High levels of ochratoxin A in blood serum and kidneys of wild boars Sus scrofa in Poland

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Source: Wildlife Biology, 18(3) : 272-279

Published By: Nordic Board for Wildlife Research

URL: https://doi.org/10.2981/11-059

Wildl. Biol. 18: 272-279 (2012)
DOI: 10.2981/11-059

www.wildlifebiology.com

Original Article

High levels of ochratoxin A in blood serum and kidneys of wild boars *Sus scrofa* in Poland

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The aim of our study was to evaluate the ochratoxin A (OTA) concentration in the blood serum and kidneys of wild boars *Sus scrofa* in two consecutive years. We took samples from wild boars hunted in five regions of northwestern Poland during November and December 2006 (N = 39) and throughout 2007 (N = 62). The body weight of the animals ranged from 35 to 100 kg. As a control, we used 20 pigs *Sus scrofa domestica* of an average body weight of 100 kg. We extracted the OTA and then purified it on immunoaffinity columns. The amount of OTA was determined using HPLC-FLD. The OTA concentration varied among individual animals, some of which had extremely high levels in their blood serum (1,170 ng/ml) and kidneys (97 ng/g). The 2006 average OTA concentration in the serum was similar to the average found in 2007 (6.15 ng/ml and 5.91 ng/ml, respectively). In 2006, the concentration of OTA in the serum of wild boars was > 3 times higher than the concentration found in the serum of pigs. We detected a higher level of OTA in the kidneys of wild boars in both 2006 (1.77 ng/g) and 2007 (2.34 ng/g) than the levels present in the kidneys of pigs (0.59 ng/g). In conclusion, the content of OTA in the serum and kidneys of wild boars changed with year and region. The OTA levels in wild boars from certain regions were much higher than in other regions, and such high levels may cause nephropathy in wild boars and thus pose a possible threat to consumers.

Key words: blood serum, kidney, ochratoxin A, Sus scrofa, wild boars

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Received 12 July 2011, accepted 24 February 2012

Associate Editor: Bjørn Olav Rosseland

Being the most frequently hunted species in Poland, the wild boar *Sus scrofa* constitutes the basis of the Polish hunting economy. Originally, their habitat was the forest only. However, they are increasingly found in fields, creating additional difficulties in the management of hunting areas. Oloff (1951) described the influence of climate, feed and reproduction on the population growth of wild boars > 60 years ago. In 1995 the number of boars in Poland was estimated to be ca 80,000 animals. However, a more recent count revealed that in 2009 their number had reached 225,000. Similar increases in wild boar populations have been reported throughout central Europe (Gethöffer et al. 2007, Happ 2007, Fonseca et al. 2011).

To compensate for the insufficiency of natural feed for wild boars, Polish hunters carry out intensive artificial (or supplementary) feeding, all year round, using mouldy by-products of low quality from grain and milling industries. This type of feed can be highly contaminated with mycotoxins, in particular ochratoxin A (OTA;Visconti & Botallico 1983). OTA is one of the most common and toxicologically important naturally occurring mycotoxins produced by *Aspergillus* and *Penicillium* species. OTA is widely distributed throughout Europe and North America and has been found as a natural contaminant in agricultural commodities, especially in cereals (Kuiper-Goodman & Scott 1989) through which it can be transmitted to animals and humans (Gareis 1996). OTA is highly

nephrotoxic, causing both acute and chronic disease of the kidneys. It is also suspected to be involved in the etiology of Balkan endemic nephropathy, a disease characterised by progressive renal fibrosis in humans, and has been implicated in the development of urinary tract tumours (Krogh 1992, Marquardt & Frohlich 1992, Stoev et al. 2002, Aslam et al. 2005, Grajewski et al. 2007). Additionally, OTA has been classified as Group 2B by the International Agency for Research on Cancer (IARC 1993) due to its carcinogenicity. The significant increase in the wild boar population has resulted in an increased prevalence of wild boar meat, offal and ready-made products in the food industry. This may pose a threat to consumers as neither the extent of OTA contamination in the tissues and organs of wild boars nor the sensitivity of wild boars to OTA have been studied.

