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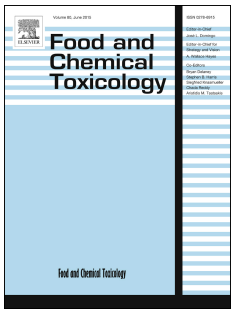

Aflatoxins ingestion and canine mammary tumors: there is an association?

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Aflatoxins ingestion and canine mammary tumors: there is an association?

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Abstract: The aim of this study was to determine the presence of mycotoxins on dogs feed and to explore the potential association between mycotoxins exposure and the chance of mamary tumors in a case-control study. The study included 256 female dogs from a hospital population, 85 with mammary tumors (case group) and 171 without mammary tumors (control group). An epidemiological questionnaire was applied to both groups, and the data were analyzed by the EpiInfo statistical package. For the study, 168 samples of the feed offered to dogs were analyzed for the presence of aflatoxins, fumonisins and zearalenone by high-performance liquid chromatography. Mycotoxins were found in 79 samples (100%) in the case group and 87/89 (97.8%) in the control group. Mycotoxins were detected in all types of feed, regardless feed quality. Level of aflatoxin B1 (p=0.0356, OR = 2.74, 95%, CI 1.13 to 6.60), aflatoxin G1 (AFG1) (p=0.00007, OR = 4.60, 95%, CI = 2.16 to 9.79), and aflatoxin G2 (AFG2) (p = 0.0133, OR = 9.91, 95%, CI 1.21 to 81.15) were statistically higher in case of mammary cancer. In contrast, neutering was a protective factor for mammary cancer (p = 0.0004, OR = 0.32, 95%, CI = 0.17 to 0.60).

Introduction

Study of mammary tumors in female dogs is a good model for clinical, pathological, diagnostic and prognostic investigations of human mammary tumors (Schneider 1970, Liu et al. 2014). Breast cancer is defined as a multifactorial disease and is the most frequent neoplasm in female dogs, while in women, it is the main cause of death throughout the world, with a high rate of malignancy in both species (Morrison 1998, Jemal et al. 2011, INCA 2013). In both species, mammary tumors arise from genomic aberrations and also, from epigenomic alterations (Liu et al. 2014). Risk factors such as age, hormonal influence, nutrition and exogenous progesterone administration can modulate neoplasm growth in female dogs (Pérez Alenza et al. 2000), whereas in women these tumors were associated with few births, early menarche, late menopause, alcoholism and smoking (INCA 2013). In dogs, ovariohysterectomy (OH) performed before the second estrus reduces the risk of developing mammary tumors (Schneider et al. 1969, Chang et al. 2005).

In the last decades an increasing market for pets' commercial feed was established (ABINPET, 2013). Sources of plant protein, such as corn, are present in all types of feed and are potential ingredients for mycotoxin contamination, including regular multi-contamination (Twomey et al. 2002, Carciofi et al. 2009, Rodrigues and Naehrer, 2012). Mycotoxin contamination is a concern since ingestion of contaminated food induces toxic and carcinogenic effects in animals and humans, contributing as an important risk factor for cancer (Maresca and Fantini, 2010, Tchana et al. 2010, Liu et al. 2015).

Association between mycotoxins and carcinogenesis is well established for aflatoxin B1 (AFB1) that is classified as a known human carcinogen by the International Agency for Research on Cancer (IARC 2002). Toxic effects include genotoxicity by the formation of AFB1-DNA adducts and the hot-spot mutation of p53 gene, and carcinogenicity, primarily causing hepatocellular carcinoma (Yao et al. 2014). Increasing evidence suggest that AFB1 cause DNA strand breakage, DNA base damage, and oxidative damage that may ultimately lead to cancer (Guindon-Kezis et al. 2014).

