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Short Communication

An alternative model for fetal loss disorders associated with mare reproductive loss syndrome

Thomas W. Swerczek

Department of Veterinary Science, University of Kentucky, Lexington, KY, 40356, United States

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ABSTRACT

Fertile chicken eggs were used as an alternative model for large animals to evaluate suspect toxic dietary ingredients for fetal loss disorders associated with mare reproductive loss syndrome (MRLS) and fetal losses in other livestock. Nitrate, ammonia, and sulfate may react with proteinaceous compounds to enable the formation of abiotic pathogenic nanoparticles which were constant findings in pathognomonic placental lesions associated with non-infectious fetal losses of previously unknown etiology in mares, chickens and other livestock. The pathogenic nanoparticles may be produced naturally by toxic elements associated with air pollution that affect pasture forages or crops, unintentionally by reactions of these elements in protein-mineral mixes in dietary rations, or endogenously within tissues of fetuses and adult animals. The nanoparticles may form niduses in small vessels and predispose animals to a host of secondary opportunistic diseases affecting the reproductive, respiratory and gastrointestinal tracts of animals. The newly recognized abiotic pathogenic micro and nanoparticles are associated with MRLS. The discovery of the pathogenic nanoparticles led to the identification of nitrate, ammonium, and sulfur, in the form of sulfate, that seemingly enable the formation of the pathogenic nanoparticles in embryonic and fetal tissues.

1. Introduction

Embryonic and fetal losses in livestock cause significant reproductive failures in livestock. There has been a lack of progress in determining the cause of non-infectious embryonic and fetal losses in mares and other livestock. Fertile chicken eggs as an alternative for large animals provides findings in multiple non-infectious disorders that are associated with mare reproductive loss syndrome (MRLS) as well as other livestock. The etiopathogenesis of non-infectious embryonic and fetal losses in livestock was found to be associated with toxic elements in air pollution and dietary ingredients. Nitrate, ammonia, and sulfate were found to induce pathogenic nanoparticles and embryonic and fetal losses in mares and other livestock.

The fetal loss syndrome associated with non-infectious abortions of unknown etiology in the mare has plagued the horse industry for over a century (Dimock et al., 1947). However, recent findings with chickens and their fertile eggs have now suggested an etiology for these non-infectious fetal losses in mares. Fertile chicken eggs as an alternative model for large animals were used to study the effects of dietary ingredients on reproductive rates in mares and other livestock. It was found that chickens were naturally infected with the same entity that is associated with MRLS and fetal losses in other livestock (Swerczek and Dorton, 2019).

There is a lack of information regarding the factors associated with fetal losses of non-infectious causes in livestock primarily because of the problematic factors and difficulties experimenting with large animals. There is a move to reduce or eliminate animals for testing potential toxic elements in dietary ingredients. Accordingly, alternative experimental research methods to replace animals, especially large animals, are a goal. Fertile chicken eggs were proven to be an excellent alternative model to replace large animals for experimental reproductive research in mares and other

E-mail address: twswer1@uky.edu.

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livestock. Fertile chicken eggs were used to test the hypothesis that nitrate toxicity and nitrate-induced pathogenic nanoparticles were associated with MRLS. The results with fertile chicken eggs are not only relevant for chickens but other animal species, including mares, with previously unknown etiologies for non-infectious reproductive losses. This report provides additional research methods that suggest that pathogenic nanoparticles in fertile chicken eggs with embryonic and fetal loss are associated with climatic elements and dietary factors in rations of poultry and diets of other animal species.

2. Review of literature on fetal losses of unknown etiologies in mares

The early fetal loss syndrome has been investigated in the mare more than other livestock affected with similar losses. Unraveling the cause of fetal losses in the mare and other animals has been unrewarding for investigators worldwide. Several retrospective studies on equine abortions were gathered from different areas of the world with fetal losses of unknown etiologies (Du Plessis, 1964, Van Niekerk, 1965, Mahaffey, 1968, Bain, 1969, Platt, 1975 Chevalier-Clement, 1989, Tengelsen et al., 1997, Ricketts et al., 2001, Smith et al., 2003. and Ricketts et al., 2003.) Also, surveys from central Kentucky (Dimock et al., 1947; Prickett, 1970; Swerczek, 1980; Swerczek, 1990; Swerczek, 1991; Hong et al., 1993; Giles et al., 1993; Swerczek, 2006) reported similar non-infectious fetal losses.

In an early study of fetal losses in central Kentucky, involving 1,150 equine fetuses from 1921 to 1947, 38% were of unknown non-infectious etiology (Dimock et al., 1947). In a more recent study of fetal losses from the same central Kentucky area from 1987 to 1991, involving 3,527 fetal losses, over 70% had an unknown non-infectious etiology (Giles et al., 1993). Within the 70% of the non-infectious fetal losses of unknown etiology, the overwhelming majority of the fetal losses included disorders associated with MRLS. These included congenital hypothyroidism, congenital anomalies, musculoskeletal malformations, contracted foal syndrome, malformations of the umbilical cord, placental edema, fetal diarrhea syndrome, among others, all of which are now suspected to be associated with nitrate toxicity, and nitrate-induced pathogenic nanoparticles. Also, others, including secondary opportunistic disorders involving bacterial, nocardiform and fungal infections of the placenta are also suspected to be associated with nitrate-induced pathogenic nanoparticles. Seemingly, the number of fetal losses of unknown etiology with pathognomonic lesions consistent with MRLS is emerging in recent years (Swerczek, 2006).

