary flow, and the increased TBL level indicated a non-specific alteration in plasma membrane integrity and permeability (6). Serum protein levels can also be used as a marker of liver function. Administration of DEN and CCl_4 caused a significant reduction in the serum albumin and total protein levels, indicating hepatocellular dysfunction. The DEN + CCl_4-induced liver damage observed in the present study was consistent with that seen in previous studies (6, 31). Our results showed that DEN and CCl_4 elicited a significant increase ($p < 0.05$) in the levels of serum tumour markers (AFP and CEA) relative to those levels in the control group. These findings coincided with those in previous studies showing increased levels of serum tumour markers,

including AFP and CEA, following treatment with DEN and CCl_4 in rats (6, 36). AFP is an oncofoetal serum glycoprotein that is normally produced by immature liver cells in the foetus but is gradually lost during development, becoming almost absent in healthy adults (37), and is an excellent marker for diagnosing HCC (38). CEA is a 180–200 kDa, heavily glycosylated protein belonging to the immunoglobulin supergene family. CEA is commonly detected at a high level in the serum of patients with liver malignancy (39). Additionally, the CEA level is elevated in the serum of patients with various other cancers, such as lung, colon, ovarian and breast cancers (40). Furthermore, administration of DEN and CCl_4 caused hepatic oxidative damage in rats, as evidenced by the reductions in antioxidant enzyme (SOD, CAT and GPx) activity and the GSH concentration, along with increased lipid peroxidation (MDA) in the livers of rats in the DEN + CCl_4-treated group relative to these parameters in control group. These results were consistent with those in previous reports (6, 36, 41). Administration of DEN has been reported to generate products of lipid peroxidation such as 4-hydroxynonenal and MDA that may interact with different molecules, resulting in oxidative stress and carcinogenesis (42). Additionally, DEN-induced oxidative stress may be due to the generation of ROS during the metabolic biotransformation of DEN (43). Oxidative stress results in carcinogenesis by several mechanisms involving damage to lipids, proteins and DNA; alterations in intracellular signalling pathways; and changes in gene expression—these events, in turn, promote abnormal cell growth and carcinogenesis (44). We observed significant ($p < 0.05$) upregulation of TNF-*a* gene expression in the livers of DEN+CCl_4-treated rats relative to that in control group. Similarly, hepatic TNF-*a* gene expression was significantly increased in DEN-treated mice (45, 46). TNF-*a* is a pro-inflammatory cytokine involved in cancer owing to its ability to activate the oncogenic transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) in epithelial cells, leading to the promotion of cell proliferation and survival. Consequently, NF-κB activation plays a vital role in tumour cell growth and invasion (47, 48). In addition, TNF-*a* acts as a growth factor in the majority of tumour cells and can affect all stages of tumour development, including initiation, promotion and metastasis (49, 50). Interestingly, co-treatment with nBE-AF ameliorated DEN+CCl_4-induced hepatic damage by significantly ($p < 0.05$) restoring the levels of liver function markers in a dose-dependent manner. Therefore, our results indicate that nBE-AF facilitates parenchymal cell regeneration in the liver, thus protecting membrane integrity by reducing enzyme leakage induced by the carcinogenic effects of DEN and CCl_4. These findings were in accordance with those of a previous study showing that the alkaloid berberine ameliorated liver damage induced by CCl_4 in rats (51). Concomitant treatment with nBE-AF and DEN+CCl_4 significantly ($p < 0.05$) decreased the levels of serum tumour markers, including AFP and CEA, relative to those in the DEN+CCl_4-treated group. This finding suggested that nBE-AF reduced the tumour incidence rate. This hypothesis was supported by the pro-apoptotic effect of nBE-AF observed in the present study, as evidenced by the increased immunohistochemical expression of Bax and decreased expression of Bcl-2 in the livers of rats in the groups co-treated with nBE-AF (III and IV). Interestingly, administration of nBE-AF mitigated DEN+CCl_4-induced hepatic oxidative damage by restoring the activity of antioxidant enzymes (SOD, CAT and GPx) and the levels of GSH and MDA towards the control values. This effect may be due to the antioxidant and free radical scavenging activities of pseurotin A, the major secondary metabolite isolated from nBE-AF (52). In a similar study, the alkaloid canadine showed antioxidant activity against oxidative stress induced by tert-butylhydroperoxide in rat hepatocytes (53). Notably, significant ($p < 0.05$) dose-dependent downregulation of TNF-α gene expression was observed in the livers of rats in the groups co-treated with nBE-AF(III and IV) relative to that in rats in the DEN+CCl_4-treated group. Similarly, the alkaloid crebanine inhibited TNF-α-induced NF-κB activation in A549 human lung adenocarcinoma cells. Additionally, crebanine suppressed the TNF-α-mediated expression of proteins implicated in cancer cell invasion, such as matrix metalloproteinase 9 (54). Our findings demonstrated that nBE-AF exerts anti-inflammatory and anticancer effects through the downregulation of hepatic TNF-α gene expression, which plays an essential role in tumour development and metastasis via NF-κB activation. Regarding the immunohistochemical expression of the pro-apoptotic Bax and anti-apoptotic Bcl-

2 proteins in the liver, these proteins are key factors that regulate apoptosis and determine cell death or survival (55). The present results showed preneoplastic nodules with a marked reduction in Bax expression in the DEN+CCl4-treated rats. However, rats in the groups co-treated with nBE-AF (III and IV) exhibited increased nuclear and cytoplasmic expression of Bax. The anti-apoptotic protein Bcl-2 is implicated in cancer initiation and progression by promoting the survival of transformed cells. Therefore, Bcl-2 is the main target for innovative specific anticancer therapeutics. In addition, the present results showed that administration of DEN and CCl_4 caused a marked increase in the expression of Bcl-2 relative to that in the control group. Consistent with our results, other studies have indicated that Bcl-2 is overexpressed in various types of cancers (56). In contrast, the groups co-treated with nBE-AF exhibited a marked reduction in Bcl-2 expression relative to that in the DEN+CCl_4-treated group. These findings were consistent with those in a similar study showing that 6-methoxydihydrosanguinarine induced apoptosis in HepG2 cells via upregulation of Bax and downregulation of Bcl-2 (57).The current findings confirmed that nBE-AF could induce apoptosis in HCC cells and eventually inhibit tumour cell proliferation in rats with DEN+CCl_4-induced HCC.