Ingestion of fumonisin (FB) contaminated diet is associated with a high incidence of human esophageal cancer in South Africa, China, and northeast Italy (Peraica et al., 1999). Fumonisins are classified as group 2B (possibly carcinogenic) by IARC. The carcinogenic mechanism underlying FB is not fully understood. Fumonisin affects sphingolipid metabolism, leading to intracellular accumulation of sphingoid bases. Changes on sphinganine and sphingosine bases disturb cell cycle, mainly DNA replication and cell proliferation (Riley et al., 2001). A recent study showed that FB_1 significantly increased the protein expression of cyclin D1 and significantly decreased the protein expression of cyclin E, p21 and p27 reinforcing the idea that FB1 may alter cell proliferation and apoptosis (Wang et al. 2014).

Zearalenone (ZEA), a mycoestrogen, induces hepatotoxic, immunotoxic, genotoxic and carcinogenic effects in a variety of *in vitro* and *in vivo* models (Zinedine et al. 2007). The association between ZEA exposition and breast cancer is controversial. Recently, a possible role for α-zearalanol, a ZEA-metabolite, in the risk of developing breast cancer was reported (Belhassen et al. 2015). In addition, female rats exposed chronically to ZEA resulted in mammary gland development and increased risk of mammary tumors (Belli et al. 2010). On the other hand, a study involving 132 patients showed no significant difference in plasma ZEA and metabolites levels between healthy subjects and women with breast or cervical cancer (Pillay et al. 2002). Although epidemiologic studies did not find ZEA in plasma of women with cancers this does not means that those women were not exposed to higher doses of ZEA long time before cancers were found.

In pet animals there is scarce data concerning a possible interaction between mycotoxins exposure and the development of mammary tumors. In addition, the effects of chronic ingestion of mycotoxins multi-contaminated diets on mammary carcinogenesis of dogs remain unknown. Epidemiological studies are a useful tool for the health-based evidence in order to intervene and reduce the damage and the prevalence of diseases. The objective of the present research was to carry out a case-control study in female dogs with mammary tumors, assess variables of interest and detect mycotoxins in feed consumed by these animals.

Materials and methods

1. *Case and control selection*

The present study was carried out in a Veterinary Teaching Hospital (HV-UEL) from October 2010 to October 2012. A total of 256 female dogs were included in the study, considering one case for every two controls, α= 5% and β= 80%. Eighty-five female dogs with mammary tumors (case group) and 171 female dogs without alterations in mammary gland (control group) were used in this study. The animals of the control group were paired according to age with the animals of the case group.

An epidemiological questionnaire was implemented, containing variables related to the environment, feeding and reproduction. These questions were asked to all the owners, who authorized the research by free and informed consent in the facilities of HV-UEL or in their residences. This study was registered and approved by the Institutional Ethics Committee for Use of Animals (number 13/10).

2. *Feed samples*

A total of 168 samples of commercial feed were analyzed. Analyzed samples were intended for feeding the female dogs in the study. Seventy-nine samples of the feed composed the samples for the case group and 89 samples composed the control group. Feeds were classified for quality as economic, premium and superpremium, according to the information declared by the manufacturer on the label and observing the levels

of crude protein, ether extract, crude fiber and mineral matter (Carciofi et al. 2009). Feed samples were collected shortly after implementing the epidemiological questionnaire. Each owner supplied 300 g of feed. Samples were weighed and placed in kraft-type paper bags, dated and stored at 4°C for up to 24 hours.

3. Determining mycotoxins by HPLC

In this study three mycotoxin families (aflatoxins, fumonisin and zearalenone) were analyzed. To determine the mycotoxins levels, 200 g of each sample was ground in a 50-mesh particle size and stored at -20°C until analysis. Aflatoxins, fumonisins and zearalenone were analyzed in triplicate by a reversed-phase isocratic high performance liquid hromatography (HPLC) system (Shimadzu LC-10 AD pump and RF-10A XL fluorescence detector) using a C-18 Luna Phenomenex column (250 x 4.6 mm, 5 µm, Scharlau, Barcelona, Spain). Data were expressed by mean ± SD.

