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REVIEW ARTICLE

Abstract

Cobalt, atomic weight 58.9, is a metallic element and environmental substance found in the animal in microgram quantities, predominantly as vitamin B12, but is also a component of at least one mammalian enzyme unassociated with B12. Cobalt is a required trace mineral and has long been administered as a dietary supplement to humans and animals. Cobalt deficiency outside of its requirement in vitamin B12 has not been reported in humans. The administration of cobalt salts was once standard treatment for anaemia in humans, owing to its ability to stimulate red blood cell synthesis. Elemental cobalt acts by stabilising hypoxia inducible factor (HIF-1α), which activates the erythropoietin gene, which in turn increases haemoglobin/red blood cell synthesis, which had led to a presumption that cobalt may be performance enhancing in athletes. Administration of cobalt in amounts sufficient to significantly increase the haematocrit are associated with risk of toxicity in humans, and the only cobalt administration study in horses showed no effect on red blood cell parameters or toxicity. Because of the perception that cobalt administration may enhance athletic performance, racing regulators have recently begun to restrict cobalt use in horseracing which has led to the introduction of cobalt thresholds in several racing jurisdictions. The International Federation of Horseracing Authorities is considering an international regulatory threshold for cobalt of 100 ng/ml in urine, based on studies performed in five different countries. In the United States, the Racing Commissioners International has recently set a primary plasma threshold of 25 ng/ml and secondary threshold of 50 ng/ml. One New York and New Jersey racetrack owner has initiated testing for cobalt and has denied his facilities to trainers whose horses tested positive for excessive quantities of cobalt. This review seeks to summarise what is known about the use of cobalt in horse racing.

Keywords: vitamin B12, horseracing, cobalt, HIF-1α

1. Introduction

Cobalt is a metallic element with chemical properties similar to those of iron and nickel. Discovered in 1735, its name comes from either 'kobold', German for 'goblin', or from the Greek 'cobalos', meaning mine (Schroeder *et al.*, 1967). German miners discovered cobalt in association with arsenic, and it is still commonly found as arsenide ore. Cobalt comprises 0.0029% of the earth's crust. Cobalt is in worldwide demand for its uses in superalloys, batteries and magnetic products (FSA, 2014). The mining of cobalt and its industrial and medical uses can increase local environmental cobalt concentrations to unpredictable concentrations. For example, in the Peruvian mining community of Cerro de Pasco, excessive cobalt exposure in combination with high altitude is reported to contribute to excessive erythrocytosis (Jefferson, 2002). As a result of its toxicity, particularly in combination with other factors, such as arsenic and high altitude, the European Food Safety Authority (2009) has suggested an acceptable safe amount for humans of 600 µg cobalt per day and the US Environmental Protection Agency (EPA) has proposed a chronic oral reference dose of 0.3 µg/kg per day.

Cobalt is present in biological systems predominantly as a component of vitamin B12 (Figure 1), which is a key cofactor in purine synthesis. Inorganic cobalt is a nutritionally required element with one of the lowest dietary requirement

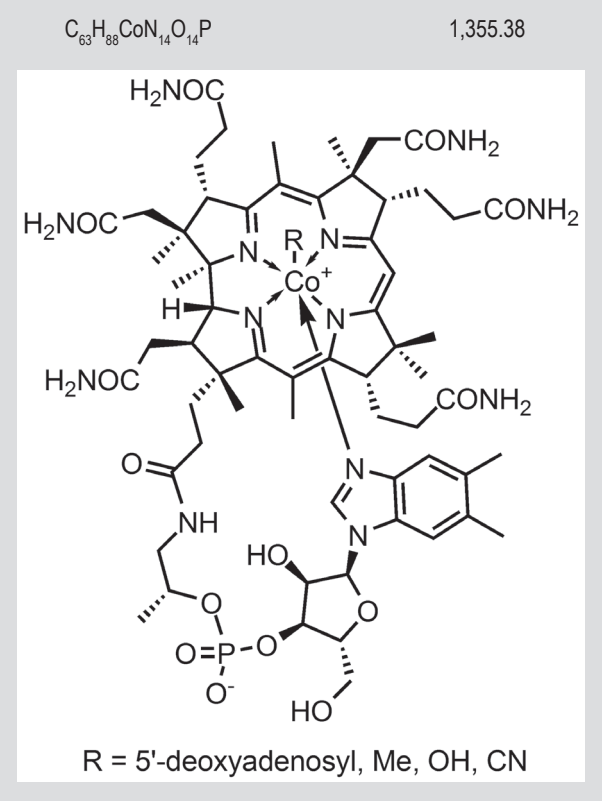

Figure 1. The structure of vitamin B12, also known as α-(5,6-dimethylbenzimidazolyl)cobamidcyanide.