The first spike in fetal losses of unknown etiology in mares was recorded in 1980 (Swerczek, 1980), and a similar unprecedented fetal loss syndrome occurred again in 2001 and was referred to as MRLS (Swerczek, 2002; Taylor, 2002; Cohen et al., 2003). A newly recognized condition in Australia and referred to as equine amnionitis and fetal loss (EAFL) (Perkins et al., 2006) has pathologic lesions and microbiologic findings similar to those reported for MRLS. Many of the fetal losses in mares of unknown etiologies have routinely occurred sporadically each foaling season in central Kentucky and elsewhere. The individual occurrences of different disorders have made it difficult to determine their etiology. However, because of the unprecedented massive fetal losses referred to as MRLS that occurred in the spring of 2001 and affected several farms at the same time with multiple fetal loss disorders, including early fetal losses in several thousand mares, a common etiology was suspected. The hypothesis for the etiology of the non-infectious fetal losses in 1980 and 2001 was nitrate toxicity associated with sudden climatic changes that affected pasture forages over a wide geographic area (Swerczek, 2006). Nitrate toxicity, associated with frosts and freeze damaged pastures, was found to be associated with nitrate-induced pathogenic nanoparticles that were found in tissues of multiple fetal loss disorders in mares and other livestock and likely the same disorders are occurring worldwide.

Even though these reports are primarily associated with fetal losses of unknown etiology in mares, other animals including chickens, were subsequently found to be naturally affected with the same entity as mares. The percentage of eggs hatching broiler chickens fell to the lowest level in the USA in a decade. This has resulted in $121 million in lost sales for the poultry industry over the first 5 months alone in 2017 (Bunge, 2017). A recent report involving fetal losses in mares from central Kentucky identified toxic levels of nitrate, ammonia, sulfate, and toxic pathogenic nanoparticles induced by these elements as the suspected etiology for these previously unknown causes of fetal losses in the mares, chickens and other livestock (Swerczek and Dorton, 2019). This report provides additional laboratory methods and findings with fertile chicken eggs to support the hypothesis that nitrate toxicity and nitrate-induced pathogenic nanoparticles are associated with MRLS.

3. Materials and methods

This study was approved by the Institutional Animal Care and Use Committee of University of Kentucky.

3.1. Fertile chickens' eggs were used as an alternative model for large animals for investigating fetal losses in mares and other livestock

Fertile chicken eggs were proven to be an ideal alternative model for large animals to study fetal loss syndrome in mares and other livestock. The fertile eggs for these studies came from 3 different sources: Farm 1, Battery-caged poultry farm; Farm 2, Free-range organic poultry farm; Farm 3, Backyard free-range organic poultry farm.

3.2. Rations used for laying hens producing fertile eggs for diagnostic investigations

Since toxic dietary ingredients were suspected to be associated with fetal losses in chickens producing fertile eggs, feed ingredients were analyzed. Farm 1 complete ration consisted of high-protein dehydrated alfalfa meal, soybean meal, and corn. The ration was supplemented with vitamins, minerals and yeast supplements. Farms 2 and 3 fed their laying hens genetically modified organisms (GMO)-free organic chicken rations. The complete rations of the 3 farms contained proprietary mineral supplements and the specific ingredients were not available. Farms 2 and 3 were not feeding any alfalfa meal as a protein source to their laying hens.

3.3. Breeding of hens for fertile eggs for diagnostic investigations

The fertile eggs from Farm 1 were from artificially inseminated hens maintained individually in battery cages. Fertile eggs form Farms 2 and 3 were from naturally bred free-range hens. Fertile eggs from Farm 1 were obtained weekly for diagnostic research investigations for suspect pathologic agents in fetal loss specimens obtained from necropsy examinations.

3.4. Laboratory diagnostic methods for investigating embryonic and fetal losses

Samples of feed supplements and fetal specimens were analyzed for pathogenic microorganisms that may affect fertility

and fetal losses. Laboratory culture methods included routine bacteriologic culture techniques for bacteria and fungi, routine tissue culture techniques for viruses, DNA analysis for pathogenic microorganisms, histopathologic and transmission and scanning electron microscopic analyses of fetal tissues and feed samples. Fertile chicken eggs were used to test for suspect uncultivable microorganisms that would not grow in routinely used cultural media.

