Journal of Animal and Veterinary Advances 17 (2): 51-60, 2018
ISSN: 1680-5593
© Medwell Journals, 2018

The Effect of Dietary Nucleotides Combined with Anti-Oxidants Supplementation on Equine Muscles Inflammation and Autophagy

[1, 2]Krzysztof Marycz, [1]Joanna Szydlarska, [1]Zuzanna Strek and [1, 3]Christine Weiss
[1]Department of Experimental Biology and Electron Microscope Facility,
The Faculty of Biology and Animal Science,
Wroclaw University of Environmental and Life Sciences, 50-631 Wroclaw, Poland
[2]Wroclaw Research Centre EIT+, 54-066 Wroclaw, Poland
[3]Postmatte 14, CH-8807 Freienbach, Switzerland

Abstract: Regular exercises not only contribute to the growth and maintenance of lean muscle mass but also bring a wide range of health benefits. On the other hand excessive training may cause oxidative stress, inflammation and metabolic abnormalities. The risk of disorders is particularly high among the young athletic horses and often leads to elimination from the sports career. The aim of the study was to investigate how exercises combined with anti-oxidants and nucleotides supplementation influenced on muscle pro-inflammatory cytokine activity, mitochondrial biogenesis and selective mitophagy. We evaluated mitochondrial morphology and distribution in skeletal muscle using transmission electron microscopy. Creatine kinase aspartate transaminase and superoxide dismutase activities were estimated by means of commercially available methods. Moreover, we determined the mRNA levels of muscle-related genes using a modified polymerase chain reaction. Our research revealed that there is a positive correlation between prolonged physical exertion in combination with well-conceived nourishment and muscle mitochondrial biogenesis as well as dynamics. Under the influence of describing dietary strategy we observed reduction of creatine kinase and asparate transaminase levels in peripheral blood. We also noticed that muscle apoptosis and inflammation in experimental groups was lowered, when compared to the control group. Obtained results indicate that physical activity combined with dietary enriched by anti-oxidants and nucleotides might increase mitochondrial number, function and efficiency in addition to reducing systemic inflammation and apoptosis at the mRNA level which might be particularly important for sport horses breeders.

Key words: Horses, nucleotides, anti-oxidants, feed supplements, transmission electron microscopy, mitochondrial biogenesis

INTRODUCTION

Exercise has a broad range of positive consequences on the body's metabolism and general health status. Regular training improves glucose homeostasis, cardiovascular health and muscle mass growth and maintenance (Booth *et al*., 2012; Agudelo *et al*., 2014). The training of young horses, besides the above mentioned benefits is also crucial for maintaining proper bioenergetics, nutrient delivery and uptake and results in tissues of superior fitness which are ready to endure the brunt of prolonged exercise. Skeletal muscle mass and physiological function are important factors which determine young horse prospects for sporting events. Muscle mass and muscle fiber growth as well as hypertrophy occur due to mechanical overload and/or anabolic hormonal stimulation during exercise. Currently, young horses that are devoted for sport career are intensively trained. Indeed, it is well known that exercise overload causes many adverse consequences including local and systemic generation of oxidative stress and inflammation in muscles, metabolic stress in the form of nutrient deficiency, calcium imbalance and general disturbances in cellular homeostasis. These in turn, result in damage of cellular constituents, muscle dysfunction, musculoskeletal system disorders and finally exclusion further sport competitions (Davies *et al*., 1982; Alessio *et al*., 1988; Rogers and Evans, 1993). Several mechanism have been proposed to be an effective in recycling of damaged organelles and/or protein

Corresponding Author: Krzysztof Marycz, Department of Experimental Biology and Electron Microscope Facility, The Faculty of Biology and Animal Science, Wroclaw University of Environmental and Life Sciences, 50-631 Wroclaw, Poland