of all of the required trace minerals as a critical component of only a few enzymes outside its role within vitamin B12. Recommended daily allowances for inorganic cobalt are not established in humans, although the RDA is about 0.1 µg/day in the form of vitamin B12 (Unice *et al.*, 2012). The National Research Council, which determines daily requirements of nutrients for animals, has determined a dietary cobalt requirement of 0.5 to 1.1 mg/day in horses and 1.2 to 2.4 mg/day for cattle, depending upon weight and metabolic state, with intake not to exceed 25 mg/kg of feed on a dry matter basis (Lawrence *et al.*, 2007).

Cobalt concentrations in drinking water typically range from 0.1-5 ng/ml, and daily cobalt intake ranges from 11-580 µg/day in humans (Schroeder, 1967; Unice *et al.*, 2012). Legumes, such as alfalfa or clover, are principal sources of cobalt in the natural diet of horses, and there can be widely varied concentrations of cobalt in forages across different geographical locations in the United States and internationally (Herbert, 1996; Kubota *et al.*, 1987). Generally, the normal diet of animals and humans contains adequate quantities of cobalt to supply that needed for synthesis of vitamin B12 and good health. However, the soils of some geographical regions (for example, some areas of New England, Florida, and the Atlantic coastal region of the United States) may be deficient in naturally occurring cobalt. Deficient soils can result in profound cobalt deficiencies in some ruminants (sheep and cattle), resulting in anaemia, weight loss and unthriftiness or 'wasting disease' (Underwood, 1975). Treatments with oral cobalt salts or vitamin B12 injections have been shown to alleviate the symptoms of this disease, indicating that the disease was a vitamin B12 deficiency resulting from lack of cobalt in the animals' diets, making it impossible for them to synthesise adequate quantities of vitamin B12. There are no scientific reports of either cobalt deficiency or toxicity in horses.

Occupational environmental exposure of humans to potentially toxic quantities of cobalt are usually due to industrial processes and occur during mining, the production of cobalt powder, the processing of cobalt alloys, and various industrial uses of cobalt, including the polishing of diamonds with cobalt containing discs, contact with cobalt powder and the use of cobalt pigments in decorative porcelain. Industrial exposure to cobalt, especially in association with other hard metals and related substances which may modulate the biological activity of cobalt, may give rise to significant long term problems (Donaldson and Beyersmann, 2005). Toxicity occasionally results from cobalt containing orthopedic implants, such as artificial hips (Bradberry *et al.*, 2014).

2. Cobalt and the B12 vitamins

In living organisms, cobalt is primarily found complexed with cobalamin, vitamin B12. The cobalamins were originally synthesised by Archaea and Bacteria about 3.5 billion years ago, and they remain the only organisms that synthesise cobalamins. Cobalt is found in vitamin B12, cobalamin (Figure 1). The B12/cobalamin structures are the largest and most complex of the vitamins, with a molecular weight of 1,355. Cyanocobalamin contains about 4% cobalt. The active cobalamins found in the human body are methylcobalamin and desoxyadenosylcobalamin. Unlike cobalt, the cobalamins are, for all practical purposes, non-toxic.

Cyanocobalamins, the generally available form of vitamin B12, and hydroxycobalamin are rapidly transformed in the body into methylcobalamin or deoxyadenosylcobalamin, the active forms. The daily requirement for an adult human is about 2-3 µg of cobalamin (Jelkmann, 2012), and hepatic cobalamin stores, about 3 mg, represent sufficient stores for about two to three years. Together with folic acid, the B12 cobalamin vitamins are essential for DNA synthesis and mitochondrial function. Cobalt is also found as a required co-factor in the active centre of at least two other cobalt containing enzymes. The first of these enzyme systems is methionine aminopeptidase-2, a ubiquitous enzyme found in humans and other mammals, where it cleaves N-terminal methionine from many newly translated polypeptide chains;

the second is nitrile hydratase, a bacterial enzyme that metabolises nitriles (FSA, 2003).