3.5. Preliminary testing for micro and nanoparticles in suspect specimens

Suspect specimens for the identification of pathogenic nanoparticles were preliminarily tested directly or diluted in commercial deionized sterile water. A small sample smear was made on a glass slide (25 mm wide by 75 mm long) and stained with bacteriologic stains, and tissue stains for the presence of biotic microorganisms and/or abiotic pathogenic micro and nanoparticles. Eosin B tissue stain was found to be an ideal tissue stain to reveal the micro and nanoparticles. The stained micro particles in coccoid and/or diplococcoid forms could be observed with a light microscope under oil emersion of 1,000× magnification. If positive, they were confirmed with further transmission electron and/or scanning electron microscopic examinations. Scanning electron microscopic examinations were ideal for demonstrating the micro and nanoparticles using high powers. For the demonstration of the pathogenic nanoparticles with scanning electron microscopes, either a direct or diluted sample in commercial deionized sterile water were placed on SEM pin stub specimen mounts and air-dried at room temperature, or a low temperature in a dehydration oven and maintained in a dehydrated state until evaluated. No stains were necessary to image the pathogenic nanoparticles (Fig. 1) with scanning electron microscopes.

3.6. Inoculation of early developing chicken embryos

The fertilized chicken eggs from each farm were incubated by standard recommended temperatures and humidity and monitored for fertility by candling each day starting on d 6 of incubation. Seven-day-old chicken embryos were inoculated in ovo into the yolk sac with 1 mL of sterile amnionic fluid from suspect fetuses with lesions consistent with MRLS. The amnionic fluid was filtered through a 0.45 Millipore filter to remove any potential bacteria or fungi. In addition to amnionic fluid other suspect infected materials, including minerals, plants, and feedstuffs ingredients, suspected to be infected with pathogenic nanoparticles, were diluted in commercial deionized sterile water, and heated for 30 min at 60 °C. The filtered fluid or heated suspect materials were tested for sterility by routine bacteriologic culture techniques for bacteria and fungi. Only processed specimens without evidence of biotic microorganisms were inoculated into fertile chicken eggs. The surface of the fertile chicken eggs was pre-sterilized with 70% ethyl alcohol and then inoculated in ovo into the yolk sac with a 1-mL sterile syringe with an attached 1.5-inch 19-gauge needle.

For ammonium sulfate and other sulfated and non-sulfonated minerals, each inoculum was first titrated to determine the minimum concentration that would not induce acute death to the chicken embryo. Ammonium sulfate, and other sulfated and non-sulfated minerals were certified laboratory grade, anhydrous, and were obtained from Fisher Scientific Laboratories. For each test, a minimum of 3 fertile eggs was inoculated for a positive or negative effect. The tests were presumed positive if all eggs developed pathognomonic lesions of edema and hemorrhage in the fetal membranes, and without any evidence of biotic infections. A minimum of 3 eggs, or more, for each test served as uninoculated controls.

4. Results

The conception rate of the fertile eggs from Farm 1 was near 100%. However, the embryonic and fetal losses from uninoculated eggs varied from month to month with minimal to marked losses of 50% or more. Starting at d 7 of incubation, fetal losses developed foci of hemorrhage around the vessels of the vitelline membrane (Fig. 2), then developed widespread hyperemia and hemorrhage progressed throughout the vessels of the vitelline membrane, amnion and allantois. The foci of hemorrhagic lesions in the vessels of the vitelline membrane were similar to those in the placental membranes of early developing equine fetuses with MRLS (Fig. 3).

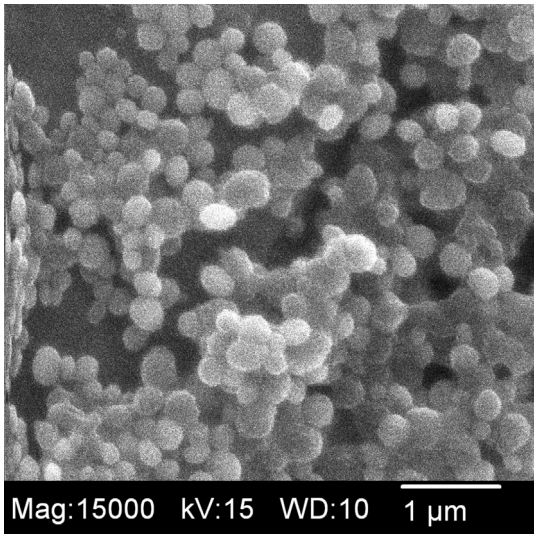

Fig. 1. Micro and nanoparticles in amnionic fluid from an equine fetus from Thoroughbred Farm A with lesions consistent with mare reproductive loss syndrome (MRLS). Note how the larger micro particles are dividing into 2 smaller particles. With each division the particles become smaller to form nanoparticles less than 100 nm in diameter. Scanning electron microscopic image. 15,000× magnification.