aggregates (Dillard *et al*., 1978). In horses used for competition, the development of a natural mechanism to recycle damaged or aged organelles and accumulated protein aggregates evolved due to the abundant generation of oxidative stress factors and damaged cellular components. Macroautophagy is one of such mechanism in which long lived misfielded or aggregated proteins are degraded and recycled (Neel *et al*., 2013). Autophagy has been linked to endoplasmic reticulum stress, apoptosis and cell death and it is well known to be a mitochondrial quality control process (Sandri, 2010). The progression of autophagy begns with phagophore formation from the bilayer of the Endoplasmic Reticulum (ER) and/or the trans-Golgi body and endosomes (Twig *et al*., 2008). Emerging data indicates that ER stress may be a potent autophagy inducer, resulting in physiological and functional recycling of a number of organelles including the mitochondria (Marycz *et al*., 2016). Many Autophagy-related genes (Atg) have been identified including Beclin-1 (Atg6) which is a key regulator during initiation of autophagosome formation and recruitment of other Atg proteins to the Class 3 Phosphoinositide 3-Kinase (PI3K) complex (Sun *et al*., 2008). Furthermore, the elongation, formation and fusion of Autophagosomes with lysosomes involves activity of Atg7, Atg9, microtubule-associated protein 1A/1B-Light Chain 3 (LC3) and Lysosomal-Associated Membrane Protein-2 (LAMP-2). In skeletal muscle, autophagy is required to maintain muscle mass and mitochondrial function (Masiero *et al*., 2009), on the other hand impaired autophagy can contribute to muscle atrophy and glycogen storage disease (Nascimbeni *et al*., 2012). However, prolonged exercise may lead to excessive autophagy and this results in protein catabolism, free radical production and inflammation (Mammucari *et al*., 2007; Penna *et al*., 2013). Thus, inhibition and/or deletion of skeletal muscle cell autophagy by supplementing certain nutrients may lead to preservation of proper muscle function and performance. In this regard, anti-oxidant supplementation is strongly recommended during intensive training of young equine athletes. Apart from anti-oxidant supplementation, additional introducing of immunomodulatory components seem to be necessary. As it is well known that excessive training can cause muscle and tissue damage, it was also found that acute exercise can cause the accumulation of hepatocyte-derived acute phase proteins such as C-Reactive (CRP) or Tumor Necrosis Factor-alpha (TNFα), Interleukin 1 Beta (IL-1β) and Interleukin 6 (IL-6) in addition to increased cortisol production. Recent studies have found that oral administration of dietary Nucleotides (NT) reduces inflammatory cell activity and cortisol response. Nucleotides are low in molecular weight intracellular compounds, synthesized endogenously which play a key role in nearly all biochemical processes (Gil, 2002). As NTs are synthesized within the organism endogenously, it is not considered an essential nutrient, although its oral administration under certain stressful conditions including prolonged exercise, seemed to be beneficial. More recently, it was shown that dietary NTs have favourable effects on the immune system (Carver and Walker, 1995).

The aim of this study was to evaluate the effects of dietary nucleotides combined with anti-oxidant supplementation on muscle autophagy, inflammation and mitochondrial biogenesis of young exercised horses. Here, we have found that exercises combined with dietary nucleotides and anti-oxidant supplementation reduces autophagy and inflammation in muscles and enhances mitochondrial biogenesis.

MATERIALS AND METHODS

All reagents used in this experiment were purchased from Sigma-Aldrich (Poland), unless indicated otherwise.

Ethical approval: All experimental procedures described in this study were conducted with the consent of the Second Local Ethics Committee of Wroclaw University of Environmental and Life Sciences (decision number: 84/2012).

Animals: The study involved 3 groups of 5 years old horses n = 18. The horse were divided into 3 groups. Two groups n = 12 each equal to 6 were exercised and third group n = 6 control was non-trained. All horses received 3 g/kg of body weight daily the commercially available feed (Muhele Ebert Dielheim, Germany, Table 1). In addition to basic ratio, one of experimental group n = 6 received additionally 150 g daily of tested additive contained the anti-oxidant and nucleotides (PSB complex®, CHEMOFORMA Ltd. Rheinstrasse 28-32 CH-4302 Augst Switzerland, www.chemoforma.com) (Table 1). All horses (experimental and control group) received commercially available timothy grass hay 1.5% per 1 kg of body weight and water *ad libitum*. All horses were housed in the same stable and were trained by the same rider in South Region in Poland. The horses were trained 40 min daily, 6 days per week and 1 day break. The training protocol was 10 min walking, 10 min trotting, 5 min galloping, 5 min trotting and 10 min walking.

Experimental materials: Three types of biological materials were obtained from the horses for examination:

Table 1: Experimental feed and additives

Ingredients	Feed	Tested additive
Crude protein	11%	24
Crude fiber	8%	40
Crude fat	7%	80
Crude ash	7%	10
Digestible energy	12.5 MJ/kg	12.5 MJ/kg
Digestible crude protein	92 kg	192 g/kg
PSB complex® (nucleotides)	---	75g
Starch	23.6%	18%
Sugar	8.2%	1.5%
Iron	200 mg	200 mg
Zinc	120 mg	1800 mg
Manganese	100 mg	700 mg
Coper	40 mg	170 mg
Cobalt	0,1 mg	30 mg
Selenium	0.5 mg	1.1 mg
Iodine	0.7 mg	2 mg
Calcium	1.2%	1.3%
Phosphorus	0.5%	0.6%
Magnesium	0.3%	2%
Sodium	0.4%	0.1%
Vitamin A	11000 IE	4500 IE
Vitamin D3	1100 IE	5000 IE
Vitamin E	320 mg	1300 mg
Vitamin B1	10 mg	2.5 mg
Vitamin B2	13 mg	4.8 mg
Vitamin B6	6 mg	2.5 mg
Vitamin B12	35 mcg	500 mcg
Biotin	400 mcg	650 mcg
Nicotinic acid	60 mg	150 mg
Folic acid	4 mg	12 mg
Lysine	---	0.7%
Methionine	---	1.2%

blood, serum and skeletal muscle. The blood samples were collected from each individual in blood collection tubes (with Ethylenediaminetetraacetic Acid/EDTA without any anticoagulant) from the external jugular vein. The skeletal muscle samples were extracted from the gluteus medius muscle of experimental and control horses by core needle biopsy using semiautomatic tru-cut needles for soft tissue biopsy (18 g and 155 mm). The tissue samples were promptly placed in a centrifuge tube containing RNA stabilization solution (RNA later) and 2.5% glutaraldehyde. The amount of collected sample was between 3-6 g from each horse while the maximum sample length was 2 cm. All biological materials were transported in a cooling complex.

