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SHORT RESEARCH AND DISCUSSION ARTICLE

Outbreak of fatal nitrate toxicosis associated with consumption of fennels (*Foeniculum vulgare*) in cattle farmed in Campania region (southern Italy)

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Abstract Nitrate and nitrite are toxicants that have become increasingly significant environmental chemicals. Increase in environmental distribution of nitrogenous compounds, especially in surface and ground water, has been attributed to the intensive use of nitrate as agricultural fertilizers and to increasing amounts of nitrogenous wastes produced by municipalities, industries, and feedlots. The purpose of this study is to illustrate a fatal nitrate toxicosis in cattle associated with the consumption of fennels (*Foeniculum vulgare*). Fifteen cows from the same farm suddenly developed weakness, muscular tremors, respiratory distress, and finally convulsions. The affected animals died within 24 to 48 h from the onset of the clinical signs. Five cows underwent a complete post-mortem examination. In all examined animals, gross lesions included presence of dark unclotted blood around the nostrils and the anal region, moderate inflammation of the gastrointestinal mucosa, and brown discoloration of the skeletal muscles and kidneys. The histological examination showed tubular degeneration and congestion of glomerular vessels in the kidney. Toxicological analysis detected nitrates at 4 672.2 ppm in the fennels used to feed the animals. The source of exposure to nitrates was identified in the fennels. The fennels were grown in a polluted area of the Campania region in southern Italy and distributed in a public market for human consumption. The waste from the sale of the fennels was fed to the cows. The accumulation of nitrates in some vegetables poses a risk not only for animal health but also for human and environmental safety.

Keywords Cattle · Fennel · Waste · Nitrate · Nitrite · Environmental pollution

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Introduction

Nitrate and nitrite are toxicants that have become increasingly significant for environmental pollution. Their increase in environmental distribution, both in surface and ground water, has been attributed to the use of agricultural fertilizers and wastes produced by municipalities, industries, and feedlots (Ozmen et al. 2003).

In Italy, the acronym SIN (Sites of National Interest) refers to those areas where the pollution of soil, surface water, and groundwater is extremely severe and represent a serious hazard to public health and the environment; SIN are usually represented by ex-industrial areas or places where industries are still active, such as ex-mines, ports, or lands nearby illegal waste dumps. Campania is a region in southern Italy that has the largest SIN in the country; moreover, the sites in question in the Campania region are located near highly urbanized and populated areas (Maselli et al. 2010).

One of the major issues in environmental risk assessment is the use of biomarkers in ecotoxicology to evaluate biological alterations in sentinel species caused by possible contaminants.

(Maselli et al. 2010). Domestic and wild animals are often studied and monitored as they may either help in revealing the presence of unknown chemicals in the environment before they cause harm to humans or help in identifying health effects due to exposure to known or suspected chemical contaminants (Roperto and Galati 1998; Maciejewski et al. 2008). As a matter of fact, disease latency periods are usually shorter in animals than in humans; conversely, animals may be exposed to higher concentrations of chemicals and manifest symptoms before humans (Perillo et al. 2009; Roperto and Galati 1998; van der Schalie et al. 1999).

Many animal species are susceptible to nitrate and nitrite poisoning; however, cattle are much more likely to be affected than other animals (Ozmen et al. 2003). The most common cause of toxicosis from these compounds in farm animals is the consumption of feed or water containing high levels of nitrates (Ozmen et al. 2003). Forage plants normally do not contain a high enough amount of nitrates to be toxic for cattle. Water also must be contaminated by exogenous sources of nitrates in order to be dangerous to both animals and humans usually (Manassaram et al. 2010). The modern agriculture system and intensive farming practice have resulted in an excessive use of nitrogen-based fertilizers which may result in accidental cases of toxicosis (Villar et al. 2003). The purpose of this study is to illustrate a fatal nitrate toxicosis in cattle associated with consumption of fennels (*Foeniculum vulgare*) and discuss the potential role of animals in monitoring environmental pollution in order to prevent diseases and toxicosis in humans.