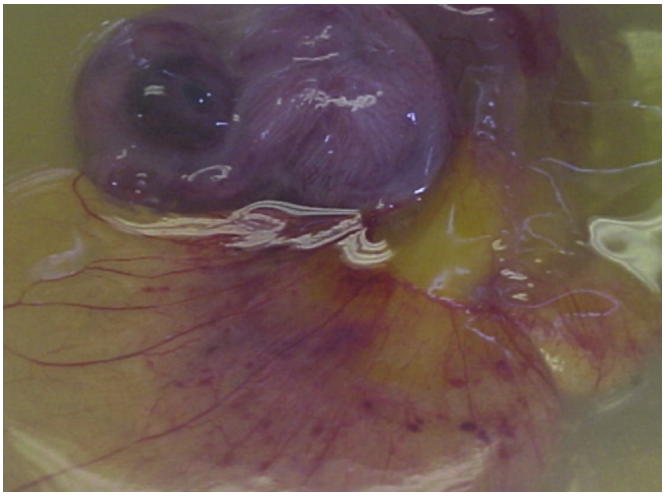

Fig. 2. Fifteen-day-old chicken fetus from Poultry Farm 1 with hemorrhage and edema in the vitelline membrane. The vascular lesions in the fetal membranes are consistent with those present in early developing equine fetuses affected with MRLS (Fig. 3). The pathogenic nanoparticles were present in fluid of the fetal membranes. MRLS = mare reproductive loss syndrome.

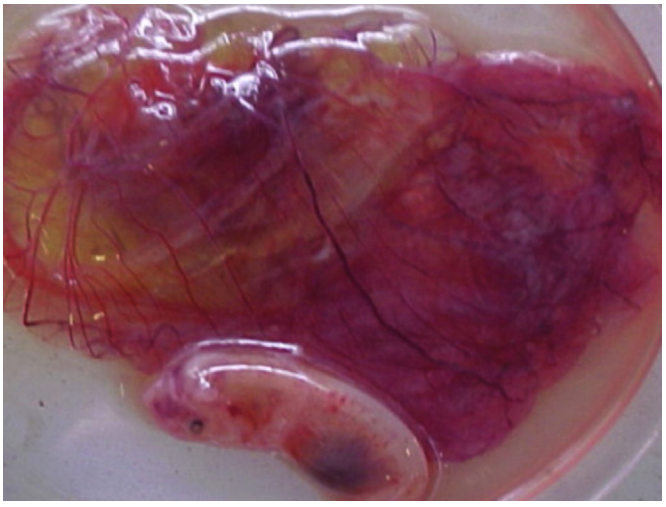

Fig. 3. Forty-day-old equine fetus with hemorrhage and edema in the amnion and allantois with lesions consistent MRLS. The pathogenic nanoparticles were present in the edematous fluids of the placental membranes. MRLS = mare reproductive loss syndrome.

Fig. 5. Term placenta from Thoroughbred Farm A. The allantochorion, upper right is congested and hemorrhagic. The amnion, lower left, is very thicken due to edema and stained with meconium from fetal diarrhea. The ammonic umbilical cord (on top of amnion and allantochorion) is thicken due to edema and funisitis. The gross lesions in the placenta are pathognomonic for MRLS. The pathogenic nanoparticles were present in the edematous amnionic fluid. MRLS = mare reproductive loss syndrome.

In severe cases, fetal death would pursue. In mild cases, the fetus would survive to normal hatching time. However, often the hatching time would be prolonged and the chicks would not hatch or would be weak at hatching with retention of their yolk sac, and very edematous amnion and allantois (Fig. 4). The retention of the yolk sac suggested that vascular lesions in the vitelline membrane prevented the absorption of its nutrient contents. The edematous amnion and allantois in affected chicken fetuses were like those present in the placenta in late-term equine fetal losses associated with MRLS (Fig. 5). In unaffected chicken fetuses, chicks were healthy at hatching with very little residual material ingredients in their yolk sac, and like unaffected equine fetuses, the amnion and allantois were very thin and not edematous or hemorrhagic.

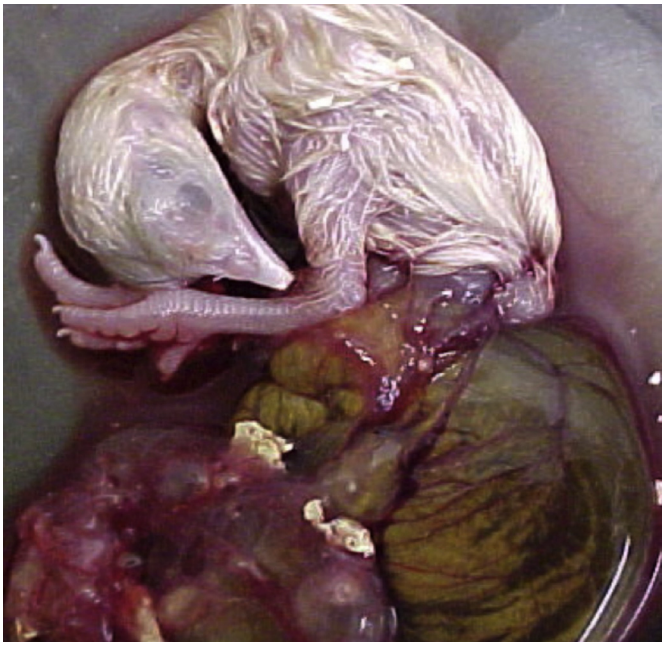

Fig. 4. Late developing chicken fetus from Poultry Farm 1 with hemorrhagic retained yolk sac, and marked edema and hemorrhage of the amnion and allantois. The vascular lesions in the placenta membranes are consistent with those found in placenta of late-term equine fetuses with MRLS as illustrated in Fig. 5. The pathogenic nanoparticles were present in the edematous and hemorrhagic fluids of the placental membranes. MRLS = mare reproductive loss syndrome.