The antioxidant, anti-proliferative and anticancer activities exhibited by nBE-AF may contribute to its chemopreventive effect against DEN+CCl_4-induced hepatocarcinogenesis in rats (13, 58).

Our aforementioned data were corroborated by the liver histopathological findings. Histopathological examination of the livers of DEN+CCl_4-treated rats revealed marked architectural changes, with highly demarcated HCC near the central vein. These findings were consistent with those of previous reports (6, 36).

Co-administration of a 1/10 LD_{50} dose of nBE-AF significantly ameliorated these pathological lesions. However, co-treatment with a 1/20 LD_{50} dose of nBE-AF produced moderate improvements in hepatocyte morphology and reduced inflammation and degenerative changes. Our results were consistent with those of similar studies showing that the alkaloid berberine exerted hepatoprotective effects against histological damage induced by CCl_4 in rats (59) and by doxorubicin in mice (60).

Conclusion

The results of this study demonstrated that nBE-AF exhibited anticancer activity on HepG2 cells *in vitro* and exerted a dose-dependent beneficial protective effect against DEN+CCl_4-induced hepatocarcinogenesis in rats by restoring the serum levels of tumour and hepatic function markers, enhancing the liver antioxidant status, downregulating the hepatic TNF-*a* gene expression and minimizing histological alterations in the liver. In addition, nBE-AF inhibited HCC cell proliferation through the induction of apoptosis via increased Bax expression and decreased Bcl-2 expression. The anti-carcinogenic effect of pseurotin A isolated from *A. Fumigatus* could be attributed to its antioxidant, anti-inflammatory, cytotoxic and pro-apoptotic activities. Therefore, nBE-AF could be a candidate chemopreventive agent for hepatic cancer.

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PSEUROTIN A IZ GLIVE *Aspergillus fumigatus* Fr. AUMC 8002 ZAVIRA RAST KARCINOMA JETER *IN VITRO* IN *IN VIVO*

G. A. Helal, F. A. Ahmed, A. Askora, T. M. Saber, S. M. Rady

Povzetek: V študiji so raziskali *in vitro* in *in vivo* protirakasto aktivnost pseurotina A, pridobljenega iz n-butanolnega izvlečka glive *Aspergillus fumigatus* Fr. AUMC 8002 (nBE-AF), na karcinom jeter. *In vitro* protirakasta aktivnost nBE-AF je bila spremljana pri humani celični liniji jetrnega karcinoma (HepG2) z uporabo analize sulforhodamin-B (SRB). Pri podganah so intraperitonealno določili povprečni smrtni odmerek (LD_{50}) nBE-AF. Jetrni karcinom je bil pri podganah sprožen z enkratno intraperitonealno injekcijo dietilnitrozamina (DEN) (200 mg/kg telesne teže), čemur so sledile podkožne injekcije ogljikovega tetraklorida (CCl_4) (3 ml/kg) enkrat tedensko 6 tednov. Po dodajanju teh rakotvornih snovi so 1/10 in 1/20 odmerka LD_{50} nBE-AF aplicirali vsak dan intraperitonealno. NBE-AF je pokazal pomembno citotoksično aktivnost proti celicam HepG2. Uporaba DEN in CCl4 je značilno povečala serumske vrednosti jetrnih funkcij in tumorskih markerjev ter znatno zmanjšala izražanje gena za faktor tumorske nekroze α (TNF- α). Poleg tega sta DEN in CCl_4 zmanjšala imunohistokemično izražanje gena Bax in povečala izražanje gena Bcl-2 v jetrih. Sočasno zdravljenje z nBE-AF je zmanjšalo z DEN + CCl_4 sprožene spremembe v jetrih v povezavi z velikostjo odmerka. Histopatološka ocena jeter je potrdila zgoraj navedene biokemične rezultate. Rezultati so potrdili, da je nBE-AF preko svojega glavnega izoliranega sekundarnega metabolita pseurotina A izkazal protirakasti učinek na jetra in bi se lahko v prihodnosti morda uporabljal kot zdravilo za zdravljenje karcinoma jeter.

Ključne besede: izvleček *Aspergillus fumigatus*; pseurotin A; karcinom jeter; protirakasto; citotoksično

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Original Research Article

DETECTION OF BOVINE LEUKOCYTE ADHESION DEFICIENCY, DEFICIENCY OF URIDINE MONOPHOSPHATE SYNTHASE, AND COMPLEX VERTEBRAL MALFORMATION IN HOLSTEIN CATTLE

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Abstract: This research aimed to evaluate the prevalence of the most common lethal diseases in the Lithuanian Holstein cattle population. Two hundred non-related (based on the documentation of origin) cattle (cows and heifers) were included in the study. DNA extraction from blood leukocytes was performed using the chloroform salt method. The cattle were tested for three inherited bovine disorders: bovine leukocyte adhesion deficiency (BLAD), deficiency of uridine monophosphate synthase (DUMPS), complex vertebral malformation (CVM). The PCR-RFLP test method was used to determine the polymorphism of the *CD18* gene, which is responsible for BLAD inherited disorder development. A recessive allele with point mutation A→G (383), causing BLAD, was found in the Lithuanian cattle population with 0.0025 frequency. CVM disease is determined by the missense mutation, which has been found in the *SLC35A3* gene. The study was performed using a sequencing method. A recessive allele with point mutation G→T (538), causing CVM, was found in the Lithuanian cattle population with 0.005 frequency. The PCR-RFLP test method was used to determine the polymorphism of the *UMPS* gene, responsible for DUMPS inherited disorder development. A recessive allele with point mutation C→T (1213), causing DUMPS, was not found in the Lithuanian Holstein cattle population. Because intensive selection programmes were performed over the previous decade, the number of heritable lethal diseases carriers has significantly decreased.

Key words: cattle; bovine leukocyte adhesion deficiency; deficiency of uridine monophosphate synthase; complex vertebral malformation; BLAD; DUMPS; CVM

Introduction

Modern breeding of dairy cattle increasingly involves programmes based on the international trade of semen from elite bulls with high genetic merit. With the widespread use of advanced reproductive technologies, including artificial insemination and multiple ovulation embryo transfer, individual bulls are able to sire thousands of heifers in many countries (1). In that way, more than ten genetic diseases have spread, some of them resulting in fatal outcomes and causing significant economic losses. These include bovine leukocyte adhesion deficiency (BLAD), deficiency of uridine monophosphate synthase (DUMPS), and complex vertebral malformation (CVM) (2, 3, 4, 5, 6, 7).