Pigs *Sus scrofa domestica* are particularly sensitive to OTA, and several studies have examined the concentration of OTA in the blood serum and kidneys of pigs (Lusky et al. 1995, Dragacci et al. 1999, Kotowski et al. 2000, Curtui et al. 2001, Jørgensen & Petersen 2002, Matrella et al. 2006). This mycotoxin plays a key role in the genesis of swine mycotoxic nephropathy, a common disease in Scandinavia (Krogh 1992). The bioavailability of OTA in monogastric species is high, with 40-60% of the orally ingested toxin being absorbed by the gastrointestinal tract (Blank et al. 1999). OTA accumulates in the blood and edible organs, especially in the kidneys (Böhm 1988, Gareis & Scheuer 2000). The elimination of OTA via urine and bile is relatively slow due to its high binding affinity with serum albumin (Hagelberg et al. 1989, Fuchs & Hult 1992, Il'ichev et al. 2002, Grajewski et al. 2008). In fact, animal tissue and animal-derived products for human consumption, particularly kidneys and organs containing blood (Jørgensen & Petersen 2002), may contain OTA residue, even though the levels of OTA in the animal feed is low.

The aim of our study was to survey the OTA concentration in the blood serum and kidneys from 101 wild boars hunted in their natural habitat (i.e. in five forest districts). Wild boar hunting was carried out in all areas between October and November mainly by a hunting drive with dogs. We compared the level of OTA in the blood serum and kidneys of wild boars with the content of toxin in the blood serum and kidneys of 20 domestic pigs.

Material and methods

Material and samples preparation

We took samples of the blood and kidneys from wild boars hunted in five regions in the western Kujawsko-Pomorskie province, Poland (Fig. 1) during

Figure 1. The Kujawsko-Pomorskie province in Poland marked with the locations of sample sites, i.e. 1) Strzelno, 2) Mogilno/Znin, 3) Gniezno/Witkowo, 4) Mrocza/Wiecbork, 5) Kcynia/Naklo, Cl-control Mogilno/Znin and C2-control Mrocza/Wiecbork.

November and December of 2006 (N = 39) and throughout 2007 (N = 62). We had selected these regions due to the large number of wild boars being shot there. In order to decrease damage to crops caused by wild boars, the hunters regularly carry out artificial feeding. We harvested blood and kidneys immediately after the shooting of the animal. We took blood directly from the heart and tapped it into two serum-separator tubes (SSTs) to clot the blood. We removed whole kidneys from each wild boar and subjected them to homogenisation. We stored all samples (blood serum and kidney homogenates) at -20°C for further analysis. We carried out determinations within a month after the sampling date.

As a control, we took samples from pigs of an average body weight of 100 kg (N=20) slaughtered in December 2006 at two slaughterhouses within the same region (i.e. Mogilno/Znin and Mrocza/Wiecbork). Blood samples were tapped into two SSTs during the slaughter and left at ambient temperature for clotting. We homogenised whole kidneys taken from each pig. All samples (i.e. of blood serum and kidney homogenates) were stored at -20°C for further analysis.

The body weight of the wild boars ranged from 35 to 100 kg. The carcasses were weighed at purchase centres. The wild boars were divided into two weight categories (> and < 50 kg) as weaners eat the same feed all the time, whereas older animals will frequently change their nutritional habitats (Oloff 1951, Spiesiwcewa 1964).

We obtained the OTA standard from Biopure (Tulln, Austria). All other chemical reagents used were of analytical grade. All solvents used were HPLC grade. Water was purified by the Simplicity 185 system from Millipore (Molshein, France).

