NZY GLUTATHIONE PEROXIDASE KIT – NEW KIT FOR DIAGNOSIS OF THE WHITE MUSCLE DISEASE IN DOMESTIC ANIMALS

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INTRODUCTION

White muscle disease, also known as nutritional myopathy or muscular dystrophy, is a degenerative disease of skeletal and cardiac muscles that affects all species, with particular emphasis on ruminants. This disease is caused mainly by lack of selenium (Se) in the diet of animals, although the lack of vitamin E also leads to a similar disease. It is a disease that affects livestock from Portugal and from many other countries, including Spain, and for which there is not an eradication program. This is due primarily to two reasons: firstly, although the lack of selenium is the main cause of the disease, its dietary supplementation is often avoided because a slight excess of it, in the order of parts per million (ppm), could be responsible for serious poisoning of the animals; secondly, because there is no simple and efficient way to monitor the status of Se in the animals, to ensure that their levels remain normal, even when the animals are subjected to dietary supplementation.

Much has been studied about the pathophysiology of Se in the genesis of several diseases, from its antioxidant effect and regulation of metabolism to its importance in infectious and cancerous diseases. Among the many contributions of Se to animal metabolism, it is worth to mention its major intervention as a cofactor of glutathione peroxidase (GPx), a group of important enzymes in fighting body oxidants, including free radicals. At least 4 different enzymes belonging to the family of selenoproteins - GPx1 to GPx4 - are known in mammals, which act at different levels: cellular, gastrointestinal and in blood. GPx catalyses the reduction of peroxides to the corresponding alcohols using stable glutathione (GSH) as a source of reducing equivalents.

At NZYTech we have developed a kit for indirect diagnosis of the status of Se in the blood of farm animals based on a simple and rapid method that determines GPx activity in blood, thus providing an indicator of the level of organic protection against the aggression by oxidative agents – NZY Glutathione peroxidase kit (Catalogue number DG00041). The determination of GPx activity is based on the method of Paglia and Valentine (1967) and its principle can be summarized as follows: oxidized glutathione (GSSG) produced by the reduction of an organic peroxide by GPx is recycled to its reduced state by glutathione reductase; the oxidation of NADPH to NADP+ is accompanied by a decrease in the spectrophotometric absorbance at 340 nm, which enables the monitorization of GPx activity in the biological sample.

IMPORTANCE OF SELENIUM

The importance of selenium became apparent in the 50's when it was shown that most of myopathies in sheep and cattle and exudative diathesis in poultry could be prevented by supplementing the diet with selenium or vitamin E. The biochemical role of selenium in animals was demonstrated in 1973, when it was discovered that this was a trace element component of glutathione peroxidase, an enzyme which catalyses the removal of hydrogen peroxide, thereby protecting cell membranes from oxidative changes. The GPx contains 4 Se atoms and forms the second line of defense, after vitamin E. GPx is also known for having a sparing effect of vitamin E, thus reducing the amount required and promoting its absorption (McDowell, 1992).

Beyond its role as an antioxidant and on strengthening the immune system, Se is equally important for its role in the production of thyroid hormones, as a component of the enzyme iodothyronine deiodonase type I that converts T4 into the bioactive T3 hormone. This enzyme is particularly important in the metabolism of brown adipose tissue of newborn ruminants, releasing T3 for use in other tissues. In adult animals, the enzyme is found mainly in the liver and kidney, but not in the thyroid gland (MacDonald *et al*, 2002).

CLINICAL SIGNS OF WHITE MUSCLE DISEASE

The most important manifestation of Se deficiency in livestock animals is muscle degeneration, which causes the white muscle disease, also known as nutritional myopathy or muscular dystrophy. In herbivores, it is more common in early spring, perhaps due to a deficiency of Se in winter feeds and the higher concentration of polyunsaturated lipids in herbs of spring, which increases the need of anti-oxidants. Myopathy mainly affects skeletal muscle, with animals showing lack of strength, difficulty in standing up, staggering gait and tremors. These symptoms may progress to complete inability to remain in a standing position and even to raise their heads. The death ultimately supervenes if no appropriate treatment is administered. If the heart muscle is affected, sudden death may occur, which happens more often in pigs, especially if subjected to accelerated growth. In lambs, the Se shortage is manifested by stiffness of the limbs, due to the sclerotic process that gets installed after the destruction of muscle cells. In addition, myopathy in chickens, due to selenium deficiency, is also displayed by a reduction in production and hatchability of eggs (MacDonald *et al*, 2002).

TOXICITY OF SELENIUM

The level of selenium in feed is highly variable and depends mainly on soil conditions where the plants grow. Values of 100 to 300 mg/kg of dry matter are common. In addition, some plant species – seleniferous - have the ability to accumulate this element in large amounts. An example of a seleniferous plant common in Portugal is the *Astragalus lusitanicus*, which is known for causing toxic episodes in sheep. The concentration of organic Se species in this genus can go up to 15 g/kg of dry matter. Selenium is a highly toxic element and, at concentrations of 5 mg/kg or 500 mg/kg in the diet or water, respectively, is potentially harmful to the animals (NRC, 2007). The toxic symptoms include dullness, joint stiffness, loss of hair and localized deformation of the nails. Death can occur after rapid intake of selenium due to respiratory arrest (Charlton and Ewing, 2005).