Most mammals, including humans, do not synthesise the B12 vitamins but rather acquire them pre-formed either from diet or by absorption of the B12 produced by intestinal bacteria. The cobalamin vitamins are specific micronutrients for humans and animals, and are absorbed by a complicated process. The salivary glands secrete haptocorrin, a glycoprotein which binds to cobalamins protecting them from degradation in the stomach. The haptocorrin-cobalamin complex is dissociated in the stomach, where the cobalamins are released and bind to 'intrinsic factor' (IF) which is a glycoprotein produced by the parietal cells of the stomach. The IF protects the cobalamin from digestion in the intestines and releases it for absorption by a pinocytotic mechanism in the ileum. A defect anywhere along this pathway results in vitamin B12 deficiency and pernicious anaemia in humans (Rojas Hernandez and Oo, 2015). This complicated human vitamin B12 absorption system is presumably more efficient than direct synthesis, likely because of the energetic costs of cobalamin synthesis. Generally, a normal diet provides sufficient quantities of cobalt to meet the needs of the animal. Cobalt deficiency, apart from its role in vitamin B12, has never been reported in humans (Taylor and Marks, 1978). As humans age, intestinal absorption of B12 vitamins declines and a significant percentage of older people (especially vegetarians) may suffer from pernicious anaemia (Jelkmann, 2012).

3. Bioavailability of cobalt

Oral absorption of cobalt salts varies depending on the dose, compound, nutritional status of the individual, and proximity in time to a meal (Tvermoes *et al.*, 2013) and cobalt is also absorbed by inhalation (Lauwerys and Lison, 1994). Gastrointestinal absorption of cobalt salts varies depending on the compound administered and the presence of other substances. For example, less than 5% of an oral dose of cobalt oxide is absorbed, compared with 30% of an oral dose of cobalt chloride, as determined from rodent studies. It has been reported that increasing the dose of cobalt does not tend to cause significant accumulation as increasing doses results in a smaller proportion of the dose being absorbed. In humans, about 80% of ingested cobalt is excreted in the urine and about 15% in the faeces (Liu *et al.*, 2008). These percentages vary with species and the cobalt compound ingested. Cobalt ions are actively absorbed across the small intestinal lumen by a divalent cation transporter (Powell *et al.*, 1999) which also transports other divalent cations, such as iron, copper and zinc, and operates under the influence of vitamin D (Schwalfenberg and Genuis, 2015).

4. Inorganic cobalt and red blood cell formation

Inorganic cobalt has other effects on red blood cell formation besides its role as a constituent of vitamin B12. In the 1930s, administration of cobalt was found to effectively stimulate red blood cell formation which led to the use of cobalt administration at doses of 25-300 mg/day as the standard medical treatment for anaemia in humans (Ebert and Jelkmann, 2014). More recently, however, this medical use of cobalt became obsolete (Reichel and Gmeiner, 2009) following the availability of human recombinant erythropoietin (rHuEPO).

The efficacy of cobalt in stimulating red blood cell production is reflected in the manner in which erythropoietin (EPO) concentration or quantity is expressed. Originally, one EPO unit (U) was defined as the quantity of EPO having the same erythropoiesis-stimulating response in rodents (historically, fasted rats) as 5 micromoles of $CoCl_2$. Today, EPO formulations are calibrated against international reference preparations of EPO, either purified from urine or standards from recombinant DNA-derived EPO (Sytkowski, 2004).

5. Mechanism of action of cobalt on red cell synthesis

Cobalt produces its effects on red blood cell synthesis by increasing blood concentrations of EPO. In sufficiently high plasma concentrations, cobalt ions act by binding to and inhibiting the enzyme which regulates the breakdown of an intracellular protein called hypoxia inducible factor (HIF-1α), resulting in increased intracellular concentrations of the HIF-1α protein. Hypoxia inducible factors are the primary mediators of the body's response to low oxygen environments, and are constitutively expressed and degraded. During hypoxia, these factors are stabilised, preventing their breakdown, increasing their intracellular concentration, which promotes the activation of a cascade of hypoxia-induced gene transcriptions (Liu *et al.*, 2012). Among the HIF-1α effects from high plasma concentration of cobalt, which have been demonstrated in some species, is direct activation of the EPO genes, resulting in increased plasma concentrations of EPO.

The consensus from the literature, including studies in a variety of species, is that chronic blood concentrations of 300 ng/ml and less are not associated with haematological or toxicological effects, whereas chronic blood concentrations in excess of 300 ng/ml are associated with both haematological and toxicological effects (Finley *et al.*, 2012). The haematological effects from cobalt doses can be impressive in laboratory animals. Daily administration of 125 mg/kg cobalt chloride to adult mice resulted in an approximate 36% increase in haemoglobin concentration (Gluhcheva *et al.*, 2011). Similarly, Fisher (1959) reported

an increase in haematocrit, haemoglobin, and haemoglobin precursors in dogs when they were injected with cobalt. Lippi *et al.* (2006) reported that human exposure to 120-150 mg/day of cobalt chloride resulted in increases in haematocrit and haemoglobin, reaching up to 20% above pretreatment concentrations. In horses, however, a single intravenous (IV) dose of cobalt chloride containing 49 mg of cobalt ion (Knych *et al.*, 20154) had no effect on EPO concentrations or red blood cell parameters, and repeated doses of IV cobalt chloride containing 810 mg of cobalt ion (Waldridge, 2015) had no effect on red blood cell parameters.