With intensive livestock development, a single breed begins to dominate in separate animal species (e.g., the Holstein breed) among dairy cattle, and a small number of reproducers are used for fertilisation in such breeds. Consequently, genetic biodiversity is significantly reduced, many genes became homozygous, and abundant genetic diseases can occur. Animals carrying various genetic defects must be eliminated from the population so that the frequency of the undesired

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gene does not increase to the critical limit, at which point it becomes homozygous and manifests phenotypically. If the animal is only the carrier of unwanted genes, this gene is not phenotypically detectable, and the study can only be performed using genetic testing methods, so it is essential to analyse breeding livestock in order to prevent the spread of the undesired gene.

Known inherited disorders in cattle are mostly caused by autosomal recessive inherited genes. Heterozygous individuals can be identified using different methods, such as examination of progeny, clinical examination or necropsy, analysis of enzyme activity in blood, and genotyping of animals by genomic analysis. Recent developments within molecular genetics have made possible the efficient and rapid identification of heterozygous animals via genomic analysis. Knowing the molecular basis of a defect, the direct detection of carriers is possible at the genetic level, thus preventing the breeding of the ineligible animal (8). At present, there are identification records for several inherited bovine disorders, including. bovine leukocyte adhesion deficiency (BLAD), deficiency of uridine monophosphate synthase (DUMPS), complex vertebral malformation (CVM), bovine citrullinaemia (BC), and factor XI deficiency (FXID).

The congenital calf immune deficiency BLAD is a lethal autosomal recessive hereditary disease manifested in Holstein breed calves. The disease was diagnosed for the first time by the American scientist Marco E. Kehrli in 1990 (9). Dale E. Shuster and other researchers have identified the *CD18* gene nucleotide sequence of the healthy bovine and mutated *CD18* gene (10). The mutation of the same gene was found in humans (11) and dogs (12). Heterozygous individuals are clinically healthy, but heterozygous bulls and cows have a 25% probability of producing homozygous calves with this disease. The BLAD disease gene is present in the BTA1 bovine chromosome. Due to the mutation, the gene responsible for the synthesis of glycoprotein-beta-integrin has been altered. This results in the synthesis of inadequate beta-integrin as in the position 128 of the protein molecule, the aspartic amine acid is replaced with glycine. Calves with two mutated alleles, which in homozygote status causes bovine BLAD, often have infections in the gastrointestinal tract and respiratory tract. Calves with BLAD are born with small body weight, grow and eat poorly, and their coat is not shiny. Calves are highly prone to infections, and are often suffering from enteritis, pneumonia, diarrhoea, ulcers, laryngitis, granulation stomatitis, gingivitis, peripheral lymphadenopathy, anaemia, and, if not treated, they usually die at 2-3 months of age. From the year 2000 to 2018 in Lithuania, the frequency of mutated allele, which in homozygote status causes bovine BLAD, decreased because of the intensive selection against this allele.

The deficiency of uridine monophosphate synthase (DUMPS) is an autosomal recessive lethal Holstein breed bovine disease characterised by early embryonic mortality (13, 14, 15). UMPS (uridine monophosphate synthase) is an enzyme that is essential in the synthesis of pyrimidine nucleotides. It is indispensable for the normal growth and development of ruminants and other species of animals. The inactivation of this enzyme is caused by a point mutation in the *UMPS* gene (C → T), 405 codon of the 5 exon. This Holstein cattle disorder is characterised by a decrease of UMPS enzyme activity in the blood (16). DUMPS causes foetal death in the early stages of pregnancy and many reproductive problems in dairy herds. The gene responsible for lack of the enzyme uridine monophosphate is found on chromosome 1 of bovine animals. In mammalian cells, the final step of the pyrimidine nucleotide synthesis comprises the conversion of orotate to urinary monophosphate and is catalysed by the UMP synthase enzyme. UMP synthase is essential for the synthesis of novel pyrimidine nucleotides, which are components of DNA and RNA. Since pyrimidines are essential for nucleic acid synthesis during embryonic development, embryos that are homozygous for a recessive allele die up to the 40th days of gestation. Embryos are often absorbed during the first two months of pregnancy, and these cows return to oestrus. This leads to increased intervals between calving (13, 14, 15). Heterozygous animals are phenotypically normal, but only half of normal UMPS enzyme activity occurs, which causes an increase in the amount of orotic acid in their milk and urine (16). To date, no live animals have been found that are homozygous for the mutated allele (15).

Complex Vertebral Malformation (CVM) is an autosomal recessive Holstein cattle disease inherited as severe spinal degeneration. The syndrome was first identified in the year 2000 in the Danish Holstein population (3, 17, 18). The

disease was also found in the Wagyu breed. The first ancestor of the cattle that had this mutation was a bull named Carlin-M Ivanhoe Bell (19). Severe Vertebral Malformation (CVM) is determined with both aborted foetuses and prematurely born dead calves. Affected calves have anomalies in the spinal column, such as not fully developed, broken, or unusually formed spinal vertebrae and ribs, scoliosis, and spinal synostosis. Low body weight and heart abnormalities are also observed (20). Such calves have shorter spine and chest areas, symmetrical contractions of meta-tarso-phalange joints on both sides and symmetrical arthrogryposis (17). Severe Vertebral Malformation (CVM) is caused by a mutation in the BTA3 chromosome, which replaces the amino acid sequence; instead of valine, phenylalanine is formed, in the 180 position of the protein uridine 5'-diphosphate-N-acetylglucosamine (21). This gene is responsible for the transportation of UDP-N-acetylglucosamine into the Golgi apparatus membrane; 80% of embryos that have inherited a gene mutation of CVM disease from both parents will be lost during the first three months of gestation. An aborted sick calve will have a shortened neck due to spinal cord injury, as well as altered ribs, limbs and interdigital joints. In addition, symptoms such as partial pulmonary hypoplasia, excessive liver segmentation, double gall bladder, as well as rectum and uterine atresia may occur (22). Heterozygous animals are carriers of the mutated gene.