SELENIUM DEFICIENCY IN PORTUGAL

The impact of selenium deficiency in extensively raised farm animals in Portugal, although recognized from a long time ago, gained new momentum with the systematic work conducted by the UTAD Investigator Filipe Silva that established unequivocally a link between the lack of selenium in Portuguese pastures with the appearance of specific diseases and a higher susceptibility to the development of numerous other diseases (Silva, 2007).

In what concerns the muscular dystrophy, studies by Silva *et al.* (2006) have shown that the implications of this nutritional disease are largely undervalued, both in its direct consequences - high morbidity and mortality of animals - and in its

indirect consequences - increased susceptibility to other diseases, reproductive failure, among others. It is urgent to correct this disastrous situation for the economy of farms and animal welfare. For this purpose, it is necessary to establish feed programs to draw for each region or exploitation through adequate forage programs, taking into account the antioxidant status of the animals, so that supplementation with selenium is effective but not excessive. The first condition for achieving this aim is to diagnose the levels of circulating selenium, which is achieved indirectly by determining the activity of glutathione peroxidase, through a method like the one proposed by NZYTech.

PREVENTION OF THE WHITE MUSCLE DISEASE

After detecting a Se deficiency in a farm or in a region, supplementation of the diets should be made properly, ensuring that the levels of the element in question in the animal are maintained within safe physiological limits. An excess is as or more undesirable than a deficiency due to its high toxicity.

Selenium sources of most feed are linked to proteins, in the form of selenomethionine and selenocysteine. Selenium supplements can be provided in the form of mineral complementary feeds containing sodium selenite, slow release capsules or yeast extract enriched with selenium.

What is then the major obstacle to guarantee that livestock animals have a proper Selenium supplementation when needed? The answer is the lack of a simple and reliable method for the determination of organic selenium in the body. Since the direct determination of the mineral is time consuming and expensive, we have to resort to indirect methods, such as the determination of the activity of enzymes that require this mineral for its action, such as GPx, which has structural selenium. However, existing indirect methods are not at all adapted to livestock species and are therefore somewhat not reproducible and reliable. Also, the high price remains an obstacle to their use in large scale.

To our knowledge, the kits available in the market for the determination of GPx activity are designed to investigate the antioxidant status of humans. These analytical kits, with some adaptations, have also been used in Veterinary Medicine. These adaptations have led to a lack of reference values between laboratories and to some discomfort of the researchers, who have been forced to use those kits due to a lack of valid alternatives.

EARLY DIAGNOSIS OF WHITE MUSCLE DISEASE - KIT OF GLUTATHIONE PEROXIDASE

According to the reasons described above, the development of a kit specifically designed for Veterinary Medicine that could be used as a generalized method was urgently needed. For this purpose, we proceeded to the cloning of two genes, one encoding glutathione reductase and the other encoding glutathione peroxidase from the genome of a bacterium, necessary for the indirect determination of the amount of selenium in the organism. After extensive tests on their activity, stability and reproducibility, we come out with a product that is suitable for use in the diagnostic of Se status of farm animals.

The GPx is an enzyme containing selenium and which main biological function is to protect the body from damage caused by oxidized chemical compounds. The biochemical function of the enzyme is to reduce hydroperoxides to their corresponding alcohols, free hydrogen peroxide and water. The activity of GPx is coupled to glutathione reductase (GR), which reduces the oxidized glutathione (GSSG) to the reduced form (GSH). In livestock, it is well established a positive correlation between GPx activity in serum and the levels of selenium. This kit is intended, therefore, to be a useful tool in monitoring the levels of GPx in farm animals thus allowing a more accurate diagnostic of the deficiencies of selenium. The principle of the method used in the kit is based on the following reactions:

$$2\ GSH + ROOH \xrightarrow{GPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \longrightarrow NADP^+ + 2\ GSH$$

In this kinetic method, based on the method described by Paglia and Valentine (1967), the cumene hydroperoxide is used as the peroxidized substrate and NADPH and GR are included in the reaction. The formation of GSSG (oxidized glutathione) catalysed by GPx is coupled to the recycling of GSSG to GSH by GR, with the concomitant oxidation of NADPH to $NADP^+$. The oxidation of NADPH to $NADP^+$ is measured spectrophotometrically by monitoring the decrease in absorbance at 340 nm (A340). Under these conditions, in which the activity of GPx limits the further reaction, the rate of decrease in absorbance at 340 nm is directly proportional to the catalytic activity of GPx.

With respect to this particular kit for determination of GPx, it was given specificity to animals most often suffering deficiency symptoms, designing and testing it specifically for these animals, unlike kits available on the market that are designed for the human species. Also, the competitive price of this kit makes it very appealing.

CONCLUSION

White muscle disease is a condition caused by the lack of selenium in livestock feed. It has recently been recognized as a major cause of economic losses in livestock farming in Portugal, either directly by the impairment that produces or indirectly by breaking the immunity in the animals. This immunodeficiency increases the risk of animal infectious and/or parasitic diseases that shows off as the effective cause of the disease and that can mask, in some cases, the underlying primary cause of the primary disease. In one way or another, the animal welfare and health are compromised, which can lead to death and economic losses.

The determination of the antioxidant status of farm animals is the only rational way to prevent this deficiency by determining the activity of a seleno-dependent enzyme, glutathione peroxidase (GPx), whose activity is directly dependent on the amount of selenium that farm animals have in their feed. The utilization of the enzymatic assay developed by NZYTech, the NZY Glutathione peroxidase kit (DG00041), allows the assessment of the nutritional status of selenium in farm animals and thus outlines a rational supplementation program, which could ensure an adequate intake of this mineral.

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