6. Red cell formation, human recombinant erythropoietin and athletic performance

When rHuEPO became commercially available for the treatment of anemia, its use was rapidly adopted by those seeking advantage in competitive athletics. Athletic performance is directly related to maximal oxygen consumption ($\dot{V}O_{2max}$), which can be artificially manipulated by increasing the oxygen carrying capacity of the blood. The ability to increase red blood cell mass, stimulated by rHuEPO, resulted in its widespread adoption for use in attempts to enhance athletic performance, both by human athletes and in horse racing, as reported in the sports media. Activation of the EPO gene, in addition to its hematopoietic effect also reduces plasma volume through an effect on the renin/angiotensin system. This action further serves to increase the haematocrit and is comparable in its effect on oxygen delivery to the effect of EPO administration on red cell numbers (Lundby *et al.*, 2007). rHuEPO had additional properties favourable to athletes seeking to avoid detection of illicit use. Early rHuEPO formulations had short plasma half-lives, 'clearing' the blood within days, or less if the dose was small, while erythrocytes with a lifespan of up to 120 days, would remain in high concentration (Mørkeberg, 2013). Thus, rHuEPO was an ideal illicit performance enhancing substance, clearing the blood within days while its performance-enhancing effects lasted for weeks. These characteristics of rHuEPO were reportedly widely taken advantage of by some human athletes (Fiedler, 2014) well into the early years of the 21st century (WADA, 2014).

7. Cobalt use in attempts to enhance athletic performance

By 2000, testing technology was developed that could detect the illicit use of rHuEPO and it became more difficult for athletes to use it without detection (Fiedler, 2014; WADA, 2014). However, it was pointed out by Lippi *et al.* (2005, 2006) that cobalt, the original treatment used to increase human haematocrits in the treatment of anaemia, also had potential to be used/misused in athletics in the same way as administration of rHuEPO. This led to further speculation about the use of cobalt for enhancing athletic performance in both human (Mørkeberg, 2013) and equine (Paulick, 2014) endeavours.

If cobalt could increase endogenous EPO production with achievable equine doses, then this would potentially confer a competitive advantage in equine sports. Typical equine doses of cobalt which might be clandestinely administered with the intent of performance enhancement cannot be accurately determined, since this information is not commonly made available. However, dose rates (based on interviews by author C.K. Fenger with trainers on the condition of anonymity) appear to range from occasional 'low' doses of 100 up to 400 mg cobalt chloride, typically diluted in 1000 ml of saline and administered by slow IV drip. According to regulators with the Kentucky Horse Racing Commission, 'intelligence' has revealed that repeated 'high' doses of as much as 4 mg/kg (1,800 mg total dose for a typical 450 kg horse) of cobalt chloride have been administered by rapid IV bolus (Waldridge, 2015). However, neither a single modest IV dose of 100 mg cobalt chloride (49 mg cobalt ion, Knych *et al.*, 2015), nor a repeated high dose of 1.8 mg/kg cobalt chloride (810 mg cobalt ion for a typical 450 kg horse) every 4 days for 7 treatments (Waldridge, 2015) resulted in any haematologic effects in horses.

8. Other biological effects of cobalt

The potential toxicity of cobalt and its sub-cellular mechanisms of action, have been used to support a perceived need for regulatory restriction of cobalt use in horse racing. While the doses of cobalt experimentally administered to horses have not been shown to increase endogenous EPO concentrations or haematologic parameters (Knych *et al.*, 2015; Waldridge, 2015), there may be other biological effects of high doses of cobalt.