Fumonisins were determined by HPLC following the method described by Shephard et al. (1990) and modified by Ueno et al. (1993). The detection limits (LOD) for fumonisin B1 (FB_1) and fumonisin B2 (FB_2) were 27.5 µg kg^{-1} and 35.3 µg kg^{-1}, respectively, defined as the minimum toxin concentration that could generate a chromatographic peak three times above the height/noise ratio of the baseline. HPLC was used to determine the ZEA levels following a method adapted from Sabater-Vilar et al. (2007), with a 3.95 µg kg^{-1} LOD. Aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 were determined by HPLC according to the method described by Miyamoto et al. (2008), with a LOD of 0.128 µg kg^{-1}, 0.027 µg kg^{-1}, 0.593 µg kg^{-1} and 0.222 µg kg^{-1}, respectively.

4. Statistical analysis

The statistical program EPI INFO 3.5.4 was used to tabulate the data obtained, calculate the relative frequency and tabulate the variables of interest. The chi-square or Fisher's exact tests, when appropriate, were used to compare proportions and, the *Kruskal-Wallis* test was applied to compare means at a 5% level of significance. The *Odds Ratio* was used as an association measure with a 95% confidence interval.

Results

1. Environmental and reproductive variables

The breed frequency for the case group was as follows: Poodle, 33.3% (15/45); Pinscher, 13.3% (6/45); Teckel and Cocker Spaniel, 11.1% (5/45). The breed frequency for the control group was as follows: Poodle, 20% (17/85); Boxer, 11.8% (10/85); Pinscher, 10.6% (9/85); Teckel and Cocker Spaniel, 9.4% (8/85). Although Poodle was the most frequent breed in both groups, there was no significant difference (p=0.0931) in relation to mammary tumors. The average age of the animals was 10.11 (±2.43 years) for the case group and 8.44 (±2.58 years) for the control group. The results of the analysis of environmental and reproductive variables are disposed on Table 1 (supplementary material section).

2. Feeding-related variables

A total of 168 dog feed samples was collected and analyzed from 17 feed manufacturing companies, consisting of 49 brands. All of the brands contained corn in its composition. Not all of the owners could identify the feed brand at the time the sample was collected. Table 2 (supplementary material section) shows the results of the analysis for the feeding-related variables.

3. Mycotoxin detection

Mycotoxins were detected in 100% (79/79) of the samples in the case group and in 97.8% (87/89) of the samples in the control group. A total of 148 feed samples were analyzed in the case and control groups for quality: economic, 48.64% (72/148); premium, 42.56% (63/148); and superpremium, 8.78% (13/148). Mycotoxin presence was detected in all of the feed categories. In the economic-type feed, 100% (72/72) contained mycotoxins, and 98.4% (62/63) of the premium feeds and 92.3% (12/13) of the superpremium feeds presented mycotoxins, but there was no significant difference between the qualities of feeds (p=0.0849) and presence of mammary tumors.

Aflatoxins (B_1+B_2+G_1+G_2) were detected in 83.1% (123/148) of the samples: 84.7% (61/72) in economic feeds, 84.1% (53/63) in premium feeds and 69.2% (9/13) in superpremium feeds. Fumonisins were detected in 70.9% (105/148) of the samples: 77.8% (56/72) in economic feeds, 71.4% (45/63) in premium feeds and 30.8% (4/13) in superpremium feeds. Zearalenone was detected in 95.3% (141/148) of the samples: 93.1% (67/72) of economic feeds, 98.4% (62/63) of premium feeds and 92.3% (12/13) of superpremium feeds. Table 3 shows the relative frequency and concentration levels for zearalenone, fumonisins (FB_1+FB_2) and aflatoxins (B_1+B_2+G_1+G_2) in the feed of 168 female dogs.

Table 3. Relative frequency and concentration levels of zearalenone, fumonisins (FB_1+FB_2) and aflatoxins (B_1+B_2+G_1+G_2) measured by HPLC technique in the feed of 168 female dogs in a mammary tumor case-control study, Londrina, PR, South Brazil, 2010-2012.