If the chicken eggs were contaminated with biotic microorganisms, fetal deaths occurred within a few days of incubation. However, with the embryonic loss syndrome, the developing chicken embryos were seemingly healthy up to 7 d incubation. In the chicken embryos that were normal up to 7 d or more of incubation, no biotic microorganisms could be isolated. In affected chicken embryos, abiotic pathogenic nanoparticles were present in yolk sac ingredients. The nanoparticles (Fig. 1) were morphologically identical to those present in fetal amnionic fluid in equine aborted fetuses with lesions consistent with MRLS.

Analyses of corn, soybean meal, alfalfa meal, and vitamins did not reveal any significant findings or evidence of nanoparticles. However, nanoparticles, similar to those found in fertile eggs with embryonic and fetal loss, were present in the mineral supplement used in Farm 1 chicken rations. It was suspected that the minerals used in the Farm 1 chicken ration were in sulfated forms, which may have enabled the formation of the pathogenic nanoparticles, but this could not be confirmed as they were proprietary. The mineral supplements present in rations from Farms 2 and 3 were in complete rations and not available individually for analysis like those used in the chicken ration from Farm 1.

The chicken eggs from Farms 2 and 3 had a fertility rate between 50% and 70%. It was suspected that the ratio between laying hens and roosters was not optimum for a higher fertility rate. The embryonic and fetal losses similar to that occurred in eggs from Farm 1 were not present making them suitable, if fertile, for investigating the entity associated with MRLS and fetal losses in other livestock.

Normal healthy fertile chicken eggs like those from Farms 2 and 3, proved to be an ideal alternative model for large animals for experimentally reproducing the pathogenic nanoparticles in developing chicken fetuses. Since sulfur was suspected to be significant in the pathogenesis for the formation of the nanoparticles in fetal tissues and feedstuffs, sulfur compounds were investigated in vitro and in vivo. When eggs from Farms 2 and 3 were used, the sulfated minerals, when inoculated *in ovo* into the yolk sac, focal hemorrhagic vascular lesions developed in the vitelline membrane followed by early embryonic and fetal losses with pathologic lesions in vessels and the formation of nanoparticles consistent with

those found in uninoculated natural cases from Farm 1. The hemorrhagic and edematous lesions in the experimentally inoculated chicken embryos were like those in early developing equine fetuses associated with MRLS. Seemingly, nanoparticles developed and multiplied in the yolk sac material after the inoculation of sulfur compounds, then they were absorbed by the vascular of the vitelline membrane and lodged in the small vessels of the amnion and allantois and produced edema and hemorrhage. Ammoniums sulfate, and to a lesser extent other sulfated mineral, were found to produce the nanoparticles in vitro and in vivo. The non-sulfated minerals in the oxide and carbonate forms did not produce the pathogenic nanoparticles when inoculated in vitro in normal chicken yolk ingredient or *in ovo* into the yolk sac of normal fertile chicken eggs. The hemorrhagic and edematous lesions in the chicken embryos and fetuses were like those in the placental membranes of equine fetuses with pathognomonic lesions consistent with MRLS.

5. Discussion

Unraveling of the etiologic factors associated with the MRLS and other non-infectious fetal losses in animals was aided by many clinical observations on affected farms, necropsy examinations of fetal losses, and helpful serendipitous events over several decades. Very little progress was made in understanding the pathogenesis of the pathognomonic lesions present in placental membranes and fetuses until the discovery that chickens were also naturally affected with the same entity as mares with MRLS. The use of fertile chicken eggs proved to be an ultimate alternative model for large animals to study the etiopathogenesis of a mysterious fetal loss syndrome that affects most livestock, primarily mares and cows, but to lesser extent sows. Exhaustive attempts over several decades failed in isolating any biotic microorganisms that were consistent with the pathognomonic lesions in placental membranes or fetuses. Because of these failures, it was suspected that the etiologic agent was an uncultivable microorganism in artificial media. For this reason, fertile chicken eggs were used as an in vivo technique in an attempt to recover the etiologic agent that seemingly was uncultivable in artificial media. However, in recent years, fertile chicken eggs that were routinely used for diagnostic investigations from Farm 1 for several years emerged with a similar fetal loss syndrome as other livestock (Figs. 2 and 4). Researchers in other departments that used the eggs from Farm 1 also found that the eggs developed similar mysterious fetal losses, making them problematic for their research investigations. Because of the fetal losses in fertile eggs from Farm 1, other sources of eggs were obtained from Farms 2 and 3, for investigating the suspect etiologic agent for the fetal loss syndrome in other livestock.