Researchers working with rodent models have shown that the administration of cobalt triggers responses beyond increased haematocrits. Studies in a variety of non-equine species show that the increased intracellular concentration of HIF-1α that results from increased cobalt concentrations activates master regulators of several hypoxia responsive genes that play key roles in facilitating adaption to hypoxia. In addition to the EPO gene, HIF-1α upregulates the genes of vascular endothelial growth factor (VEGF) (Suzuki, 2004), which is responsible for increasing muscle capillary density, myoglobin (Mb) whose main function is storage of oxygen in the muscle, and glucose transporters 1 and 3, which facilitate the transport of glucose into cells. HIF-1α also upregulates a wide array of key factors in glycolysis and energy metabolism as well as factors important for cell growth and viability. The resultant multitude of changes within the organism are designed to maximise energy availability and transfer in an oxygen deprived

environment (Semenza, 2014; Simonsen *et al.*, 2012). These resultant changes that permit HIF-1α to pre-condition the animal for survival in an oxygen poor environment have the potential to act in concert to affect athletic performance. The preconditioning of the tissues with HIF-1α, and presumably cobalt, enhance those tissues' ability to subsequently tolerate the stresses of intense exercise, a phenomenon which is amplified when cobalt is combined with exercise training (Saxena *et al.*, 2010; Suzuki, 2004). This preconditioning by cobalt is accomplished by the activation of up to 300 genes in addition to the EPO gene, a significant number of which could be directly related to athletic performance (Jelkmann, 2012). It is unclear whether typical doses reportedly administered to horses are capable of producing any of these effects.

9. Adverse reactions following cobalt exposure

Adverse reactions following cobalt administration in humans include nausea, vomiting, heart failure, hypothyroidism and goiter (Jelkmann, 2012). Cobalt given intravenously can cause increased blood pressure, slowed respiratory rate, tinnitus and deafness. With cobalt intake greater than 10 mg/day, signs of cardiomyopathy and congestive heart failure can be seen in humans. Animal studies have also shown myocardial degeneration from cobalt injections (Goyer and Clarkson, 2001). The normal human body burden of cobalt is about 43% in muscle, 14% in bone and the remainder in soft tissue. Organs with the highest reported concentrations of cobalt include the liver, kidneys, adrenals and thyroid. Oral administration of sufficient doses of cobalt to humans for the treatment of anaemia may cause goiter. Human necropsy data on cobalt treated humans have shown high cardiac cobalt concentrations. Smith *et al.* (2014) report that exposure to cobalt compounds produces cytotoxic and genotoxic effects in human lung fibroblasts depending on particle size and solubility of the cobalt compound.

Adverse reactions in horses from cobalt administration have not been specifically reported in the scientific literature. However, tremors, hyperhidrosis and colic have reportedly been associated with rapid IV bolus doses of 1,800 mg of $CoCl_2$ intravenously (RMTC, 2015; Waldridge, 2015). There are unsubstantiated anecdotal reports of sudden death on the racetrack among Standardbreds which reportedly had high concentrations of cobalt at post-mortem examination. However, any causal relationship between equine sudden death and cobalt has not been established.

10. Beer drinkers' cardiomyopathy and metal on metal implants

Two examples highlight the dangers of cobalt toxicity. In the early 1960s some North American brewers began adding cobalt to beer in order to stabilise the foam. Within months, a new type of cardiomyopathy was recognised. This 'beer-drinkers' cardiomyopathy' syndrome was soon recognised in various locations around the world (Kesteloot *et al.*, 1968). Based on post-mortem examinations of tissue, cobalt was a suspected toxin and Canadian medical investigators subsequently identified beer as the source of cobalt. This suggested that large numbers of individuals were at risk, since about 25% of the beer sold in metropolitan centres in the US during 1964-1966 contained cobalt (Alexander, 1969). The pathology of 'beer drinkers' cardiomyopathy' was distinct from and more complicated than simple cobalt toxicity. Factors considered important in contributing to the cardiotoxicity of the cobalt-laced beer were malnutrition, inadequate protein and vitamin (especially thiamine) intake, zinc depletion, and prior heart damage (Alexander, 1974). The total amount of cobalt to which the affected individuals were exposed was much less than those typically associated with cobalt cardiotoxicity, suggesting a synergistic effect from alcohol.

More recently, some metal-on-metal (MoM) hip replacement implants have been identified as a source of cobalt toxicity (Jones *et al.*, 1975). In two such cases, patients experienced symptoms such as dizziness, disorientation, nausea, vomiting, weakness, decline in cognitive function and memory, and hypertension. Serum cobalt concentrations were more than 100 times the expected range (Mao *et al.*, 2011).