Materials and methods

A group of Italian Podolica breed of dairy cattle, between 3 and 6 years of age, from the same farmhouse, suddenly developed anxiety, weakness, muscular tremors, and ataxia; these animals had been recently fed with fennels. The fennels were produced in a polluted area of Campania and distributed in a public market for human consumption. The waste from the sale of the fennels were collected and fed ad libitum to cows. Fifteen out of the entire herd of 50 cows died within 24 to 48 h from the onset of the first clinical signs, which were evident after 2–4 h from the consumption of fennels; cattle showed mainly convulsions associated with dyspnea and tachypnea. On field, necropsies were performed in five of those cows by the pathologists of the Unit of Pathology of University of Naples Federico II (OP and AC). The brownish color of the blood and the cyanosis of the visible mucosa found at the necropsy helped us rule out all other diseases associated with the clinical signs shown by the animals. The anamnesis, the clinical signs, and the anatomopathological lesions led to a presumptive diagnosis of toxicosis by methemoglobinizing agents. For histopathology, tissues from all the examined organs were collected, fixed in 10 % buffered formalin, and embedded in paraffin. Five micron tissue sections were collected and stained with hematoxylin and eosin.

The liver, kidney, and blood samples and the ruminal content from the cattle were collected for toxicological examination. Assuming an alimentary intoxication of the animals, the samples from the cattle feed were submitted in order to check the presence of methemoglobinizing agents focusing mainly on nitrates and chlorates. The samples were collected in disposable plastic bags, cooled at refrigeration temperature of 4 °C by a cooling media during the transportation to the laboratory which took about 3 h, and then frozen at −30 °C.

Nitrate was detected according to the regulations of the Association Française de Normalisation (AFNOR 1974). Briefly, 10 g of the samples (liver, kidney, and blood samples; ruminal content and fennels) was homogenized and macerated with 5 ml of Borax solution and 70 ml of hot (over 70 °C) deionized distilled water. The supernatant was heated in boiling water for 30 min and then 2 ml of potassium ferrocyanide trihydrate reagent and 2 ml of zinc acetate dehydrated in glacial acetic (220 g of zinc acetate in 30 ml of glacial acetic and adjusted to 1 l with distilled water) were added. The pH was adjusted to 8.3, and the volume was adjusted to 200 ml with distilled water. The mixture was centrifuged at 3,000 rpm (rotations per minute) for 10 min, and the supernatant was filtered. Fifty milliliters was taken, and animal carbon was added for discoloring. Nitrate in the extract was assayed following the Griess cadmium reduction method (Green et al. 1982), with the formation of an azoic compound through the reaction with sulfanilic acid and a-naphthylamine chloride after reduction by means of a cadmium column. To establish the percentage of recovery from the samples, three different samples spiked with pure nitrate solution were ran; the mean recovery was 85 %. The nitrates added for the recovery control were subtracted from each value. The absorbance was measured at 540 nm with a spectrophotometric method. The nitrate content of the different samples was calculated using the calibration curve.

Results

In the necropsies, the animals were in a good nutritional status; visible mucosae appeared cyanotic (Fig. 1a,b), and there was brownish unclotted blood around the nostrils and the anal regions. Furthermore, we observed a brown discoloration of the skeletal muscles and kidneys. The lungs, heart, kidneys, and liver showed blood stasis and disseminated small hemorrhages (Fig. 1c,d). Moreover, severe and diffuse edema was also detected in the lungs. Micro-hemorrhages were also