Since chickens and their fertile eggs from Farm 1 were found to be naturally affected with seemingly the same entity that is associated with MRLS, their use became problematic, unless non-affected eggs from other non-affected farms could be obtained for use in an attempt to recover the suspect abortigenic agent of unknown characteristics. Therefore, other diagnostic methods, including electron microscopic examination were used determine the nature of the suspect abortigenic agent. After many transmissions and scanning electron microscopic evaluations of fluids from affected fetal losses with MRLS, pathogenic nanoparticles were consistently present in pathognomonic placental lesions consistent with MRLS.

The findings with fetal losses with fertile chicken eggs from Farm 1 proved to be valuable regarding the etiology for the fetal loss syndrome for mares and other livestock. Also, the relatively recent occurrence of the fetal losses in fertile chicken eggs from Farm 1 suggests that it is an emerging syndrome, like MRLS, that may be associated with climate changes that are affecting plants and crops, and/or ingredients in rations and methods by which dietary rations are currently being formulated. Since fertile chicken eggs from Farm 1 were found to be naturally infected with the entity causing embryonic and fetal losses, chicken eggs from a source free of fetal losses were found to be an ideal model for isolating and confirming the suspect etiologic agent that was naturally affecting chickens and other livestock.

Based on epidemiologic investigations over several years, the fetal loss syndrome in mares and other livestock was found to be associated with high-protein feedstuffs, and protein-mineral supplements, but the nature of toxicity was unknown. Analyses of pasture forages and feedstuffs were of little value as potentially toxic elements were endless. However, analysis of pond water from rainwater runoff from a cattle feedlot that cattle drank provided a valuable serendipitous hint. Cattle broke out of the nearby feedlot and drank feedlot runoff rainwater in a containment pond, and were returned to the feedlot, but all acutely developed opportunistic diseases similar to those found in fetuses with MRLS and other the fetal loss disorders in livestock. The cattle within the feedlot that did not drink the contaminated pond water were not affected. Analysis of the seemingly toxic pond water revealed high levels of potassium, nitrate, ammonia, and sulfur. No other potentially toxic elements were found in the pond water. The hint that high-sulfur in pond water may be toxic concurs with similar findings found on Thoroughbred Horse Farm A that had high sulfur drinking water for their pregnant mares. Also, the horse farm also had a history of feeding high-protein alfalfa hay, which is also high in sulfur, to their pregnant mares. Farm A had a history of several mysterious equine fetal losses, consistent with MRLS, including congenital hypothyroidism, congenital anomalies, malformed umbilical cords, musculoskeletal abnormalities, contracted foal syndromes, fetal diarrhea syndrome, placental edema, among others that included apparent secondary opportunistic bacteria, nocardiform and fungal infections of the placenta. Following these clues that sulfur may be significant and associated with fetal losses in livestock, retrospective analyses of suspected toxic high-protein feedstuffs and protein-mineral supplements associated with fetal losses of unknown etiology were performed. These suspected feedstuffs also contained high levels of the same elements, especially sulfur, similar to those found in the contaminated, high-sulfur pond water.

Toxic levels of potassium, nitrate, and ammonia were previously suspected to be associated with the fetal loss syndrome, but sulfur was not routinely tested for like the other elements and was previously overlooked. Then, sulfate, along with nitrate and ammonia, in dietary ingredients were shown by in vitro testing in normal equine amnionic fluid to be associated with the formation of the insoluble pathogenic nanoparticles that were found to be associated with fetal losses in mares and other livestock. Also, sulfate was recently confirmed to be an essential element by other international investigators to be associated with the formation of nanoparticles in air pollution (Wang et al., 2016). Nitrate, ammonia or sulfate alone likely would not produce the pathognomonic lesions that are seen with the mysterious fetal loss syndrome since the lesions are suggestive of a pathogenic microorganism, and not a toxic element *per se*. However, these elements together, like ammonium sulfate, may react with other proteinaceous compounds. Insoluble, toxic pathogenic nanoparticles may form and induce pathognomonic lesions identical to those found in naturally and experimentally infected chicken eggs and fetal tissues in mares and other livestock.

Natural nanoparticles may be inorganic or organic matrixes of minerals and a complex of amino acids, peptides, proteins, or other compounds that are less than 100 nm in diameter. Seemingly,

nanoparticles associated with fetal loss syndrome are endogenously formed matrixes that are induced by toxicities to proteinaceous components of amnionic fluid and plasma of developing fetuses. The composition of the toxic pathogenic nanoparticles has yet to be determined but they likely consists of an insoluble matrix of mineral and nitrogenous elements. Their formation in vivo in the amnionic fluid and plasma may be due to unintended consequences of sulfate mineral-induced precipitation of amnionic and serum proteins by toxic levels of ammonium sulfate and other sulfated minerals present in the diet of affected animals.