Mycotoxins (µg/kg)	Case (n=79[a])	Control (n=89[a])	*P* value	OR (IC 95%)
Zearalenone				
Yes	74	86	0.2959[b]	0.51 (0.11-2.23)
No	5	3		
Mean (±SD)	38.87 (±59.38)	38.66 (±40.68)	0.3226[c]	
Median	23.20	26.47		
Amplitude	(4.07-442.25)	(4.45-208.91)		
Fumonisin				
Yes	62	60	0.1521	1.76 (0.87-3.53)
No	17	29		
Mean (±SD)	184.81 (±170.00)	130.70 (±113.45)	0.0212[c]	
Median	150.00	99.37		
Amplitude	(30.00-1014.72)	(0.09-503.02)		
Total Aflatoxin				
Yes	71	69	0.0529	2.57 (1.06-6.22)
No	8	20		
Mean (±SD)	1.32 (±1.13)	1.16 (±0.64)	0.7014[c]	
Median	1.15	1.37		
Amplitude	(0.16-6.16)	(0.19-3.36)		
Aflatoxin B1				
Yes	71	68	0.0356	2.74 (1.13-6.60)
No	8	21		
Mean (±SD)	1.01 (±0.97)	1.03 (±0.59)	0.1481[c]	
Median	0.67	1.37		
Amplitude	(0.16-4.99)	(0.19-2.08)		
Aflatoxin G1				
Yes	33	12	0.00007	4.60 (2.16-9.79)
No	46	77		
Mean (±SD)	0.53 (±0.24)	0.52 (±0.29)	0.3731[c]	
Median	0.44	0.43		
Amplitude	(0.39-1.46)	(0.38-1.5)		
Aflatoxin B2				
Yes	5	1	0.1004[b]	5.94 (0.67-52.03)
No	74	88		
Mean (±SD)	0.37 (±0.15)	1.84 (±0.00)	0.1432[c]	
Median	0.27	1.84		
Amplitude	(0.25-0.61)	(1.84-1.84)		
Aflatoxin G2				
Yes	8	1	0.0133[b]	9.91 (1.21-81.15)
No	71	88		
Mean (±SD)	0.41 (±0.34)	1.63 (±0.00)	0.1213[c]	
Median	0.28	1.63		
Amplitude	(0.24-1.25)	(1.63-1.63)		

[a]Total number of feed samples in cases and controls, any variation was due to the absence of response from interviewed owners.
[b]Fisher's exact test (two-tailed). [c] Kruskal-Wallis.

Discussion

Mammary tumors were the most common type of neoplasms affecting intact female dogs worldwide (Merlo et al. 2008, Sleeckx et al. 2011) and also in Brazil (Oliveira Filho et al. 2010). Malignant behavior is most frequent in mammary tumors (Misdorp 2002). In the last years, multiple factors were associated with increased risk of mammary neoplasms as increased age, intact status, ovariectomy after 2.5 years of age, progestagen treatment as well as homemade diet (Perez Alenza et al. 2000). The present study suggests that ingestion of diets contaminated with underline regulatory levels of aflatoxins is associated with an increased chance of developing mammary tumors in female dogs. Recently, a case-control study showed in women an association between zearalenone exposition and risk of breast cancer (Belhassen et al 2015). To the best of author´s knowledge there is no previous report evaluating canine mammary tumors and mycotoxins exposition.

Previous studies reported an association between environmental factors and neoplasms incidence. Sugar refinery and poultry production workers are indirectly exposed to dust that is frequently contaminated with aflatoxins, resulting in an increased risk of hepatocellular carcinoma (Lai et al. 2014, Viegas et al. 2012). In addition, progesterone administration, number of pregnancies, breed, age and diets rich in saturated fat were considered a risk factor for mammary tumors in women and female dogs (Itoh et al. 2005, Lima et al. 2008, Pérez Alenza et al. 1998, 2000, Russo et al. 2012, Rutteman 2003). In this study no association between these variables and risk of mammary tumors were observed.