Extraction of OTA

We extracted the OTA from the serum by first adding 5 ml of a 1:1 mixture of 0.2 M magnesium chloride and 0.1 M hydrochloric acid and 3 ml of chloroform to 1 ml of serum. The flask was shaken for 30 minutes at 200 rpm and then centrifuged at 2,500 rpm for 20 minutes at 10°C. The water phase was removed and the organic phase was dried at 40°C using nitrogen. The dry extract was dissolved in 250 µl of methanol and placed in an ultrasonic bath for two minutes. Then 2 ml of phosphate buffered saline (PBS) was added to the extract. The whole extract was applied to an OCHRAPREP® (R-Biopharm Rhone Ltd., Glasgow, UK) column. Next, the column was washed, using 3 ml of PBS and 20 ml of water, and dried by passing air through the column. For the elution of OTA, 1 ml of methanol:acetic acid (98:2) was used followed by 1 ml of water. The extract was collected in a sample vial and mixed by vortexing.

The OTA was extracted from kidneys by first homogenising 20 g of kidney for two minutes with 27 ml of acetonitrile and 13 ml of water. Then the sample was centrifuged at 4,000 rpm for 10 minutes. Of the supernatant, three ml was diluted in 33 ml of PBS and then filtered. Afterwards, 24 ml of diluted extract were applied to an OCHRAPREP® column. The column was washed using 20 ml of water and air-dried. For the elution of OTA, 1.5 ml of methanol: acetic acid (98:2) was used followed by 1.5 ml of water. The extract was collected in a sample vial and mixed by vortexing.

Chromatographic analysis

The OTA concentration was determined using a LaChrom ELITE (Merck-Hitachi, Darmstadt, Germany) liquid chromatograph equipped with a pump (L-2130), an autosampler (L-2130) and a fluorescence detector (L-2480). Separation was performed on a LiChrospher 100 RP-18 (250 × 4 mm, 5 µm; Merck, Darmstadt, Germany) chromatographic column. A mobile phase composed of a 70:30 mixture of acetonitrile:acetic acid (2%) was used at a flow rate of 1 ml/minute. Fluorescent detection of OTA was obtained using 330 and 460 nm as wavelengths for excitation and emission, respectively. The retention time of OTA was 3.5 minutes.

Statistical analysis

The original OTA content data were $\log_{10}$-transformed and tested for normal distribution by the Shapiro-Wilks W test ($P < 0.05$). Boars from the five forest districts were selected at random. The highest 10% of the data within each region was discarded according to Grubbs's test before performing an analysis of variance. We used one-way ANOVA with random factors in a completely randomised model to determine ($P < 0.05$) the effect of origin (the five regions) on the OTA levels in the blood serum and kidneys of wild boars for each year, separately and overall.

We separated the means by an unequal N HSD test at $P = 0.05$ and used student's t-test with separate variance estimation in comparisons between female and male wild boars, between the two body weight groups of wild boars, between wild boars and pigs and between wild boars from the two different years of the study. We computed the correlation between

the OTA content in blood serum and kidneys for $\log_{10}$-transformed data, using Pearson's coefficient and the linear regression equation. The relationship between the body weight of wild boars and the OTA content in serum or kidneys, respectively, was estimated using non-linear regression. All analyses were computed using Statistica 8.0 (StatSoft Inc., Tulsa, Oklahoma, USA).

Results

We examined a total of 39 wild boars in 2006 and 62 in 2007 for the presence of OTA. We detected large variations among individuals as to the OTA content in the blood serum and kidneys. The standard deviations (SD) for the OTA content (ng/ml and ng/g) were higher than the average values within every single region, thus causing the Pearson's coefficient of the variation to exceed 100%. Additionally, we found huge departures in the median values from mean values, which indicated that 50% of individuals did not reach the mean OTA content, neither in blood serum nor in kidneys. Some individuals had extremely high OTA levels in their blood serum (1,170 ng/ml) and kidneys (97 ng/g).

In both years, there were significant differences in the OTA content in blood serum among the regions ($P = 0.01$; Table 1). The mean OTA content in the blood serum was significantly higher in boars from the Gniezno/Witkowo region (15.8 ng/ml) than in the blood serum of boars from Strzelno or Kcynia/Naklo (1.95 and 1.98 ng/ml, respectively; see Table 1). The region did not influence the OTA content in kidneys, which ranged from means of 1.16 to 3.74 ng/g (see Table 1).