Remarkably, the abiotic pathogenic nanoparticles mimic pathogenic biotic microorganisms in their morphology and their ability to produce pathologic lesions. Unlike biotic microorganisms, the abiotic particles lack organelles and DNA, but due to their minute size, they may lodge in small vessels and induce vascular lesions and/or niduses for a host of secondary opportunistic diseases. Only the secondary opportunistic microorganisms were isolated from fetal loss specimens and the pathogenic nanoparticles were overlooked as the primary etiologic agent The pathognomonic lesions associated with the fetal loss syndrome resemble that of a specific pathogenic microorganism, but etiopathogenesis does not fit a biotic pathogenic microorganism. The reason being, biotic microorganisms are often species-specific and likely would not concurrently affect multiple different animal species worldwide. However, universally occurring toxic elements in air pollution (Wang et al., 2016) that were found to be associated with the formation of nanoparticles may concurrently affect multiple animal species.

Common bacterial species associated with the pathogenic nanoparticles and MRLS were *Streptococcus* spp., primarily non-beta hemolytic and alpha hemolytic streptococci (Swerczek, 1980). These bacteria and other sulfur-oxidizing bacteria may be playing a role in oxidizing sulfur compounds to sulfate, which along with ammonia from excess nitrate are enabling the formation of pathogenic nanoparticles. Biological oxidation by bacteria of sulfur compounds to sulfate is one of the major reactions of the global sulfur cycle (Friedrich et al., 2001).

Nitrogenous elements, including nitrate, ammonia, and urea have been suspected to be associated with early embryonic and fetal losses in livestock and they may induce fetal losses (Taylor, 2002; McEvoy et al., 1997; Hammon et al., 2002). Seemingly, these nitrogenous elements are involved in fetal losses, but in addition sulfate is necessary for the production of toxic and pathogenic nanoparticles. The endogenous nanoparticles are suspected to be associated with a host of mysterious opportunistic disease syndromes in plants and animals. Seemingly, early developing embryos and fetuses of herbivores and other animals are very susceptible to the pathogenic effects of the toxic pathogenic nanoparticles. It is suspected that pathogenic nanoparticles from air pollution and dietary supplements may also be associated with the decline of insects, including honeybees, and birds, as they are exposed to the same environmental toxic elements and dietary ingredients as livestock (Swerczek and Dorton, 2019).

Fertile chicken eggs from Farm 1 were used for diagnostic investigations since 1970, and before by other investigators. The fetal loss syndrome in chicken eggs seemingly emerged to a significant degree during the early 2000s and coincides with the emergence of fetal losses in mares during the same period. It is suspected that the ingredients in the chicken ration for the Farm 1 laying hens were changed, or the processing of the ingredients was changed and promoted the production of the pathogenic nanoparticles. The fetal loss syndrome, since it is affecting several different species, is also suspected to be associated with the increase in universal toxic elements in the environment that affect climate change and pasture forages and subsequent production of pathogenic nanoparticles in mares and other livestock.

There has been a recent increase use of fermentation by-products as an alternative source of protein. In addition to nitrogenous compounds, these alternative sources of protein may contain excessive sulfur compounds. Also, sulfated minerals have recently replaced some of the non-sulfated minerals in mineral supplements. The reason being, they are thought to be more readily available, however, they are more soluble and reactive, and may be reacting with nitrogenous compounds, to form toxic and insoluble pathogenic nanoparticles. The increase in nanoparticles in the environment may also be associated with high levels of nitrate, sulfur dioxide and sulfate in air pollution and rainwater that may affect pasture forages and crops.

Without finding the potentially toxic nanoparticles, it would have been problematic to determine which toxic elements were significant for the etiology of the mysterious fetal loss syndrome, and other secondary opportunistic diseases of livestock. By experimentally reproducing similar nanoparticles in vitro and in vivo in proteinaceous substrates with the suspect toxic elements led to the identification of nitrate, ammonia, and sulfate as necessary elements in dietary ingredients and the environment to enable the pathogenic nanoparticles to form.

In livestock, primarily mares and cows, based on extensive epidemiologic investigations, in addition to the high nitrogenous forages and feedstuffs, animals supplemented with high-protein alfalfa were found to be more susceptible to fetal losses. Alfalfa contains abundant sulfur-containing amino acids and other sulfur-containing compounds (Steward et al., 1951). The feeding of high-protein alfalfa, nitrate, ammonia, and sulfate would be formed by the microbiome in the gut of animals. These elements would be available to produce the toxic and pathogenic nanoparticles. The laying hens from Farm 1 were being fed a high-protein alfalfa meal and high-protein soybean meal while their fertile eggs were experiencing embryonic and fetal losses. Laying hens from Farms 2 and 3 were not being fed any alfalfa meal and were not experiencing embryonic and fetal losses. The feeding of high-protein feedstuffs and alfalfa to laying hens may be associated with the production of excessive nitrate and ammonia that would react with sulfate, and nitrogenous compounds, in the intestinal tract of chickens. Excessive ammonia in the feces is a common toxic element in poultry and other livestock, like horses, that are fed high-protein diets, especially high-protein alfalfa. Seemingly, ruminants can tolerate more ammonia than simple-stomach animals. That may explain why chickens and mares are more susceptible to fetal losses than ruminant animals if they are fed high-protein diets.

