

ASCORBATE-GLUTATHION AFFILIATION AND THE TREATMENT OF NUTRITIONAL MUSCULAR DYSTROPHY IN LAMBS WITH SPECIAL REFERENCE TO THE OXIDATIVE STRESS

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Received December 18, 2009

ABSTRACT - This study was applied on a total number of 40 native breed lambs from Dakahlia governorate, their ages being ranged from 5 to 26 days, in order to declare the effects of nutritional muscular dystrophy as an oxidative stress in lambs and the effect of vitamin C administration in enhancing the treatment of Nutritional Muscular Dystrophy (NMD), decreasing oxidative stress and their correlation with glutathione in diseased lambs. The used lambs were allotted into four groups. Group 1 included apparently clinically healthy animals (n-10) and was the control group. The 2nd group (suffering from NMD) was treated by Vitamin E-selenium alone (n-10). The 3rd group (suffering from NMD) was treated by vitamin E -selenium and vitamin C (n-10) and the 4th group (suffering from NMD) was treated by vitamin C only (n-10). Two blood samples were obtained from each lamb, before and after treatment, and subjected to the laboratory investigation. The results revealed that there was significant increase in the oxidative stress markers with

significant reduction in the antioxidant levels of diseased lambs. After the treatment, there were improvement of the general health condition and disappearance of the clinical signs within 4-7 days in group 2 and group 3, in contrast with the 4th group in which the clinical signs persisted even after treatment. The obtained results concerning blood and serum biochemical analysis revealed that NMD was playing an important role as an oxidative stressor in lambs. Moreover, antioxidant mixture of vitamin E-selenium and ascorbate proved to be more powerful and effective in the treatment of diseased lambs with NMD and decreasing the resulted oxidative stress.

Key words: lambs, Vitamin C, NMD, oxidative stress, antioxidants

REZUMAT – Legătura dintre ascorbat și glutation în tratamentul miodistrofiei nutriționale la miei, cu referire la stresul oxidativ. Studiul a fost aplicat la 40 de miei, crescuți în Guvernoratul Dakahlia (Egipt),

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cu vârste cuprinse între 5 și 26 zile, pentru a demonstra efectele miodistrofiei ca factor de stres oxidativ la miei și efectul administrării vitaminei C și legătura cu glutatiunul în creșterea eficienței tratamentului miodistrofiei nutriționale, diminuând, astfel, stresul oxidativ la miei bolnavi. Miei studiați au fost grupați în patru loturi: lotul 1 a inclus animalele clinic sănătoase (n-10) și a fost folosit drept lot martor; lotul 2 (miei bolnavi de miodistrofie nutrițională) a fost tratat doar cu vitamina C și seleniu (n-10); lotul 3 (cu miodistrofie nutrițională) a fost tratat cu vitamina E, seleniu și vitamina C (n-10), iar lotul 4 (cu de miodistrofie nutrițională) a fost tratat doar cu vitamina C (n-10). De la fiecare miel au fost obținute două mostre de sânge, înainte și după tratament, care au fost supuse studiului în laborator. Rezultatele au demonstrat o creștere semnificativă a stresului oxidativ și reduceri importante ale nivelului antioxidanților la miei bolnavi. După tratament, s-a observat o ameliorare a stării generale a sănătății și dispariția semnelor clinice la 4-7 zile, la loturile 2 și 3. În opoziție, la lotul 4, semnele clinice au persistat, chiar și după tratament. Rezultatele privind analiza biochimică a sângelui și a serului au demonstrat că miodistrofia nutrițională reprezintă un factor de stres oxidativ important pentru miei. Amestecul antioxidant de vitamină E, seleniu și ascorbat s-a dovedit a fi mai puternic și mai eficient în tratamentul miodistrofiei la miei, diminuând stresul oxidativ.

Cuvinte cheie: miei, vitamina C, miodistrofie nutrițională, stres oxidativ, antioxidanți

INTRODUCTION

Nutritional muscular dystrophy is a degenerative rather than a dystrophic disease of striated muscles, which occurs without neural

involvement in a wide range of animal species. Lesions were probably initiated by free radical damage (Underwood and Suttle, 1999).

The affected animals exhibit muscular stiffness, arrhythmia, tachycardia and abdominal breathing (Hidiroglou *et al.* 1995). Furthermore, Radostits *et al.* (2007) stated that recumbency, inability to stand, stiffness, trembling of the limbs, muscle tremors, dyspnea and abdominal breathing were the obvious signs in cases of NMD.