In animal breeding, genetic disorders are one of the most imperative issues for breeders. Due to the negative influence of such disorders on animals, through abnormal anatomy or reduced production, breeders and breeding associations need to control the impact on the population.

Material and methods

The animals for this study were selected from dairy herds, in which breeding record and cattle productivity control were carried out. The farms were located in four different regions of Lithuania. In each dairy herd, 50 non-related (based on the documentation of origin) cattle (cows and heifers) were selected. In total, 200 dairy cattle were tested. They were healthy, and kept and fed according to hygiene norms; the conditions for keeping and feeding were in line with veterinary requirements.

Blood for genetic testing was collected in aseptic conditions from the cephalic vein in vacuum tubes (Vacutainer) with K_2EDTA (ethylenediamine tetraacetate) anticoagulant. The blood was stored at +4 °C until the test. DNA extraction from blood leukocytes was performed using the chloroform salt method, according to Miller et al. (23). Genomic DNA content and purity were determined using the spectrophotometric method (DNA/RNA Reader, Pharmacia).

BLAD genetic disease PCR-RFLP test method (10)

For the identification of mutation c.383A>G (g.145114963A>G, rs445709131) in the *CD18* gene, causing a change of adenine to guanine in the 383 position of the gene and manifesting as BLAD in cattle, the PCR-RFLP test method was used. We targeted a 357 bp long fragment of the fifth exon of the gene of the BLAD disease locus (Table 1).

10µl of DNA and 15µl of PCR mixture were poured into the tube. The PCR reaction was performed using a thermocycler (G-storm, United Kingdom). Reagents used: PCR Mix - 2.95µl ddH_2O; 2.5µl 10xPCR buffer; 2.5µl of dNTP (2 mM); 1.5µl of $MgCl_2$; (50 mM); 2.5µl of the forward primer (20 pmol); 2.5µl of the reverse primer (20 pmol); 0.25ml BSA; 0.3µl *Taq* polymerase (Thermo Scientific, Lithuania); in two places in the same column 10µl of the PCR product was digested with 10µl of the restriction mix (7.5µl ddH_2O, 2µl 10 × buf., 0.5µl *TaqI*). Samples were left in a thermostate for 1 hour at 65 °C. The digested PCR products were fractionated using electrophoresis in 2.5% agarose gel, 100 V 40 min. The gel was stained with ethidium bromide for 15-20 minutes and analysed in UV light (wavelength 300 nm) with MiniBisPro Video Documentation (Herolab) (Table2).

DUMPS genetic disease PCR-RFLP test method

Testing method for nonsense (stop-gain) mutation c.1213 C>T in *UMPS* gene (g.69756880C>T) causing a change of cytosine to thymine in the 405 codon of the gene of the fifth exon and manifesting as DUMPS in cattle (Table 3).

10µl of DNA and 15µl of PCR mixture were poured into the tube. PCR reaction was performed

Table 1: Primers, PCR profile, PCR product size and restriction enzymes used for identification of genetic disease - BLAD

Genetic defect	Primers	PCR profile			PCR product size	Restriction enzyme
BLAD	F: 5‘ GAATAGGCATCCTGCATCATATCCACCA 3‘ R: 5‘ CTTGGGGTTTCAGGGGAAGATGGAGTAG 3‘	94°C	3 min		357 bp	*TaqI*
		94°C	30 s	33 cycles		
		65°C	30 s			
		72°C	30 s			
		72°C	5 min			

Table 2: *CD18* gene c.383A>G DNA fragments sizes in bp after digestion with restriction endonuclease

Bp	Homozygous for normal allele	Heterozygous	Homozygous for disease allele
357	—	—	
201		—	—
156		—	—

Table 3: Primers, PCR profile, PCR product size and restriction enzyme used for identification of genetic disease – DUMPS

Genetic defect	Primers	PCR profile			PCR product size	Restriction enzyme
DUMPS	F: 5‘ GCAAATGGCTGAAGAACATTCTG 3‘ R: 5‘ GCTTCTAACTGAACTCCTCGAGT 3‘	94°C	5 min		108 bp	*AvaI*
		94°C	60 s	40 cycles		
		58°C	60 s			
		72°C	90 s			
		72°C	5 min			

Table 4: *UMPS* gene c.1213 C>T DNA fragments sizes in bp of cattle DUMPS causing gene after digestion with restriction endonuclease

Bp	Homozygous for normal allele	Heterozygous	Homozygous for disease allele
89	—	—	
53		—	—
36		—	—
19	—	—	—

Table 5: Prevalence of BLAD, DUMPS and CVM diseases in Lithuanian Holstein population

Genetic disease	Gene	Genotype frequency			Allele frequency	
		Homozygous for normal allele	Heterozygous	Homozygous for disease allele	Normal	Mutated
BLAD	*CD18*	0.995	0.005	0	0.9975	0.0025
CVM	*SLC35A3*	0.99	0.01	0	0.995	0.005
DUMPS	*UMPS*	1	0	0	1	0

using a thermocycler (G-storm, United Kingdom). Reagents used: PCR Mix - 2.95ml ddH_2O; 2.5ml 10xPCR buffer; 2.5ml of dNTP (2 mM); 1.5ml of $MgCl_2$; (50 mM); 2.5ml of the forward primer (20 pmol); 2.5ml reverse primer (20 pmol); 0.25ml BSA; 0.3ml *Taq* polymerase (Thermo Scientific, Lithuania). 10µl of the PCR product was digested with 10µl of the restriction mix (7.5µl ddH_2O, 2µl 10 × buf., 0.5µl *AvaI*). Samples were left in a thermostat for 10 hours at 37 °C. The digested PCR products were fractionated using electrophoresis on 4% agarose gel, 100 V 40 min. The gel was stained with ethidium bromide for 15-20 minutes and analysed in UV light (wavelength 300 nm) using MiniBisPro Video Documentation (Herolab) (Table 4).

CVM genetic disease test method

Investigations of polymorphism analysis of the gene for CVM disease in cattle was performed at the Neogen Genomics Corporation Laboratory (USA). The study was performed using a sequencing method. CVM disease is determined by the missense mutation, present in the *SLC35A3* gene. In the third cattle chromosome, a modified *SLC35A3* gene with a single base conversion c.538G>T (g.43412427G>T, rs438228855) was detected.