11. Blood and urine concentrations of cobalt

Cobalt is bound extensively to albumin in the serum, with 5-12% of the cobalt in human serum present in the free ionised state (Simonsen *et al.*, 2012). Cobalt is taken up by red blood cells via calcium channels, where it binds irreversibly to haemoglobin (Simonsen *et al.*, 2011), a characteristic which could be exploited for forensic analysis of cobalt administration. There is not a significant difference between serum and plasma concentrations of cobalt in humans (Kasperek *et al.*, 1981). Typical serum/plasma cobalt concentrations in humans are less than 0.4 to 1 ng/ml, depending upon the study population (Barceloux, 1999; De Smet *et al.*, 2008; Tvermoes, 2013) and a population of research horses had baseline equine serum cobalt concentrations of less than 1 ng/ml (their LOQ) prior to cobalt administration (Knych *et al.*, 2015). Adverse health effects such as cardiomyopathy and vision or hearing impairment have been reported with human blood concentrations over 700 ng/ml. Reversible hypothyroidism and polycythemia were reported in humans at concentrations of 300 ng/ml and higher (Paustenbach *et al.*, 2013). Krug *et al.* (2014) reported typical human urinary concentrations of cobalt of less than 2.2 ng/ml. Human test subjects receiving oral 500 µg doses of cobalt chloride daily via dietary supplements had urine concentrations of cobalt between 40 and 318 ng/ml. Daily

doses of 500 µg of vitamin B12 did not produce urinary cobalt concentrations above 2 ng/ml. Some common and legitimate human dietary supplements were shown to raise urinary cobalt concentrations above 75 ng/ml and/or serum concentrations above 2 ng/ml. Krug *et al.* (2014) proposed different thresholds for race-day and out-of-competition samples in order to avoid any problems from elevated cobalt concentrations due to dietary supplements.

Cobalt is commonly measured in human serum to assess cobalt toxicity or metallic prosthetic implant wear. Serum cobalt concentrations of 1 ng/ml or greater suggest possible environmental or occupational exposure. Prosthesis wear, especially with MoM implants, is known to result in increased circulating concentrations of metal ions. If cobalt is ingested, serum concentrations greater 5 ng/ml suggest major exposure and likely toxicity. Modest increases (4-10 ng/ml) in serum cobalt concentration are likely to be associated with a prosthetic device in good condition. Serum cobalt concentrations greater than 10 ng/ml in a patient with cobalt-based implants suggest significant prosthesis wear (De Smet *et al.*, 2008).

Given recent publicity regarding the potential for use/misuse of cobalt in competitive athletics and the tendency of manufacturers to include cobalt in both human and animal dietary supplements and energy drinks, there has been increasing interest in the absorption, distribution, metabolism, and elimination of cobalt after its oral administration. Furthermore, given the perception on the part of some regulators that cobalt concentrations may need to be monitored and perhaps regulated, review of the literature in the human arena may well be helpful as the racing industry approaches the problem in horses.

Unice *et al.* (2012) developed a biokinetic model of cobalt uptake, blood concentrations and clearance in humans that provides the most satisfactory interpretational basis for cobalt toxicokinetics available to date. Following 190 days of oral supplementation in amounts from 0.4 to 1 mg/day, predicted blood concentrations ranged from 1.7 to 10 ng/ml, and urinary concentrations from 20 to 120 ng/ml. In humans, in the absence of supplementation, plasma concentrations of cobalt averaged about 0.4 ng/ml. Supplementation at 0.4 mg of cobalt per day drove plasma concentrations from baseline to 3.6 ng/ml, with a range of 1.7 to 10 ng/ml, and urinary concentrations from 20 to 120 ng/ml. Based on this model they estimated that supplementation at 1 mg/day for 1 year or more would yield blood concentrations from about 6 to 13 ng/ml and urinary concentrations from 65 to 150 ng/ml, consistent with published data, and suggesting that urinary cobalt concentrations will be about ten-fold greater than the corresponding plasma concentrations. These data, developed in 4 healthy adult males, were consistent with an earlier pharmacokinetic model.

Knych *et al.* (2015) administered a single 100 mg dose of $CoCl_2$ (49 mg cobalt ion) to 16 exercised horses and measured the serum and urinary concentrations of cobalt ion. Serum cobalt concentrations were best described by a three compartment model with an initial rapid decline with rapid alpha and beta phase half-lives, followed by a much longer terminal (gamma phase) half-life of approximately 156 hours. Intravenous administration of this dose of cobalt had no effect on serum erythropoietin concentrations, heart rates or red blood cell parameters, and no adverse responses were noted in any of the treated horses. By 10 days post administration, the last time point at which samples were taken, the average serum concentration for cobalt (more than 10 ng/ml – estimated from Knych's Figure 1) remained substantially above the pre-administration baseline values of less than 1 ng/ml (the limit of quantification). Based on their data they concluded that regulatory agencies should easily be able to detect excessive cobalt administration by measuring the cobalt concentrations of equine samples.