Selenium levels and the activities of AST, CPK, LDH and GSH-PX were of paramount in the diagnosis of clinical and subclinical cases of NMD in lambs (Sobbiech and Kuleta, 2002).

Ascorbic acid markedly stimulates collagen synthesis without affecting synthesis of other proteins (Pinnell, 1982; Joseph *et al.*, 1990 and Mohan and Venkataramana, 2007). Moreover, Zern *et al.*, (1985) concluded that the ascorbate stimulated protein synthesis in fetal calf smooth muscle and exhibited the production of an endogenous collagen rich matrix. Meanwhile, Mukhopadhyay and Chatterjee (1994) found that collagen degradation was completely prevented by ascorbate and not by catalase. Moreover, Linetsky *et al.* (1999) reported that ascorbate increases the generation of superoxide anion, which is important for a major portion of the oxidation of sulphur amino acids seen during aging.

TREATMENT OF NUTRITIONAL MUSCULAR DYSTROPHY IN LAMBS

Mahmoodian and Peterkofsky (1999) concluded that vitamin C deficiency has caused morphological changes in the endothelial and smooth muscle compartments of blood vessels due to the changes in laminin and elastin synthesis.

Ascorbic acid, retinoic acid and dihydrocytochalasin B were proved to increase the growth, protein synthesis and maturation of fetal bovine epiphyseal chondrocytes with induction of collagen synthesis and an increase in alkaline phosphatase activity as stated by David, 1993 and Freyria *et al.* (1999).

Malondialdehyde (MDA) - the product of polyunsaturated fatty acid oxygenation is a reliable and commonly used biomarker for assessing lipid peroxidation (Moore and Roberts, 1998). Recently, there has been growing interest in the use of MDA as a marker of lipid peroxidation in various kinds of diseases (Sheu *et al.*, 2003).

This study was carried out in order to proclaim the effects of nutritional muscular dystrophy as an oxidative stressor in lambs and the effect of vitamin C administration in enhancing the treatment of Nutritional Muscular Dystrophy (NMD), decreasing oxidative stress and the correlation with glutathione in diseased lambs.

MATERIAL AND METHODS

Animals. A total number of 40 native breed lambs of both sexes (5-26 days old) from the Dakahlia Governorate (in the period from March 2007 to

September 2008) were used in this study. The feeding program of the examined lambs was random suckling and the dams were raised on grazing. Lambs were categorized into four groups. Group 1 (n-10) involved those clinically and apparently healthy lambs used as control group. Group 2 t included 10 lambs suffering the clinical signs of vitamin E & selenium deficiencies and treated by vitamin E & selenium. Group 3 (n-10) included lambs suffering vitamin E & selenium deficiencies and that were treated by vitamin E & selenium and vitamin C. Group 4 involved 10 lambs suffering vitamin E & selenium deficiencies and were treated by vitamin C. The selected animals have been kept under close observation and were examined clinically according to Kelly (1984).

Samples and sampling protocol.

Blood samples were obtained in heparinized tubes for obtaining whole blood and on non-heparinized tubes for obtaining blood serum, which was kept frozen until the biochemical analysis of selected parameters. The blood samples were taken in two occasions: one before treatment and the second after treatment.

Blood serum samples were used for determination of catalase enzyme (CAT), reduced glutathione (R.GSH), super oxide dismutase (SOD) and ascorbic acid according to the methods described by Aebi (1984), Beutler *et al.*, (1963), Nishikimi *et al.*, (1972) and Harris & Ray (1935), respectively using commercially available test kits supplied by Biodiagnostic Egypt. Moreover, blood serum samples were subjected to the biochemical analysis for the determination of creatin phosphokinase (CPK), aspartat amino transferase (AST) and lactate dehydrogenase (LDH) according to the methods described by Hare, (1950), Ritman and Frankel (1957)

and Wroblewski *et al.* (1955) respectively, using commercially available test kits supplied by Biodiagnostic Egypt. Furthermore, the MDA levels were estimated using commercially available test kits supplied by Biodiagnostic Egypt according to the methods described by Satoh, (1978) and Ohkawa *et al.* (1979). Selenium level was carried by atomic absorption spectrophotometer (wave length 136.03nm, Band path: 1.0 Um, lamb current 6.0mA. Flame "acetylene air" Air flow: 4.5 ib/inch and gas pressure 2.1 ib/inch) according to the method described by Fernadez and Kahr (1971).