Results

We tested cows for lethal autosomal recessive disorders widely distributed in the Lithuanian Holstein cattle population: bovine leukocyte adhesion deficiency (BLAD), deficiency of uridine monophosphate synthesis (DUMPS) and vertebral malformation (CVM). A recessive allele with point mutation c.383A>G, causing BLAD, was found in the Lithuanian cattle population with 0.0025 frequency; 0.5% of individuals were heterozygous. The recessive allele with point mutation c.1213 C>T, causing DUMPS, was not found in the Lithuanian cattle population. A recessive allele with point mutation c.538G>T, causing CVM disease, was found in the Lithuanian cattle population with 0.005 frequency; 1.0% of individuals were heterozygous (Table 5).

In the year 2000, the percentage of Lithuanian dairy cattle population who were heterozygote animals, BLAD disease gene carriers, was 5%; this percentage was 4% in 2002, and 2% in 2004. In 2018, we found 0.5% mutated allele carriers and distributors of disease genes in the population (Figure 1).

Discussion

Genomic selection is carried out not only to improve the genetic potential of cattle productivity, reproduction, and milk quality but also by assessing the signs of bovine health. In the dairy cattle population, due to the international breeding of males with high genetic value, despite also being the carriers of recessive genetic disease alleles, genetic diversity has been significantly reduced. As a result, many genes have become homozygous, producing abundant genetic diseases among the dairy cattle population, which causes significant economic losses, as many of them are lethal. After a retrospective assessment of the prevalence of the lethal genetic diseases of BLAD, DUMPS, and CVM in the dairy cattle population, the following was established.

CVM and BLAD have become some of the most common hereditary genetic defects of the Holstein breed in recent decades. Researchers found that in the year 2000 the mutant allele for BLAD disease frequency was 24%, and the rate of mutated alleles for CVM in the German Holstein population from 2001 to 2007 ranged from 9% up to 16% (6).

The mutated allele causing BLAD, c.383A>G, was found at a frequency of 0.0025% in the Lithuanian dairy cattle population; with 0.5 % of cattle being heterozygous while no homozygous animals with BLAD were found. Since 1999, all sires and selected cows used for breeding in Lithuania have the genotype for this disease recorded in the pedigree information. In Lithuania in the year 2000, 6.7% of key areas of the altered gene leading to BLAD disease were found among selected cows. Of the 146 bulls tested, 4 carriers were found. In 2002, 3% of selected cows were carriers, and in 2004, 2% of selected cows and 1 young bull were carriers. All breeding bulls and selected cows that are newly introduced among breeding animals are compulsorily tested for BLAD disorder. A similar mutated allele frequency as in our study was reported in Czech cattle: 0.82% (24), and in Chinese Holsteins (0.69%; 25). However, a much larger mutated allele frequency was reported for Indian Holstein Friesian cattle (2.99%) (26),

Iranian Holsteins (3.3%) (27), American Holsteins (8.2%), Polish Holsteins-Friesian (7.9%) (28) and Turkish Holstein cows (4.0%) (29).

The mutated allele causing the DUMPS genetic disorder in cattle (c.1213 C>T), manifested as early mortality of cattle embryos due to the shortage of uridine monophosphate synthase, was not found in the Lithuanian population. No carrier animals of the DUMPS genetic disorder were found in Turkey (2, 29, 30). Similar results were obtained in the research done by other scientists in Poland (13), the Czech Republic (24), Germany, India, Iran and Romania. However, the mutant DUMPS disease allele with a 1–2% frequency was found in the Holstein breed in the USA, 0.96% in Argentine Holstein bulls, and 0.06% in Chinese Holsteins.

The mutated allele causing CVM cattle disease (c.538G>T), manifested in malformations of the foetal spine, was found in the population of Lithuanian dairy animals at the frequency 0.005, while the number of heterozygous bovines was found to be 1%, and no homozygous bovine affected by CVM was found. In Turkey, the frequency of Holstein bovine CVM mutated allele carriers was 3.4% and 3.86% in the Chinese dairy population. A high frequency of mutated allele was found in Denmark with 31.0% of all cattle affected (21), in Poland at 24.8% (31), in Japan at 32.5% (32), in Sweden at 23.0% (33), in Germany, at 13.2% (34), and in China 15% (19). Since the year 2000, breeding programmes in most counties have been implemented to reduce the prevalence of CVM carriers. However, in some Holstein populations, the incidence of CVM disorder carriers remains high (Denmark, Poland, Japan). A study of Iranian Holstein cattle did not identify heterozygous bovine animals (18). This can be associated with the use of a small number of bulls carrying the mutated allele for breeding, during the formation of the breed, and further selection.

In the Lithuanian dairy cattle population, lethal bovine diseases caused by recessive genes in the population were reduced because of an intense selection programme, eliminating the BLAD disease gene carriers and avoiding the use of BLAD, DUMPS, and CVM heterozygous bulls for breeding. The number of mutated allele carriers dropped from 6% in 2000 to 0.5% in 2018.

The incidence of hereditary diseases causes not only direct economic losses to livestock breeders but also leads to reductions in the genetic diversity of animal populations as a result of extensive culling of disease carriers. The identification of carriers with the use of molecular diagnostic tests is an important step in reducing the frequency of detrimental alleles and consequently lowering the incidence of hereditary diseases in the herd. This is particularly important when considering bulls inserted into progeny testing programmes because they can potentially sire thousands of progeny before the incidence of affected progeny can be associated with a particular animal.

Conclusions

An investigation of cows for the prevalence of the lethal genetic disorders BLAD, DUMPS, and CVM in the Lithuanian dairy cattle population showed 0.5% BLAD disease gene carriers and 1.0% CVM disease gene carriers. No DUMPS disease gene carriers have been found. In the Lithuanian Holstein cattle population, the number of carriers of heritable lethal diseases caused by recessive genes decreased, because of an intensive selection programme, eliminating mutated gene carriers, and avoiding the use of heterozygous bulls for the diseases BLAD, DUMPS, and CVM.