More concerning with the regulation of cobalt in horse racing is that there is no standardised technique upon which the international testing community can agree. In September 2014, Popot *et al.* (2014) reported on an international 'Ring Test' in which five laboratories tested five samples of urine and plasma for cobalt, in an investigation to determine the repeatability of cobalt testing between testing laboratories. All five laboratories used different testing methods, and one lab (Hong Kong Jockey Club) tested for free ionised cobalt, while the others tested for total cobalt. The conclusion of this study was that the international standard must be in urine, because there was insufficient agreement between laboratories when testing for cobalt in plasma to establish an international standard (Popot *et al.*, 2014).

12. Cobalt and horse racing

The potential uses of inorganic cobalt in human athletics were first noted in the scientific literature by Lippi *et al.* (2005). Because of the potential toxicity of cobalt, Lippi *et al.* (2006) questioned whether testing for cobalt in sports should be implemented by regulators. The first formal recognition by racing regulators of the potential of cobalt for misuse in horseracing occurred in Ontario in 2009, when the Ontario Horse Racing Commission noted in a communication to horsemen that cobalt 'used in safe and appropriate formulations' was 'known to have certain blood building qualities' and also noted that 'speculation about performance enhancing capabilities are doubtful' (ORC, 2009).

To our knowledge the next mention of cobalt in association with horseracing is the first administrative action taken on cobalt in North American racing. These actions were not taken by a Racing authority but by the owner of three

racetracks in New Jersey and New York, who had samples taken out of competition from horses at one or more of his tracks which were sent to the Hong Kong Jockey Club laboratory for analysis. Based on the results of the Hong Kong testing two trainers were banned from the Meadowlands racetracks in New Jersey (TH, 2014).

Since the cobalt used in horse racing is administered in private, little is known about dosage and administration and there are little published equine data. Also, any adverse effects which may be expected when large doses are administered to racehorses are unlikely to be reported. However, through discussions with horse trainers who spoke with one of us (author C.K. Fenger) on the condition of anonymity, we have determined that 'low' dosages range from 100 mg to 400 mg diluted in a litre and administered IV two days prior to racing. While no trainers admitted to using 'high' dosages, researchers with the Kentucky Horse Racing Commission have claimed that doses as high as 4 mg/kg cobalt chloride (about 1,800 mg) are used (Waldridge, 2015).

Significant effects resulting from cobalt mediated changes in gene transcription, such as possible increased red blood cell production, seem unlikely to be in place by the time of a race if cobalt is administered only two days before competition. However, there is a perception, unsubstantiated by the scientific literature, among some industry participants that certain Standardbreds experience improved racing performance pursuant to cobalt administration (TH, 2014). If such reports are true, then one possible reason (or suggestion for further research) may be the mechanism of action of the cobalt through its effect as a calcium channel blocker (Simonsen *et al.*, 2012). Standardbreds are susceptible to recurrent exertional rhabdomyolysis (RER), or 'tying-up' during racing (Isgren *et al.*, 2010; Knoepfli, 2002). The underlying mechanism of this condition is unclear, other than it is probably a heritable condition (Collinder *et al.*, 1997), and likely mediated by sarcoplasmic calcium channels (López *et al.*, 1995). Many of the current preventative therapies for RER involve calcium channel blockers, such as Dantrolene (López *et al.*, 1995) or another divalent cation, magnesium (C.K. Fenger, unpublished observation). Since RER is prevalent among Standardbreds, and preventative medications for this condition are prohibited on race day, any possible performance effect of cobalt administration two days before racing may simply be prevention of RER. Based on one author's clinical experience (C.K. Fenger), the administration of 200 to 400 mg cobalt salts will prevent RER in a susceptible horse for 1-2 weeks.

13. Cobalt regulation in horse racing

One difficulty with regulating cobalt use in racing is that cobalt is a naturally occurring environmental substance present in all horses. Given this circumstance, a positive cannot be called unless a defined regulatory threshold is exceeded. While equine regulatory organisations have set thresholds for many endogenous, dietary, and environmental substances (Tobin *et al.*, 2012), cobalt has no generally accepted threshold as it has only become of regulatory interest in horseracing in 2009.

Hong Kong has set an 'in-house' urinary cobalt threshold of 100 ng/ml, whereas in Australia, NSW Harness racing officials introduced a urinary threshold of 200 ng/ml pursuant to post race samples in certain harness horses exceeding 3,500 ng/ml (Bartley, 2014). Research is currently under way by a number of North American equine organisations or jurisdictions to establish normal concentration values and suitable regulatory threshold(s) for cobalt in horseracing.