The simple correlation between the OTA content in blood serum and in kidneys was positive and high

Table 1. OTA concentration in blood serum (in ng/ml) and kidneys (in ng/g) of wild boars by region, studied in two consecutive years.

		2006					
		Blood serum			Kidneys		
Region	N	± SEM	Maximum	Median	± SEM	Maximum	Median
Strzelno	6	196.0 ± 194.8	11700	104	16.57 ± 16.07	969	60
Mogilno/Znin	8	1.67 ± 0.88	692	0.52	0.52 ± 0.32	26	20
Gniezno/Witkowo	12	15.99 ± 8.09	971	8.75	4.14 ± 2.06	235	179
Mrocza/Wiecbork	7	34.70 ± 33.89	2380	8.20	2.96 ± 2.82	199	20
Kcynia/Naklo	6	4.07 ± 2.93	185	104	0.86 ± 0.43	227	39
	39						
		2007					
		Blood serum			Kidneys		
Region	N	± SEM	Maximum	Median	± SEM	Maximum	Median
Strzelno	13	1.32 ± 0.61	783	20	0.46 ± 0.22	241	0
Mogilno/Znin	16	1.68 ± 1.11	153	0	0.21 ± 0.16	245	0
Gniezno/Witkowo[1]	0	-	-	-	-	-	-
Mrocza/Wiecbork	11	9.97 ± 6.35	716	139	2.12 ± 0.99	907	20
Kcynia/Naklo	22	1.08 ± 0.41	783	10	0.44 ± 0.19	241	0
	62						
		Overall mean for two years (excluding the top 10% of the data)					
		Blood serum			Kidneys		
Region	N	± SEM, ng/ml		Median	± SEM, ng/g		Median
Strzelno	17	1.95 ± 0.63 [a]		128	1.21 ± 0.26 [a]		129
Mogilno/Znin	22	3.71 ± 1.80 [ab]		284	1.16 ± 0.40 [a]		78
Gniezno/Witkowo	11	15.80 ± 5.13 [b]		835	3.74 ± 1.68 [a]		268
Mrocza/Wiecbork	16	10.41 ± 6.28 [b]		601	2.68 ± 1.14 [a]		42
Kcynia/Naklo	26	1.98 ± 0.53 [a]		117	1.48 ± 0.31 [a]		146
	92						

[1] Wild boars were not sampled.
[ab] The letters group together regions that did not differ significantly using unequal N HSD test, at $P < 0.05$.

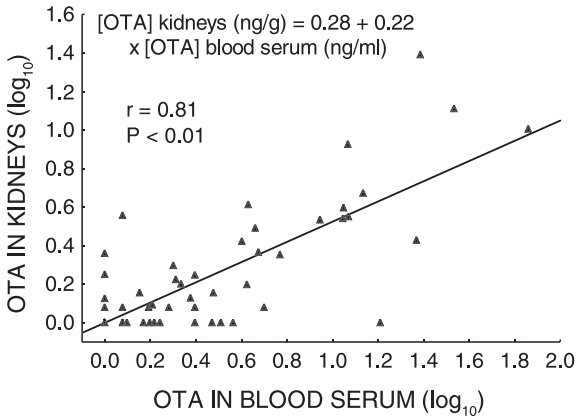

Figure 2. Correlation between the OTA content in blood serum and kidneys of wild boars from the Kujawsko-Pomorskie region, Poland.

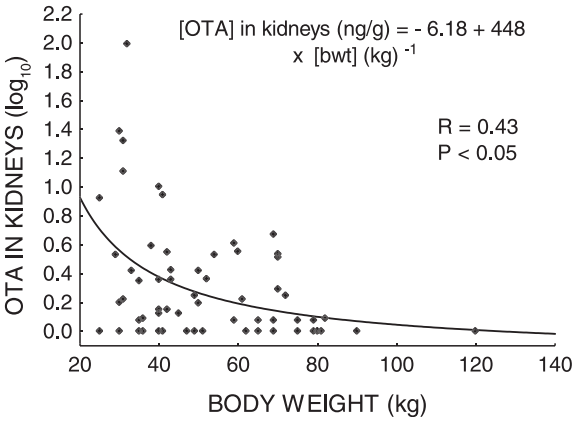

Figure 4. Relationship between body weight and the OTA content in the kidneys of wild boars from the Kujawsko-Pomorskie region, Poland.