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UGOTAVLJANJE POMANJKLJIVE ADHEZIJE GOVEJIH LEVKOCITOV, POMANJKANJA ENCIMA URIDIN MONOPOSFAT SINTETAZE TER KOMPLEKSA MALFORMACIJE VRETENC PRI HOLŠTAJNSKEM GOVEDU

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Povzetek: Namen raziskave je bil oceniti razširjenost najpogostejših smrtnih bolezni v populaciji litvanskega holštajnskega goveda. V študijo je bilo vključenih dvesto nesorodnih (na podlagi dokumentacije o poreklu) krav in telic. Izolacijo DNK iz krvnih levkocitov smo izvedli z metodo izolacije s soljo in kloroformom. V vzorcih DNK krav in telic smo preverili prisotnost mutacij v treh genih, ki povzročajo naslednje bolezni: motnjo prilepljanja govejih levkocitov (BLAD), pomanjkanje encima uridin monofosfat sintaze (DUMPS) ter kompleksno malformacije vretenc(CVM). Metoda PCR-RFLP je bila uporabljena za določanje polimorfizma gena *CD18*, ki je odgovoren za razvoj dedne bolezni BLAD. Recesivni alel s točkovno mutacijo A → G (383), ki povzroča bolezen BLAD, je bil ugotovljen v populaciji litvanskega goveda s frekvenco pojavljanja 0,0025. Bolezen CVM je povzročena z drugačno-pomensko mutacijo v genu *SLC35A3*. Prisotnost mutacije v tem genu smo izvedli z metodo sekvenciranja. Recesivni alel s točkovno mutacijo G → T (538), ki povzroča CVM, je bil ugotovljen v populaciji litvanskega goveda s frekvenco pojavljanja 0,005. Testna metoda PCR-RFLP je bila uporabljena za določanje polimorfizma gena *UMPS*, ki je odgovoren za razvoj dedne bolezni DUMPS. Recesivni alel s točkovno mutacijo C→ T (1213), ki povzroča DUMPS, ni bil najden v litvanski populaciji holštajnskega goveda. Ker so se v preteklem desetletju izvajali intenzivni selekcijski programi, se je število prenašalcev dednih smrtnih bolezni znatno zmanjšalo.

Ključne besede: govedo; moteno prilepljanje govejih levkocitov; pomanjkanje encima uridin monofosfat sintaze; kompleksna malformacije vretenc; BLAD; DUMPS; CVM

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Case Report

Hafnia paralvei ISOLATED FROM AN EMPHYSEMATOUS PYOMETRA IN A BITCH

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Abstract: We report the case of a 9-year-old female Labrador retriever, presented to the Veterinary Teaching Hospital with a history of bloody/purulent and malodourous vulvar discharge, decreased appetite and progressive abdominal enlargement for about 20 days. Clinical examination showed a distended and painful abdomen and tympanic sounds on percussion. The patient also presented systemic inflammatory response syndrome (SIRS) and a leukemoid reaction with leukocyte count of 77.9 x 10^9/L due to 62.32 x 10^9/L lymphocytes. The abdominal radiography showed distended tubular structures occupying most of the abdomen; dorsal displacement of the colon in the left-right lateral projection was also found. The ultrasonographic study also revealed tubular structures in the mid-abdomen that contained flowing fluid presenting mixed echogenicity and hyperechoic particles in suspension. The exploratory celiotomy showed that the uterine horns were distended and contained a mixture of gas and liquid, hence an ovariohysterectomy was performed. The uterine fluid was collected with aseptic technique into a syringe and submitted to the clinical pathology laboratory for aerobic and anaerobic culture and antibiotic sensitivity. *Hafnia alvei* (now classified as *Hafnia paralvei*) was identified as the causative bacterial agent. Pyometra caused by gas-producing bacteria is a rare condition and is known as emphysematous pyometra. There are only six reports in the literature of this condition in bitches. A wide range of vaginal bacteria has been found in dogs with pyometra, but to our knowledge this is the first report of emphysematous pyometra caused by *Hafnia paralvei* in dogs.

Key words: veterinary surgery; uterus; emphysematous pyometra; *Hafnia paralvei*

Introduction

Pyometra is a common condition that affects intact adult bitches in dioestrus (1). It is a local infection, but is often accompanied by sepsis and/or systemic inflammatory response syndrome (SIRS) (2). Open cervix pyometra is most common, but the closed cervix pyometra have a poorer prognosis (3), and affects about 25% of old nulliparous bitches (4).

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Emphysematous pyometra is a rare entity, and has been described in only 6 reports (5-10).

Although the most commonly isolated bacteria in dogs with pyometra is *E. coli*, several other bacteria found in the normal vaginal flora have been reported in canine pyometra (11-13).

Hafnia paralvei (formerly *Hafnia alvei*) is a gram-negative, mobile, rod-shaped facultative anaerobe. It is part of the normal microbiota of animals (14). In humans, it behaves as an opportunistic pathogen (15), but it is a rare cause of infection in humans and animals (15-17). However, in veterinary medicine, it has been

reported in equine abortion (18), as a secondary infection in a cat with a nasal tumor (19), and was also isolated from the wound of a dog (20).

In the past, the genus consisted of a single species, *Hafnia alvei*. However, in 2010, organisms previously called *Hafnia alvei* HG 2 were categorized as a novel species, *Hafnia paralvei* (21).

The objetive of this study is to present a rare case of an emphysematous pyometra in a bitch, in which was isolated *Hafnia paralvei.*

Case

A 9-year-old Labrador retriever nulliparous bitch was submitted to our hospital for evaluation. The animal had a history of vaginal bleeding and a purulent and foul-smelling vulvar discharge. The owner reported that the patient had been hyporexic and that the abdomen had progressively increased in size. The animal weighed 25.36 kg and the last heat had occurred 2 months before.

The clinical evaluation revealed a heart rate of 120 beats per minute, respiratory rate of 40 breaths per minute, and a dehydration rate of 7%, with pale mucous membranes, delayed capillary refill time (>3 seconds) and a temperature of 40°C. Moreover, the patient showed abdominal pain on palpation and tympanic sound on percussion. In addition, the bloody-purulent vulvar discharge was confirmed.

The abdominal left-right lateral and ventro-dorsal projection showed distended tubular structures occupying most of the abdomen (Figure 1). The presence of coprolites was confirmed in both projections.

The abdominal ultrasonography showed tubular structures in the mid-abdomen presenting mixed echogenicity; a flow of hyperechoic particles in suspension and reverberation lines superimposed on the image of mixed echogenicity were also found.