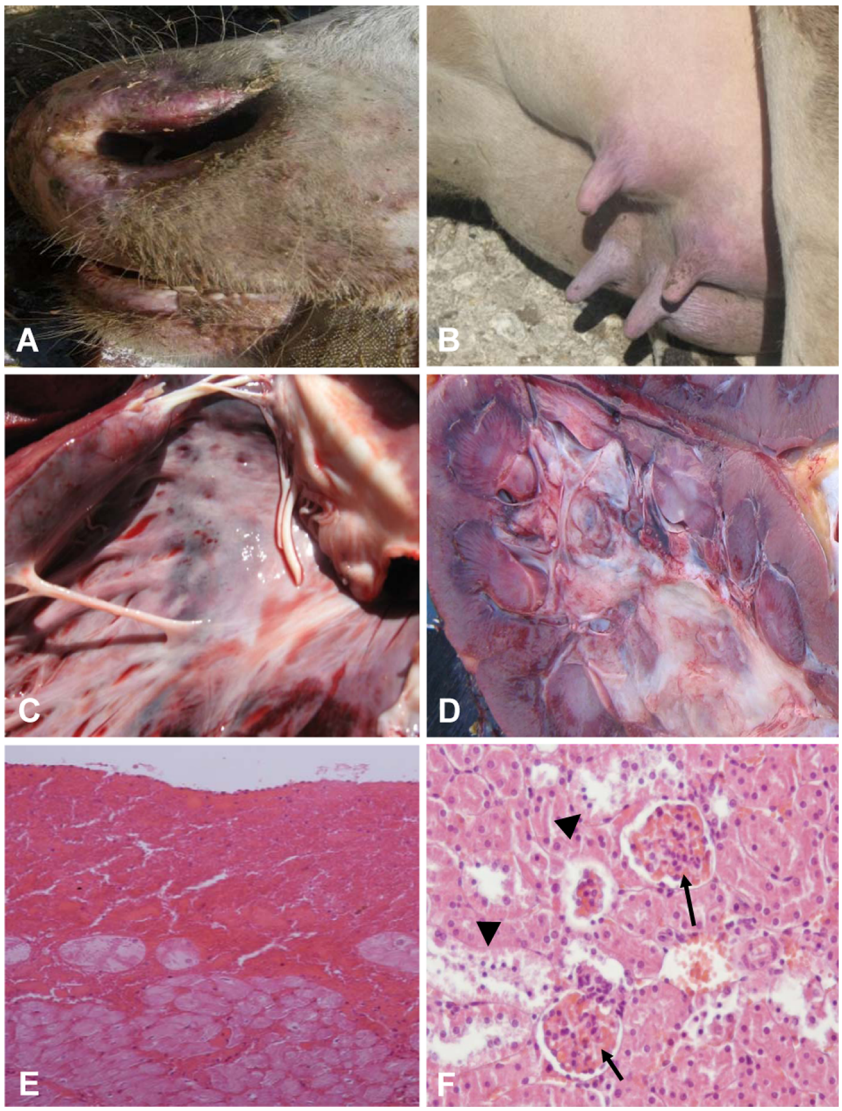

Fig. 1 Cow: representative pictures of necropsy findings. **a** Bluish color of the oral and nostril mucosa and skin around them. **b** Cyanotic aspect of mammary gland. **c** Severe and diffuse subendocardial hemorrhages in the right ventricles. **d** The kidney appears congested with brown discoloration of the cortex and medulla. **e** Histological section of the myocardium showing diffuse subendocardial hemorrhage and hemorrhage in between cardiomyocytes (hematoxylin–eosin stain, ×40 original magnification). **f** Histological section of the kidney showing degeneration and necrosis of the renal tubular epithelium (*arrow heads*) and congestion of glomerular vessels (*arrows*) (hematoxylin–eosin stain; ×40 original magnification)

detected on the serosae of the lungs, and mild signs of inflammation were observed in the rumen which was characterized by a friable, easy to remove, brownish-colored mucosa of papillae and hyperemic gastrointestinal mucosa.

Histopathological findings were characterized by congestion, hemorrhages, and edema in the gastrointestinal mucosa, associated with mild diffused infiltration of neutrophils in the mucosa. In the spleen, erythrophagocytosis and hemosiderosis were noted. The lungs showed widening of the alveolar septal wall with the congestion of the alveolar capillaries and features of edema characterized by cell-free fluid in the alveoli. Furthermore, many hemorrhages were observed in the lungs and myocardium (Fig. 1e). In the kidneys, the degeneration and necrosis of the renal tubular epithelium associated with the presence of cellular debris and traces of protein in the tubules and congestion of glomerular vessels were evident (Fig. 1f).