($r = 0.81$). The coefficient of the line regression was 0.22, indicating an increase of OTA in kidneys per 1 ng/ml increase of OTA in blood serum (Fig. 2). We estimated the hyperbolic regressions for the relationship between body weight and OTA in both serum and kidneys with the coefficients of curve correlation R of 0.36 and 0.43, respectively. As body weight increased within the range of 22-120 kg, the OTA content would decrease rapidly, and animals with a body weight $>$ 70 kg can be considered to have only trace amounts of OTA in their blood serum and kidneys (Figs. 3 and 4).

The average content of OTA in the blood serum in 2006 (6.15 ng/ml) was similar to the average content found in 2007 (5.91 ng/ml; Table 2). In 2006, the content of OTA in the blood serum of wild boars was $>$ 3 times the OTA content in the blood serum of slaughtered pigs (see Table 2). Significant differences in the OTA content of wild boar kidneys were measured for the two years, where the content in 2007 (2.34 ng/g) was higher than the content recorded for 2006 (1.77 ng/g). Furthermore, the mean OTA content in wild-boar kidneys was three times higher than in the kidneys of pigs (see Table 2).

We found no significant difference in the OTA content between female and male wild boars (Table 3).

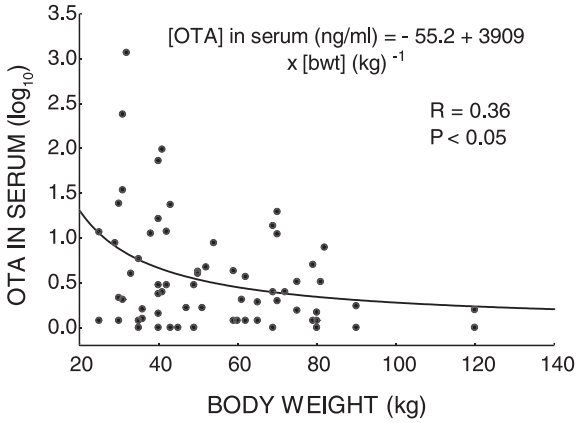

Figure 3. Relationship between body weight and the OTA content in the blood serum of wild boars from the Kujawsko-Pomorskie region, Poland.

Discussion

Many studies have been conducted to determine the level of OTA in the blood serum, kidneys and livers of domestic pigs (Kotowski et al. 1993, Langseth et al. 1993, Gareis & Scheuer 2000, Jørgensen & Petersen

Table 2. OTA content in blood serum and kidneys of wild boars and control pigs.

		Overall mean for all regions			
		Blood serum		Kidneys	
Years	N	± SEM, ng/ml	Median	± SEM, ng/g	Median
2006 - wild boars	34	6.14 ± 1.57 [1,2]	542	1.77 ± 0.57 [1,2]	32
2006 - pigs	20	1.91 ± 0.41[1]	108	0.59 ± 0.09 [1]	36
2007 - wild boars	58	5.91 ± 1.12 [2]	306	2.34 ± 0.22 [2]	20

[1] Indicates significant difference ($P < 0.05$) between wild boars and pigs using Student's t-test.
[2] Indicates significant difference ($P < 0.05$) between wild boars sampled in 2006 and 2007 using Student's t-test.

Table 3. Effect of sex of wild boar on the OTA content in blood serum and kidneys. No significant difference between females and males of wild boars were found according to Student's t-test.