Fertile chicken eggs from Farm 1 were from hens in a closed caged environment and fed high-protein diets that were complete and not varied. The likelihood of excessive ammonia in a confined environment would be more likely than from hens from Farms 2 and 3 that were housed in an open range environment where the diet was varied and fresh air with less ammonia.

The pathogenic nanoparticles are widespread and ubiquitous, and are insoluble complexes of minerals and proteins, less than 100 nm in diameter, and are capable of inducing pathologic lesions in various body organs (Gatti and Montanari, 2008). The minute pathogenic nanoparticles may lodge in small vessels in the placenta and fetal organs, including the heart, inducing niduses for a host of opportunistic bacterial and fungal organisms like those commonly found with fetal losses in livestock. Heart failure in early developing equine embryos and fetuses was found to be the first clinical sign of impending death in fetuses with MRLS. The pathogenic nanoparticles, the inflammatory lesions in tissues of fetuses, because of the nature of the vascular lesions and edema present in affected tissues, suggested a primary pathogenic biotic microorganism, rather than a toxic element *per se* was suspected. The abiotic pathogenic nanoparticles explains the lack of success in isolating a

primary biotic microorganism from fetuses affected with the mysterious fetal loss syndrome.

Industrial-produced nanoparticles and extensive nanotechnology applications are relatively new (Gatti and Montanari, 2008). Their impact on human health has yet to be determined (Gatti and Montanari, 2008; McClements and Xlio, 2017). Endogenously produced abiotic pathogenic nanoparticles like those associated with pathogenic disease disorders associated with MRLS are newly recognized phenomena. The endogenously produced coccoid and diplococcoid suspect abortigenic agent was first observed in fetuses during the 1980 foaling season when there was a spike in fetal losses consistent with MRLS. The coccoid and diplococcoid suspect abortigenic agent was subsequently confirmed to be pathogenic micro and nanoparticles by scanning electron microscopic imaging.

Our recent findings suggest the mixing of minerals, especially the sulfate minerals, with proteins and protein by-products may result in unintended consequences due to the formation of the toxic and pathogenic nanoparticles within the mineral-protein mixes. This may occur naturally with the toxic environmental elements like nitrate, ammonia, and sulfate that are associated with air pollution (Wang et al., 2016) or unintentionally when these elements are formulated in complete feedstuffs. It is plausible that reactions that enable the production of the pathogenic nanoparticles may be exacerbated if dietary ingredients, like fermentation by-products, are heated, or if feedstuffs are heated during the pelleting of complete feeds.

Regarding nitrate toxicity, nitrate per se may not be toxic, as reported in the literature, but the metabolic products of nitrate, like nitric oxide, are. Excessive nitric oxide may lead to anoxia due to methemoglobinemia. Other metabolic products of nitrate, including ammonia, may react with sulfates and proteinaceous compounds to form pathogenic nanoparticles. Therefore, the toxic effects of nitrate toxicity are much more extensive than previously reported and are associated with a host of mysterious fetal losses, congenital abnormalities and nitrate-induced pathogenic nanoparticles that are associated with pathologic lesions in fetuses and adults. Also, the nitrate-induced pathogenic nanoparticle may induce secondary opportunistic disease disorders in many species, including mares and other livestock.

6. Conclusions

Mare Reproductive Loss Syndrome was found to not only affect horses but also chickens and other livestock. Accordingly, fertile chicken eggs were used as an alternative model for large animals to study the etiologic factors associated with fetal losses of unknown etiology in mares and other livestock. In mares, the several mysterious equine fetal losses, consistent with MRLS, include congenital hypothyroidism, congenital anomalies, malformed umbilical cords, musculoskeletal abnormalities, contracted foal syndromes, placental edema, fetal diarrhea syndrome, among others that include apparent secondary opportunistic bacteria, nocardiform and fungal infections of the placenta.

Epidemiological investigations, necropsy findings and experimental studies with fertile chicken eggs as a model have shown that these disorders associated with MRLS are consist with nitrate toxicity and nitrate-induced pathogenic nanoparticles, that are unique and novel findings. High-protein dietary ingredients, including nitrate byproduct ammonia, along with sulfur-containing compounds seemingly enabled the production of endogenously produced pathogenic nanoparticles. Nitrate toxicity has previously been reported to be associated with reproductive failure in livestock. However, the mechanism of action was poorly understood. The findings presented in this study confirm that nitrate toxicity indeed is a major cause of reproductive losses in livestock.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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