The complete blood count (CBC) findings showed a decrease in hemoglobin values 7.1 g/dl (range 12-18 g/dl), hematocrit 0.204 L/L (range 0.37-0.55 L/L), erythrocytes 3.53 x 10^{12}/L (range 5.5-8.5 x 10^{12}/L) and platelets 160 x 10^{9}/L (range 175-500 x 10^{9}/L). The mean corpuscular hemoglobin (MCH) 34.9 g/dl (range 31-37 g/dl) and mean corpuscular volume (MCV) 57.9 FL (range 60-72 FL) were within normal ranges. The patient also presented a highly elevated leukocyte count of 77.9 x 10^{9}/L (range 5.5-16.9 x 10^{9}/l), with 62.32 x 10^{9}/L lymphocytes (ranges 1-4.9 x 10^{9}/L).

Based on the imaging and laboratory findings we conducted an exploratory celiotomy with a presumptive diagnosis of emphysematous pyometra. The preanaesthetic medication protocol was an intravenous dose of the commercial combination of tiletamine/zolazepam (5 mg/kg) (Zelatol, Fort Dodge, Spain) plus tramadol (3 mg/kg, IV) (Tramadol Jet, Norvet, Mexico) for analgesia. For the induction of anaesthesia an intravenous dose (4 mg/kg) of propofol (Fresofol, Fresenius Kabi, Austria) was used. The inhalant anaesthesia was maintained with 2.5% inspired fraction of isoflurane.

Exploratory celiotomy confirmed the presence of distended uterine horns containing gas and fluid (Figure 2), therefore ovariohysterectomy was performed. Further pre- and postoperative medication included cefazolin (25 mg/kg, IV) (Cefazolin, Apotex Corp, USA), q12 h, gentamicine (6 mg/kg, IV, q 24 h) (Genta Ved, Vedco, USA), metronidazole (15 mg/kg, IV, q 12 h) (Flagyl, Sanofi, Mexico), tramadol (3 mg/kg, IV, q 12 h) and carprofen (2 mg/kg, PO, q 24 h for 7 days) (Novox, Vedco, USA).

Uterine and ovarian tissue samples were submitted to the histopathology laboratory. The histopathological report revealed cystic endometrial hyperplasia and suppurative metritis/endometriosis**.** Fluid from the uterus was collected via aseptic technique into a syringe, air was removed and the syringe was sealed. The sterile sample was sent to the clinical pathology laboratory for aerobic and anaerobic culture and antibiotic sensitivity. *Hafnia paralvei* was recovered in *pure culture* of the uterine fluid.

Two days after surgery, the patient was discharged with oral cephalexin (22 mg/kg, PO, q 12 h) (Ceporex, Glaxo Smithkline, Mexico) metronidazole (15 mg/kg, IV, q 12 h), and tramadol (5 mg/kg, PO, q 12 h).

The patient recovered completely within a week. Laboratory control tests were taken, being all the biochemical and haematological results within the normal range.

Discussion

Canine pyometra is a common disease of intact bitches; with previous reviews indicating that approximately 75% of bitches with pyometra are nulliparous. A delay in onset of treatment of this condition may result in toxaemia, septicaemia

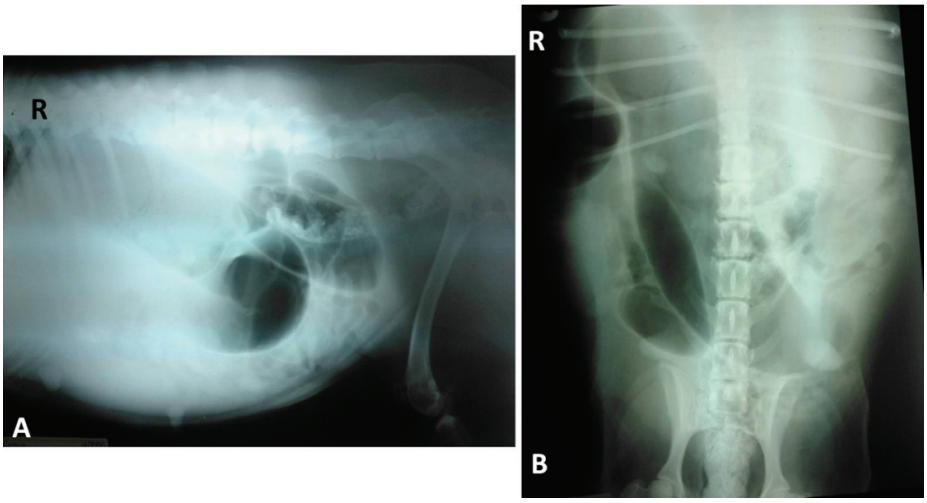

Figure 1: A) Left-right lateral and B) Dorsal-ventral view of the abdomen of a 9-year-old Labrador retriever bitch with emphysematous pyometra. Notice the large tubular structure filled with gas displaced to the ventral side, the presence of coprolites, and complete and incomplete spondylosis deformans from T-10 to L-4

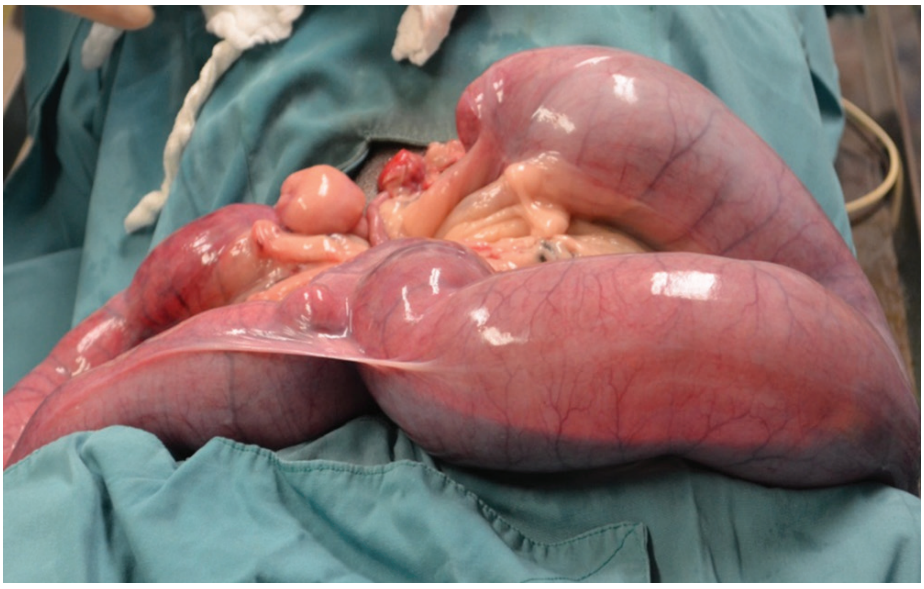

Figure 2: A 9 year old Labrador Retriever bitch with emphysematous pyometra. Notice the uterus filled with gas and liquid on the inside