The effect of neutering female dogs is commonly associated with a significant reduction in the risk of mammary tumors (Schneider et al. 1969, Rutteman and Kirpensteijn 2003). In the present study, dogs of the case group spayed prior to mastectomy represented 20.2% (17/84) of the cases in contrast with 43.7% (73/167) of the controls. Castration showed a protective effect (p=0.0004. OR= 0.32. 95%CI=0.17-0.60) against mammary tumors. Considering the period, no dogs had been spayed until the second estrus in the case group compared to control group (20.3%), even so there was no statistically significant results (p=0.0621). Other studies have indicated that when carried out before the first estrus, early castration decreases the incidence of mammary tumors (Schneider et al. 1969, Rutteman et al. 2001). The protective effect conferred by OH disappears or becomes more moderate when performed after two and a half years of age (Fonseca and Daleck 2000, Sorenmo et al. 2000, Rutteman and Kirpensteijn 2003). On the other hand, a systematic review for the risk of bias in published results about this association was performed and, concluded that the evidence of the advantage of neutering and the age at neutering in reducing the risk of mammary neoplasia are not a sound basis for firm recommendations (Beauvais et al. 2012).

The quality of dried feed for cats and dogs commercialized in Brazil is considered good in both nutritional and health aspects (Carpim and Oliveira 2009). In the present study, 98.6% of the 168 samples of dog feed assessed, regardless the quality classification, were contaminated with mycotoxins. However, the levels of contamination of all mycotoxins analyzed were below the maximum tolerance limit (MTL) established by Brazilian legislation for animal and human consumption. In corn grains and byproducts the MTL for total aflatoxins in Brazil is the same value established for human consumption: 20 μg/kg total aflatoxins (BRASIL 2002, ANVISA 2011). In the United States, Canada and Europe, the MTL for total aflatoxins for animal feed is 20 μg/kg (Leung et al. 2006). No legislation is established for animal consumption in relation to fumonisins and zearalenone, meanwhile feed industries follow a recommendation of MTL of 4000 μg/kg of fumonisins (B_1+B_2) and 100 μg/kg zearalenone (ABINPET, 2013). However, one should be aware of the contamination of feed with mycotoxins and its chronic effects in dogs.

In the present study, aflatoxin B_1 was detected in 89.9% of the feeds in the case group and in 76.4% of the control group. Similar results were reported previously (Sharma and Márquez 2001). Aflatoxin is considered one of the most potent natural carcinogens, and its oncogenic, immunosuppressive, hepatotoxic, nephrotoxic and anti-coagulant effects are the most abundant and toxic (IARC, 2002). In this case-control study, an association was found between the presence of aflatoxins in the feed and mammary tumors for aflatoxin B_1 ($p=0.0356$), aflatoxin G_1 ($p=0.00007$) and aflatoxin G_2 ($p=0.0133$), suggesting that exposure to these mycotoxins might lead to tumor development. Although levels below MTL were detected, the cumulative effect due to exposure time and mycotoxins co-occurrence should be taken into consideration. Mycotoxins multi-contamination is commonly reported in feed and food natural contamination (Bohm et al. 2010, Rodrigues and Naehrer 2012). Prospective epidemiological studies are necessary to confirm these findings.

AFB1 is highly reactive and is considered the main genotoxic metabolite because it attaches to DNA and induces mutations. The most studied mutations associated with AFB1 involve the p53 growth suppressor gene and the *ras* proto-oncogene (Eaton and Gallagher 1994). There are few experimental studies regarding the genotoxic and mutagenic effects of other metabolites (AFSSA 2009). Most epidemiological studies show a tendency toward correlation between chronic exposure to aflatoxins in the feeding regimen and the prevalence of primary hepatic cancer (Stoloff 1987, Lai et al. 2015). However, this relationship is modulated by other factors, such as infection by hepatitis B virus (Lai et al. 2015). To the best of author´s knowledge there are no data regarding the association between aflatoxins and mammary tumors in women and animals.

Conclusions

Epidemiological and experimental data indicate that mycotoxins can induce cell neoplastic transformation. Despite these data, studies investigating carcinogenic effects of mycotoxins on pet animals are scarce. This is the first study that evaluates aflatoxins, fumonisins and zearalenone levels in feed of female dogs presenting mammary tumors with the aim to associate mycotoxin ingestion and the chance of mammary neoplasms. An association between aflatoxin B1, G1 and G2 concentrations and increased chance of mammary tumors was observed. These results suggest a potential role for chronic ingestion of aflatoxins in the risk of developing mammary neoplasms. Further prospective studies are necessary to elucidate the role of mycotoxins in mammary cancer.