Treatment trials. The second group was treated by vitamin E & selenium (Seletoc ® supplied by Schering/Plough Company, USA in a dose rate of 1ml/9 kg body weight, one injection and repeated after 3 days). The composition of this preparation was selenium 1gm as sodium selenite and vitamin E 68 IU in 10ml vial. The 3rd group was treated by vitamin E & selenium (Seletoc* in a dose rate of 1ml/9kg body weight one injection and repeated after 3 days, in addition to vitamin C (Cevaryl ampoules, supplied by Memphis company, Egypt and contains 20% ascorbic acid) in a dose rate of 30 mg/kg body weight once daily/3 successive days, and the 4th group was treated by vitamin C only in a dose rate of 30 mg/kg body weight once daily for 3 successive days.

Statistical analysis. The mean values and the standard error were calculated for the obtained data and the significance for all means have been carried out by applying one-way ANOVA test using SPSS computer program. The values have been calculated according to Snedecor and Cochran (1989).

RESULTS

The clinical examination of the lambs in the second, third and fourth groups showed the clinical signs of vitamin E-selenium deficiencies including, sternal recumbency and inability to stand but with obvious desire to stand in most cases. Stiffness, trembling and muscle tremors if the lambs were forced to stand, the affected muscles (shoulder & gluteal) felt hard and swollen.

After treatment, there was an improvement of the general health condition and disappearance of the clinical signs within 4-7 days in group 2 and group 3. In contrast with the 4th group in which the clinical signs persisted even after treatment.

Regarding the blood and serum biochemical analysis in group 2, there was a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, catalase and ascorbic acid with a significant ($P \leq 0.05$) increase in the levels of CPK, AST, LDH and MDA before treatment and a significant ($P \leq 0.05$) improvement of all parameters was recorded seven days after treatment (*Table 1*).

Concerning the blood and serum biochemical analysis in group 3, there was a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, CAT and ascorbic acid. In contrast, there was a significant ($P \leq 0.05$) increase in the levels of CPK, AST, LDH and MDA before treatment and a high significant ($P \leq 0.05$) improvement of all parameters

TREATMENT OF NUTRITIONAL MUSCULAR DYSTROPHY IN LAMBS

was recorded seven days after treatment (Table 2).

Regarding the blood and serum biochemical analysis in group 4, there was a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, catalase and ascorbic acid with a significant ($P \leq 0.05$) increase in the

levels of CPK, AST, LDH and MDA before treatment. While after treatment, there was a significant ($P \leq 0.05$) increase in the levels of R.GSH and ascorbic acid, with a significant ($P \leq 0.05$) decrease in the level of MDA seven days after treatment (Table 3).

Table 1 - The mean values \pm standard error of some blood biochemical parameters of lambs suffering NMD in group (2) compared with clinically healthy lambs before and after treatment

Biochemical parameters	Control group (1)	Group (2) before treatment	Group (2) after treatment
Ascorbic acid (mg/dl)	2.27 \pm 0.122	0.75 \pm 0.227**	1.30 \pm 0.12*
R. GHS (nmol/ml)	8.38 \pm 0.18	3.57 \pm 0.29**	6.5 \pm 0.15*
CAT (nmol/L)	49.45 \pm 0.19	33.17 \pm 0.29**	39.60 \pm 0.352*
SOD (μ mol/L)	1.73 \pm 0.15	0.58 \pm 15*	1.17 \pm 0.07**
Selenium (ppm)	66.40 \pm 0.520	18.0 \pm 0.84**	61.08 \pm 0.287*
CPK (IU/L)	66.8 \pm 0.32	1542.8 \pm 2.14**	177.5 \pm 3.16*
AST (IU/L)	65.80 \pm 0.78	1320.16 \pm 1.13**	107.5 \pm 3.7*
LDH (IU/L)	591.1 \pm 2.13	1703.3 \pm 145.0**	815.5 \pm 4.81*
MDA(μ mol/L)	0.4720 \pm 0.013	1.6 \pm .043**	0.87 \pm 0.035*

*Means significantly differ at the level ($p \leq 0.05$)

** Means significantly differ at the level ($p \leq 0.01$)

Table 2 - The mean values \pm standard error of some blood biochemical parameters of lambs suffering (NMD) in group (3) compared with clinically healthy lamb before and after treatment