and even death of the patient. This condition is more common in older bitches (average age of 7.25 years) (22). Pyometra usually occurs during dioestrus, except when the infection progresses slowly, and dioestrus ends before the diagnosis is confirmed (23). The history and clinical findings in our case matches those commonly described in canine pyometra: age, breed, nulliparity and the resumption of oestrus 2 months prior to the onset of the disease.

The physical examination and laboratory results suggested SIRS in this dog, which has previously been described in 57% of bitches with pyometra, causing the production and release of inflammatory mediators (2). Patients with SIRS can be identified when presenting at least two of the following four criteria: heart rate greater than 160 beats per minute, temperature greater than 39.7 °C or less than 37.7 °C, respiratory rate over20 breaths per minute or a carbon dioxide pressure lower than 32 mm/Hg, and a WBC count over 12.000/µl or below 4,000/µl or more than 10% of band neutrophils (24). Our patient matched three of these parameters: temperature of 40°C, 40 breaths per minute and a leukocytosis of 77,000/µl. This markedly high leukocyte count is known as leukemoid reaction; this condition has been described in canine pyometra. In the same form that another inflammatory conditions, pyometra stimulating the formation of cytokines

such granulocyte colony stimulating factor and granulocyte/macrophage colony stimulanting factor. This cytokines, stimulating granulopoiesis because promote release of neutophils in the bone marrow (25). In acute cases, a myeloid leukemoid reaction with neutrophilia and toxic neutrophils has been described (26), although this was not the scenario found in our case. In the present case, the bitch presented an uncommon lymphoid leukemoid reaction.

Although the most common bacterial agent associated with pyometra in bitches is *E. coli*, several aerobic and anaerobic bacteria have been reported to be involved (10-13). Pyometra caused by gas-producing bacteria is a very rare condition and is also known as emphysematous pyometra. This condition has only been described six times in the literature (5-10). Therefore, this is the first report of an emphysematous pyometra caused by *Hafnia paralvei*, a gram-negative, mobile, facultative aerobe bacillus capable of producing gas (27). Other studies have isolated different vaginal bacteria in healthy dogs, but in none of them *Hafnia paralvei* was identified (28, 29). It is the only species of *Hafnia* in the *Enterobacteriaceae* family. This bacterium is widely distributed in water, soil and food and is also part of the normal intestinal microbiota of mammals, marsupials, birds, reptiles, fish, invertebrates and insects (15, 30, 31). In humans, it is described as an unusual opportunistic pathogen causing nosocomial (18) or community infections (32, 33). This bacterium is not a common pathogen in animals (15), although it has been isolated in some cases (18-20, 34), but never has been isolated from a pyometra.

In the majority of bitches that develop pyometra, vaginal contamination is the source of infection (35). However, a previous report showed a strong correlation between the normal canine intestinal bacteria and those found in the uterus of dogs with pyometra (36). Although we did not perform faecal culture in this animal, we suspect that the bacteria arrived to the uterus via ascendent faecal contamination, as previously described for other causative agents of pyometra (37).

We conclude that, even though emphysematous pyometra is uncommon in dogs, it should be regarded as a presumptive diagnosis in patients presenting with tubular structures filled with gas in abdominal radiographs, especially in bitches with vulvar discharge presented during dioestrus. Finally, in the present clinical report we describe for the first time a case of canine emphysematous pyometra caused by *Hafnia paralvei.*

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Hafnia paralvei IZOLIRANA IZ EMFIZEMAZOTNE PIOMETRE PRI PSICI

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Povzetek: V članku poročamo o primeru 9-letne samice pasme labradorec, ki je bil sprejet v Bolnišnici za veterinarsko medicino z anamnezo gnojnih in smrdljivih izločkov iz vulve, zmanjšanim apetitom in postopnim povečevanjem trebuha, kar je trajalo približno 20 dni pred obiskom veterinarja. Klinični pregled je pokazal napihnjen in boleč trebuh in timpanične zvoke ob pretrkavanju. Pacientka je imela tudi sindrom sistemskega vnetnega odziva (SIRS) in povečano skupno število levkocitov (77.9 x 10^9/L) zaradi povišanega števila limfocitov (62.32 x 10^9/L). Rentgenska slika trebuha je pokazala razširjene cevaste strukture, ki so zasedale večino trebuha; na stranski projekciji (levo-desno) pa so ugotovili tudi dorzalni premik debelega črevesa. Ultrazvočni pregled je prav tako pokazal cevaste strukture v sredini trebuha, ki so vsebovale tekočino mešane ehogenosti s hiperehoičnimi delci v suspenziji. Preiskovalna celiotomija je pokazala, da so bili rogovi maternice raztegnjeni in so vsebovali mešanico plina in tekočine, zato je bila izvedena ovariohisterektomija. Tekočino iz maternice so z aseptično tehniko zbrali v injekcijsko brizgo in jo predali kliničnemu patološkemu laboratoriju za ugotavljanje prisotnosti aerobnih in anaerobnih bakterij ter bakterijske občutljivosti na antibiotike. *Hafnio alvei* (sedaj razvrščeno kot *Hafnia paralvei*) so opredelili kot bakterijskega povzročitelja piometre. Piometra, ki jo povzročajo bakterije, ki proizvajajo plin, je redko stanje in je znana kot emfizematozna piometra. V literaturi je opisanih le šest primerov pri psicah. Pri psicah s piometro so našli širok spekter vaginalnih bakterij, vendar je to prvo poročilo o emfizematozni piometri, ki jo povzroča *Hafnia paralvei* pri psicah.

Ključne besede: veterinary surgery; uterus; emphysematous pyometra; *Hafnia paralvei*

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