Nitrates were detected in the submitted samples (Table 1), and no chlorates were detected in the same samples. The detection of high levels of nitrate in the liver, kidney, and blood samples and in the ruminal content and fennels, along

Table 1 Results of toxicological analysis

Sample	NO_3 level detected (ppm) on dry weight basis
Liver	112.73
Kidney	281.97
Blood	3,662.22
Ruminal content	4,354.72
Fennels (green part of the plant)	4 672.2
Fennels (white part of the plant)	2,915.2

with other compatible post-mortem findings, confirmed the diagnosis of death due to nitrate intoxication.

Discussion

The presence of nitrate in water and foods is a serious threat to both human and animal health. Nitrate per se has very low toxicity, but if ingested, approximately 5 % is converted by bacterial enzymes present in the gastrointestinal system into the more toxic nitrite (Santamaria 2006). By the way, both nitrite and *N*-nitroso compounds, formed before or after ingestion, are very toxic and can be the cause of severe pathologies even in humans (Santamaria 2006). Thus, the assessment of the health risk for humans and animals of nitrate should include the potential toxicity associated to both nitrite and *N*-nitroso compounds (Santamaria 2006). The most important effect of nitrite is its ability to react with hemoglobin (oxyHb) to form methemoglobin (metHb). As a consequence, the formation of metHb leads to an impairment of red blood cells to deliver oxygen to tissues, and once the proportion of metHb reaches 10 % of normal Hb levels, clinical symptoms (from cyanosis to asphyxiation) occur (Santamaria 2006).

Furthermore, nitrite is a powerfully oxidizing species, and peroxidase-catalyzed nitrite oxidation could induce biomolecular modifications in host tissues and contribute to cell or tissue injury as a result of increased nitric oxide production (van der Vliet et al. 1997). Nitric oxide (NO) is a highly reactive molecule which interacts with oxygen, transition metals, and reactive oxygen species (ROS), especially with superoxide anion (Gryglewski et al. 1986). The interaction of NO and O_2– results in a formation of peroxynitrite ONOO– that can further decompose into highly toxic products, such as hydroxyl radical (OH) (Beckman et al. 1990), or in the presence of thiols, it may be transformed to nitrate or *S*-nitrosothiols (Beckman and Koppenol 1996). Those molecules have been proven to have pro-inflammatory and cytotoxic effect (van der Vliet et al. 1997) and could explain, in part, the inflammation, edema, hemorrhages, and acute tubular necrosis that we found.

Moreover, nitrite and nitrate may react with amines or amides to form *N*-nitroso compounds that are highly carcinogenic chemicals (Santamaria 2006). Nitrates and nitrites may be involved in the accidental intoxication of farm animals; one of the most frequent causes of poisoning by these compounds is the ingestion of plants, which have absorbed great quantities of nitrates from the soil, or the consumption of water containing high levels of nitrate nitrogens (McKenzie et al. 2009).

Vegetables that easily concentrate nitrate include some cereal grasses like oat (*Avena sativa*), some varieties of millet, rye (*Secale cereale*), and corn (*Zea mays*); moreover, some kind of weeds, like pigweed or lamb's quarter, usually concentrate high levels of nitrate. Furthermore, soils containing high levels of nitrate, environmental pollution, some weather conditions, and agricultural techniques (for example, the inappropriate use of certain fertilizers) tend to increase nitrate content in forage (Schneider 2012; Cardenas-Navarro et al. 1999).

Nitrates are chemical compounds of low toxicity for animals. They exert their effects in respect to their toxic chemical properties of chemicals locally irritating the mucous membrane of the gastrointestinal tract, causing gastroenteritis (Schneider 2012). Moreover, they can be very dangerous as they can be reduced to nitrites in the rumen by ruminal microorganism (Kennett et al. 2005). The metabolic pathway of nitrate and nitrite compounds normally leads to the production of ammonia, which is used to synthesize microbial proteins (Ozmen et al. 2003). If the nitrate intake exceeds the reduction capacity of the rumen, the process can lead to the accumulation of nitrites which enter the bloodstream and cause the oxidation of ferrous hemoglobin to ferric methemoglobin, which is unable to bind and transport oxygen to the tissues (McKenzie et al. 2009). The lack of oxygen in tissues leads to several injuries including pulmonary edema, caused by an increase in alveolar capillary hydrostatic pressure due to vasoconstriction (Russell et al. 2008), degeneration and necrosis of epithelial cells lining the renal tubules (Máthé et al. 2007), and endothelial dysfunction (Gori and Parker 2008).