Biochemical parameter	Control group (1)	Group (3) before treatment	Group (3) after treatment
Ascorbic acid (mg/dl)	2.27 \pm 0.122	0.75 \pm 0.227**	2.016 \pm 0.169**
R. GHS (nmol/ml)	8.38 \pm 0.18	3.57 \pm 0.29**	7.7 \pm 0.13**
CAT (nmol/L)	49.45 \pm 0.19	33.17 \pm 0.29**	46.96 \pm 0.36**
SOD (μ mol/L)	1.73 \pm 0.15	0.58 \pm 0.15*	1.45 \pm 0.09**
Selenium (ppm)	66.40 \pm 0.520	18.0 \pm 0.84**	63.25 \pm 0.27**
CPK (IU/L)	66.8 \pm 0.32	1542.8 \pm 2.14**	121.0 \pm 3.20**
AST (IU/L)	65.80 \pm 0.78	1320.16 \pm 1.13**	86.8 \pm 0.56**
LDH (U/ml)	591.1 \pm 2.13	1703.3 \pm 145.0**	625.9 \pm 3.46**
MDA(μ mol/L)	0.4720 \pm 0.013	1.6 \pm .043**	0.64 \pm .028**

*Means significantly differ at the level ($p \leq 0.05$)

** Means significantly differ at the level ($p \leq 0.01$)

Table 3 - The mean values \pm standard errors of some blood biochemical parameters of lambs suffering NMD in group (4) compared with clinically healthy lamb before and after treatment

Biochemical parameter	Control group (1)	Group (4) before treatment	Group (4) after treatment
Ascorbic acid (mg/dl)	2.27 \pm 0.122	0.75 \pm 0.227**	1.9 \pm 0.18**
R. GHS (nmol/ml)	8.38 \pm 0.18	3.57 \pm 0.29**	6.2 \pm 0.15**
CAT (nmol/L)	49.45 \pm 0.19	32.12 \pm 0.53**	32.27 \pm 0.60
SOD (μ mol/L)	1.73 \pm 0.15	0.58 \pm 0.15*	0.6 \pm 0.14
Selenium (ppm)	66.40 \pm 0.520	17.9 \pm 0.84**	18.1 \pm .67
CPK (IU/L)	66.8 \pm 0.32	1542.8 \pm 2.14**	1529.5 \pm 9.56
AST (IU/L)	65.80 \pm 0.78	1320.16 \pm 1.13**	1316.4 \pm 1.29
LDH (IU/L)	591.1 \pm 2.13	1703.3 \pm 145.0**	1703.3 \pm 502.3
MDA(μ mol/L)	0.4720 \pm 0.013	1.6 \pm .043**	1.025 \pm 0.035*

*Means significantly differ at the level ($p \leq 0.05$)

** Means significantly differ at the level ($p \leq 0.01$)

DISCUSSION

It was postulated that sulphur-containing amino acids and vitamin E act synergistically to protect tissues from oxidative damage. It is evident that glutathione peroxidase, which depend on dietary selenium, plays a major role in detoxifying lipid peroxides by reducing them to non-toxic hydroxy fatty acids, and vitamin E prevents fatty acid hydroperoxide formation (Mohan and Venkataramana, 2007; Radostits *et al.* 2007). On the other hand, Scrivanek *et al.* (1990) declared that ascorbate supplementation led to the formation of an extracellular matrix consisting primarily of collagen and that matrix influenced the biosynthetic capacities of the cell.

In the current study, NMD affected rapidly 5-26 day old growing lambs in spring season, especially at

the turnout from winter to spring pasture, which is low in selenium and vitamin E but very rich in myopathic agent (polyunsaturated fatty acids). This picture was previously described by Smith *et al.*, 1994 and Miller *et al.*, (1995). Moreover, Andress *et al.* (1996) founded that selenium concentration and erythrocytic GSH-PX activities were higher in summer and autumn than in winter and spring. Moreover, Maas (1990) reported that turnout from winter to spring pasture led to inactivity of the ruminal microflora and inability to hydrogenate fully the poly unsaturated fatty acids (PUFA) in the rumen at the adaptation of new diet leading to accumulation of plasma PUFA within first three days of turnout and oxidation of them to fatty acids hydroperoxides, which caused destruction of muscle cells in the absence of selenoenzymes (GSH-PX)

TREATMENT OF NUTRITIONAL MUSCULAR DYSTROPHY IN LAMBS

and vitamin E deficiency, which are responsible for removal of these peroxides. Moreover, in this study feeding on human wastes as main diet for dams may be contributing factors in the occurrence of NMD in lambs and this bad management may through the light on the effects of dams feeding on the incidence of NMD in their lambs.