On March 4, 2014, the California Horse Racing Board (CHRB) issued a notification that they were about to commence monitoring cobalt concentrations. The CHRB indicated that cobalt in excess of 5 ng/ml (ppb) in the blood or 25 ng/ml in the urine would be a cause for an investigation. In addition, the top 10% of cobalt concentrations obtained at necropsy will 'be a source for investigation'. Dr. Rick Arthur, speaking on behalf of the California Horse Racing Board, said that although there have been no reported cases of racehorses with cobalt toxicity, the CHRB will consider administration of cobalt as 'a potential equine health and safety issue' (Larson, 2014). When the CHRB actually implemented their regulatory threshold, it was put in place at 25 ng/ml in blood. Indiana horseracing authorities have also recently introduced a 25 ng/ml regulatory threshold in blood. On May 20, 2014, the Maryland Racing Commission announced that Maryland will begin testing horses for cobalt. The chairman of the board indicated that there was 'no definitive threshold' concentration for cobalt at that time. There are also no specific rules in place in Maryland relating to cobalt, and no comments were made on how a horse presenting with high concentrations of cobalt might be addressed (Vespe, 2014).

As this communication goes to press the International Federation of Horseracing Authorities (IFHA) is considering a proposed international urinary cobalt threshold of 100 ng/ml (Popot *et al.*, 2014). This proposed threshold is based on an international collaborative study involving over ten thousand samples from horses racing in Europe, France, Hong Kong, New Zealand, and the United Kingdom (Ho *et al.*, 2015). This threshold carries with it a statistical risk of an overage frequency of less than one in 62,000 samples (in horses not administered cobalt) and is proposed for

application in the context of race-day testing. Additionally, the threshold comes with a recommendation that the use of cobalt containing supplements, especially injectables, be banned at least 24 hours prior to racing, or at all times if this threshold is to also be applied to non-race-day samples. This study also noted that an approved testing technology for cobalt analysis had not yet been identified at the time of their report.

In April 2015 the Association of Racing Commissioners International (ARCI) board of directors approved and the ARCI Model Rules Committee later approved a regulatory threshold of 50 ng/ml of cobalt in plasma, exceeding which threshold would be considered a Class B penalty, with a recommended 15 day suspension, a 500 $ fine and disqualification of the horse in question. The ARCI also recommended that horses testing above 25 ng/ml be placed on the Veterinarians List until the horse tests below 25 ng/ml. On January 1, 2016 Kentucky implemented the following cobalt rules: (1) for a finding between 25 and 50 ng/ml in serum, a written warning to a 500 $ fine and the horse placed on the Veterinarians List until its serum cobalt is below 25 ng/ml. (2) For cobalt concentrations above 50 ng/ml in Kentucky, the first cobalt finding incurs a 30 day suspension and a 500-1,500 $ fine, with the horse being disqualified and purse monies redistributed. The horse is also placed on the Veterinarians List until its serum concentration of cobalt drops below 25 ng/ml.

14. Conclusions

Regulation of cobalt use in horse racing faces a number of challenges. First, the testing technology, which is new to equine testing laboratories, must be rigorously validated and, though inductively coupled plasma mass spectrometry (ICP/MS) has been used by several laboratories to measure cobalt concentrations, a fully validated and standardised analytical methodology is not yet available. Second, the availability of dose response information is negligible in man and laboratory animals (Baselt and Cravey, 2011) and at this time only one pharmacokinetic study in horses has been published in the scientific literature (Knych *et al.*, 2015). A further complication is that cobalt concentrations have been reported to depend on multiple factors, such as age, sex and even menstrual cycles in humans. Much is known about the properties of cobalt, and yet little is known about any of its potential effects in horse racing or any other athletic endeavour. Despite this, horseracing regulatory agencies are now setting cobalt thresholds in order to control its use in racing. Regulatory efforts before understanding of dose-response characteristics of any naturally occurring substance are premature and should be undertaken with great caution.

Conflict of interest statement

Dr. Fenger is a Board Certified Veterinary Internist who practices veterinary medicine on race horses and has administered cobalt in the course of this practice. Dr. Fenger is the Secretary of the North American Association of Racetrack Veterinarians and has offered expert opinions in cobalt matters. Dr. Tobin is a Veterinarian, Pharmacologist and Toxicologist and is the Veterinary advisor to the National Horseman's Benevolent Protective Agency and has offered expert opinions in cobalt matters. Dr. Maylin is a Veterinarian and Pharmacologist and Director of the New York Equine Drug Testing Program at Morrisville State College, Morrisville, NY and performs regulatory cobalt testing for New York State Gaming Commission and conducts cobalt research funded by the United States Trotting Association.

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