According to Adams et al. (2012), stored forages containing more than 7,500 ppm of nitrates may cause acute toxicity; moreover, forage containing 5,000 to 10,000 ppm nitrate ion (NO_3) can be considered potentially toxic if it is provided as the only feed (Adams et al. 2012; Whittier 2011). However, even forage concentrations of 1,000 ppm NO_3 dry weight basis have been lethal to hungry cows engorging themselves in a single feed within an hour, so the total dose of nitrate ingested is a deciding factor (Schneider 2012). In our study, the concentration of NO_3 in the fennels was 4 672.2 ppm on dry weight basis, and considering that was the only feed provided to the cows, this amount was enough to determine the acute toxicosis.

Clinical signs of acute poisoning vary depending on the amount of ingested nitrates and on the quantity of nitrites that bind to hemoglobin (Cockburn et al. 2013); clinical signs include muscular tremors, weakness, dyspnea, and cyanosis leading to death (Cockburn et al. 2013). The aim of the therapy of nitrate and nitrite poisoning is to reconvert ferric methemoglobin into ferrous hemoglobin by a reducing agent such as methylene blue. The dosage will depend on the severity of the exposure (Burrows 1980).

The increase in environmental distribution of nitrogenous compounds has been attributed to the intensive use of nitrates as agricultural fertilizers and to the increasing amounts of nitrogenous wastes produced by municipalities, industries, and feedlots (Ozmen et al. 2003). Nitrates and nitrites are also used in the alimentary industry to preserve the organoleptic

qualities in meat, poultry, and fish products (Chow and Hong 2002). Thus, humans and animals may be exposed in many ways to significant nitrate and nitrite levels in food and water (Chow and Hong 2002). In humans, the ingestion and inhalation of nitrates and nitrites are the most frequent causes of acute methemoglobinemia. The most sensitive people are infants and ill adults. The World Health Organization indicates that the toxic dose for humans is 0.4–200 mg/kg of body weight, and the lethal dose is 33–250 mg/kg of body weight (Speijers and van den Brandt 2006). The symptoms of nitrate poisoning are characterized by cyanosis, headache, dizziness, tachypnea, tachycardia, and general weakness. With the increase of methemoglobinemia at values higher than 60 %, loss of consciousness and death can occur (Matteucci et al. 2008).

Nitrate toxicosis is very common in cattle, but, to the authors' knowledge, this is the first study in which the source of nitrates is represented by vegetables such as fennels (*F. vulgare*). Fennel (*F. vulgare*), like many other vegetables, may accumulate high quantity of nitrates from the soil in certain conditions, and it is very important to consider that these vegetables are also consumed by people. Thus, not only farmers and veterinary nutritionists should be aware and consider the safety of the feed administered to livestock but also physicians should consider the clinical signs of acute or chronic exposure to nitrate and nitrite in people that consume these vegetables. When sudden death occurs, a necropsy may be necessary for a definitive diagnosis; in addition to histopathological findings, laboratory analysis of feed and water as well as assays of body fluids, including serum and urine and aqueous humor or cerebrospinal fluid, may be considered to confirm the suspect of nitrate and nitrite poisoning (Villar et al. 2003).

This report focuses on the environmental risk of nitrate and nitrite accumulation, which is considered hazardous both for humans and animals since these compounds may accumulate in fruits, vegetables, and drinking water (Katan 2009). Even if the data on nitrate and nitrite residues in meat or milk are very scarce, it is thought that their accumulation in muscle tissues and animal products is very low in normal condition (Cockburn et al. 2013). Nevertheless, we are still concerned that the concentration of nitrate and nitrite in animal products could be a problem for human health.

The use of sentinel animal data can be very helpful in monitoring the pollution in certain areas and in evaluating the long-term impact of some chemicals on human health. Environmental pathologists may compare the frequency and the pattern of some diseases in animals with those corresponding in humans and help detect new polluted areas and monitor the environmental pollution impact in already known SIN.

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