Concerning the clinical picture of NMD, the affected lambs showed a sternal recumbency and inability to stand but with obvious desire to stand in most cases. Stiffness, trembling and muscle tremors if the lambs were forced to stand, the affected muscles (shoulder & gluteal) felt hard and swollen. This clinical picture was in harmony with those described by El-Kabbani (1985), Nassif and EL-Kataway (1997), Underwood and Suttle (1999) and Radostits *et al.* (2007). This clinical picture was attributed to the deficiency of vitamin E & selenium which resulted in a wide spread of tissue lipoperoxidation that consequently led to hyaline degeneration and calcification of muscle fibre as authorized by Radostits *et al.* (2007).

Regarding the blood and serum biochemical analysis in group 2, there was a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, CAT and ascorbic acid. A significant ($P \leq 0.05$) improvement of all parameters was observed seven days after treatment as shown in Table 1. These results were in concern with those obtained by Pinnell, (1982), El-Kabbani (1985), Nassif and EL-Kataway (1997), Smith, (1997) and

Radostits *et al.* (2007). These changes were attributed to the role of vitamin E and selenium in preventing oxidative damage of sensitive lipoprotein membrane by decreasing hydroperoxide formation and protecting cellular membranes from lipoperoxidation.

The blood and serum biochemical analysis of group 3 revealed a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, catalase and ascorbic acid. While a significant ($P \leq 0.05$) increase in the levels of CPK, AST, LDH and MDA before treatment were recorded. Treatment trial resulted in a highly significant ($P \leq 0.01$) improvement of all parameters toward normal levels (Table 2). These results were in harmony with those obtained by Mahmoodian and Peterkofsky (1999).

The rapid and highly significant improvement of enzymatic activities in response to a combination of vitamin E-Selenium and Vitamin C was attributed to the role of vitamin C in the encouragement of collagen synthesis and regeneration of vitamin E, as previously proved by Pinnell (1982) and Scrivanek *et al.* (1990). In addition to the rapid repair of morphological changes in the endothelial and smooth muscles compartment of the blood vessels due to enhancement of collagen, laminin and elastin synthesis. In addition to the role of ascorbate in amplification of GSH activity as GSH-dependant reduction of dehydroascorbate to ascorbate is catalyzed by mammalian

glutaredoxine and protein disulfide isomerase. This study presented strong evidence that this reaction takes place in lambs. Moreover, the marked decrease of ascorbate and increase of dehydroascorbate produced by glutathione depletion is impressive and indicated that the reduction of dehydroascorbate was an important physiological function of GSH and this was supported by the finding that treatment of GSH-deficient lamb with ascorbate led to significant sparing of GSH (Martensson and Meister, 1991 in rats and Mohan and Venkataramana, 2007 in humans).

Regarding the blood and serum biochemical analysis in group 4, there was a significant ($P \leq 0.05$) decrease in the levels of selenium, R.GSH, SOD, CAT and ascorbic acid with a significant ($P \leq 0.05$) increase in the levels of CPK, AST, LDH and MDA before treatment. Treatment trial resulted in a significant ($P \leq 0.05$) increase in the levels of R.GSH and ascorbic acid & a significant ($P \leq 0.05$) decrease in the levels of MDA (Table 3). These results were in concern with those obtained by Martensson and Meister (1991). These changes were attributed to the role of vitamin C in increasing and sparing the synthesis of GSH-PX.

Ascorbate spars glutathione indicating that these compounds have a similar antioxidant actions. Although glutathione normal functions is to maintain ascorbate, α tocopherol and other cellular components in reduced states,

ascorbate may serve as an essential antioxidant in the presence of severe glutathione deficiency.

From this study, it could be concluded that feeding on poor pasture was a main cause of NMD in the studied lambs. NMD plays an imperative task in elevating the levels of oxidative stress markers in diseased lambs. A mixture of vitamin E-selenium and ascorbate is more powerful and effective in the treatment of vitamin E-selenium deficiency and decreasing lipid peroxidation in diseased lamb than vitamin E-selenium alone or vitamin C alone.

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TREATMENT OF NUTRITIONAL MUSCULAR DYSTROPHY IN LAMBS

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