		Blood serum	Kidneys
Sex	N	± SEM, ng/ml	± SEM, ng/g
Female	44	6.19 ± 1.93	2.80 ± 1.91
Male	48	6.12 ± 1.57	1.27 ± 0.43

2002, Milicevic et al. 2009). Various porcine nephropathy cases from different countries have also been studied (Krogh 1992). However, there is no published data on the OTA contamination of organs in wild boars living in their natural habitat or on the extent of mycotoxin contamination of wild-boar meat and offal. To the best of our knowledge, this is the first report on the concentration of OTA in the blood serum and kidneys of wild boars.

Our study revealed that the blood and internal organs of wild boars can be contaminated with high levels of OTA. We also found that animals weighing < 50 kg accumulated much OTA in their blood serum and kidneys (see Figs. 3 and 4). Adult wild boars migrate in search of food (Oloff 1951) resulting in the ingestion of more diversified (i.e. varied) feed and in a reduction of the time they spend in each feeding area. However, hungry young pigs consume large quantities of feed at a time which makes them subject to a more rapid mycotoxin poisoning (Spiesiwcewa 1964).

For the two subsequent years of our study, the mean content of OTA in the blood serum and kidneys of wild boars from the examined regions was several times higher than the content found in pigs. The levels of the toxin were fairly uniform within each region. Particularly high OTA concentrations were recorded in wild boars from the Gniezno/Witkowo region, which is mainly covered by pine forest, where intensive artificial feeding is carried out during both autumn and winter. Due to a decrease in the wild boar population we were, however, unable to collect samples from this region in the second year of our study. The other regions are also covered by forest, but with a higher prevalence of oak and beech. The level of the OTA contamination in the studied regions could be affected by the type of artificial feed in combination with the availability of acorn and beechnut in a given year. Since wild boars are fed throughout the year, it is difficult to determine the extent of milling waste used for this purpose and for how long the remains will be left. As wild boars will eat in several feeding areas during a single night, it can be difficult to determine the level of their OTA intake.

Hult et al. (1979) suggested that the determination of OTA in pigs' blood would be the best reference for assessing the rate of nutrient contamination. Based on results from administering the crystalline form of the toxin, they proposed that the OTA concentration in a feed (in ng/g) could be 1.5 times higher than the mycotoxin content in the blood (ng/l). A more recent study found an OTA level in the blood of four times the level of natural cereal feed (Lusky et al. 1995). Taken together with our blood serum results, it can be concluded that, in some regions, the feed was extremely contaminated with OTA.

Chronic intake of OTA by pigs will lead to ochratoxin nephropathy. Krogh (1992) observed degeneration of pigs' renal ducts after the pigs had ingested feed with a high OTA concentration for four months. In general, OTA is excreted via the urine and bile, and it can remain in the body for several days. Impairment of the organs responsible for the distribution of the toxin may be assumed to be present in several of the individuals with high OTA levels included in our study. The relative distribution of OTA in organs and tissues is, in descending order: blood, kidneys, muscles and fat (Harwig et al. 1983). While there are few studies on the level of toxicity and the metabolic distribution of OTA in humans, OTA can be carried over to wild-boar meat products and can also be present in the muscle tissue of wild boars that have not been sufficiently bled. While, for many food products, permissible levels for some mycotoxins have been legally set, such regulations do not apply to meat products. Considering the amount of OTA present in many feeds, legal regulations should be laid down, rather than just recommended (Commission Recommendation 2006/576/EC).

Conclusions

In Poland, it is very common to feed wild boars with milling by-products, mouldy bread and poor-quality silage leftovers. The results of our study suggest that the high levels of OTA found in the majority of blood-serum and kidney samples of wild boars may be due to intensive artificial feeding with poor-quality feed. The increase in the wild boar population is correlated with an increase in the consumption of wild boar meat, which may involve a risk of OTA carry-over to consumers. A wider screening of such meat products is extremely essential. Continued intensive feed of wild boars in forests using low-

quality feed will continue to intensify wild boar meat contamination with OTA. We found a higher level of OTA in the kidneys from wild boars than in the kidneys of pigs, and in wild boars from some regions the OTA level was sufficiently high to cause possible nephropathy. This could pose a threat to consumers.

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