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Conflicts of interest

The authors declare no conflict of interest.

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Highlights (mandatory)

- Aflatoxins (AF) are associated with an increased risk of mammary tumors in dogs.
- Chronic intake of AF, even in low levels, can predispose to canine mammary cancer.
- Neutering has a protective effect against canine mammary tumors.

Table 1. Results of environmental and reproductive variables in a case-control study of mammary tumors in 256 female dogs in a hospital population, Londrina, PR, South Brazil, 2010-2012.

Variables	Cases (n=85[a])	Controls (n=171[a])	*P value*	OR (IC 95%)
Breeding				
Yes	45	85	0.7565	0.88
No	40	85		(0.52-1.49)
Age (years)				
Mean (±SD)	10.11 (± 2.43)	8.44 (±2.58)	-	-
Median	10	8		
Amplitude	(3-18)	(3-16)		
Dogs residence				
Urban area	82	167	0.6884[b]	0.65
Rural area	3	4		(0.14-2.99)
Agricultural area				
Yes	2	7	0.7221[b]	0.57
No	81	164		(0.11-2.84)
Lives close to a factory				
Yes	22	30	0.1768	1.61
No	63	139		(0.86-3.02)
Smoker in the house				
Yes	21	41	0.9180	1.01
No	63	125		(0.55-1.86)
Mammary gland tumor in the family				
Yes	19	34	0.7849	1.15
No	66	136		(0.61-2.17)
Other animals in the house				
Yes	60	127	0.6344	0.83
No	25	44		(0.46-1.48)
Spayed				
Yes	17	73	0.0004	0.32
No	67	94		(0.17-0.60)
When spayed				
By the second estrus	0	14	0.0621	0
After the second estrus	17	55		(0-1.32)
Contraceptive administration				
Yes	16	30	0.9372	1.09
No	68	139		(0.55-2.13)
Frequency of contraceptive administration				
Once	3	9	0.5272	-
Twice	3	5		
Three or more times	9	11		
Pregnancy				
Yes	34	73	0.8107	0.90
No	49	95		(0.52-1.53)
How many pregnancies				
≤ three	26	63	0.7293[b]	0.72
≥ four	4	7		(0.19-2.67)

[a]The variation in the total number of cases and controls was due to the absence of response from interviewed owners.
[b]Fisher's exact test (two-tailed).

Table 2. Results of the feeding-related variables in a case-control study of mammary tumors in 250 female dogs in a hospital population, Londrina, PR, South Brazil, 2010-2012.

Variables	Cases (n=85[a])	Controls (n=171[a])	*P* value	OR (IC 95%)
Food				
Home-made food	1	4	0.3365	-
Feed	35	55		
Home-made food + feed	49	111		
Feed ingested since				
Puppyhood	68	123	0.0749	-
Adult	9	9		
Other	7	32		
Where feed was purchased				
Grocery store	20	26	0.2259	-
Agricultural store	17	44		
Pet shop	45	93		
Purchased loose				
Yes	21	50	0.2227	0.68
No	57	98		(0.39-1.18)
Feed purchase frequency				
Weekly	13	35	0.6588	-
Every 2 weeks	20	41		
Monthly	32	53		
Other	17	36		
Feed package stored open				
Yes	22	26	0.0671	1.91
No	60	136		(1.00-3.65)
Covered storage place				
Yes	83	166	1.00[b]	0.50
No	1	1		(0.03-8.09)
Humid storage place				
Yes	18	35	0.9010	1.01
No	66	130		(0.53-1.92)
Feed Classification				
Economic	42	64	0.1334	-
Premium	26	59		
Superpremium	4	17		

[a]The variation in the total number of cases and controls was due to the absence of response from interviewed owners.
[b]Fisher's exact test (two-tailed).