CK, AST analysis: Frozen serum was used for the establishment of Creatine Kinase (CK) and Aspartate Transaminase (AST) presence and quantification. The analysis was performed by a Commercial Veterinary Diagnostic Laboratory (Vetlab, Wroclaw, Poland).

Determination of SOD activity: Superoxide Dismutase (SOD) activity analysis was conducted using a SOD assay kit. The procedure was performed in accordance with the manufacturer's instructions as described earlier.

Transmission Electron Microscopy (TEM); Mitochondrial morphology and distribution in muscle fibres: Mitochondrial morphology and distribution analysis in muscle fibres was performed using he Transmission Electron Microscope (TEM). The tissue samples were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 6.8) as described previously. After fixing, tissue samples were washed 3 times with cacodylate buffer and post-fixed for 1 h at 4°C using 2% osmium tetroxide/1.5% potassium ferricyanide in 0.1 M cacodylate buffer. Thereafter, biological material was washed thrice in buffer and twice in ultrapure water, the samples were then centrifuged at 300×g for 10 min. Then samples were incubated overnight at 4°C in 1% uranyl acetate. After this procedure, tissue samples were washed three times with ultrapure water, dehydrated using a graded ethanol series 50-99.8% and embedded in agar low viscosity resin (Kit from Agar Scientific Ltd., Stansted, Essex, UK). Ultrathin sections were cut using an ultramicrotome (Leica EM UC7) and collected on copper grids. Samples were observed under the transmission electron microscope (Tecnai) at 120 kV acceleration voltage.

Analysis of gene expression; Quantitative real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR): The tissue samples were homogenized using 1 mL of TRI reagent. Total RNA was isolated according to a single-step method, originally described by Chomczynski and Sacchi (1987). The resulting total RNA was diluted in DEPC-treated water was analyzed for quantity and quality by means of nanospectrophotometer (VPA biowave II). Enzymatic digestion of genomic DNA (gDNA) and complementary DNA (cDNA) synthesis was conducted using Takara PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) following the manufacturer's instructions using a T100 ThermoCycler (Bio-Rad). Traces of gDNA were digested with 150 ng of total RNA for each reaction. Quantitative RT-PCR was performed using the SensiFast SYBR Green Kit (Bioline) in a total volume of 20 μL. The reaction mixture contained 2 μL of each matrix. Primer concentration was 0.5 μM. The primer sequences used are listed in Table 2. The reactions were conducted with the CFX Connect™ Real-Time PCR Detection System (BioRad). Relative gene expression analysis (Qn) was calculated in relation to the GAPDH housekeeping gene. The ratio between BCL-2 and BAX expression in the control and experimental groups was evaluated by dividing Qn of BCL-2 by Qn of BAX.

Table 2: Sequences of primers used in qRT-PCR

Genes	Abbreviations	Sequence 5'-3'	Amplicon length	Accesion No.
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	F: GATGCCCCAATGTTTGTGA	250	XM_014866500.1
		R: AAGCAGGGATGATGTTCTGG		
Microtubule-associated protein 1 Light Chain 3	LC3	F: TTACTGCTTTGCTCTGCCAC	213	XM_014835085.1
		R: AGCTGCTTCTCCCCCTTGTA		
Forkhead box protein O1	FOXO1	F: ATTGAGCGCTTGGACTGTGA	311	XM_014732057.1
		R: CGCTGCCAAGTTTGACGAAA		
PTEN-induced putative kinase 1	PINK1	F: GCACAATGAGCCAGGAGCTA	298	XM_014737247.1
		R: GGGGTATTCACGCGAAGGTA		
Parkin RBR E3 ubiquitin protein ligase	PARK2	F: TCCCAGTGGAGGTCGATTCT	218	XM_014858374.1
		R: CCCTCCAGGTGTGTTCGTTT		
DNA damage inducible transcript 3	CHOP	F: AGCCAAAATCAGAGCCGGAA	272	XM_001488999.3
		R: GGGGTCAAGAGTGGTGAAGG		
PKR-like Endoplasmic Reticulum Kinase	PERK	F: GTGACTGCAATGGACCAGGA	283	XM_014731045.1
		R: TCACGTGCTCACGAGGATATT		
Lysosomal Associated Membrane Protein 2	LAMP2	F: GCACCCCTGGGAAGTTCTTA	147	XM_014831347.1
		R: ATCCAGCGAACACTCTTGGG		
Coiled-coil myosin-like BCL2-interacting protein	Beclin1	F: GATGCGTTATGCCCAGATGC	233	XM_014833759.1
		R: AACGGCAGCTCCTCTGAAAT		
Pyruvate Dehydrogenase Kinase 4	PDK4	F: GCTGGTTTTGGTTATGGCTTGC	137	XM_001493731.5
		R: TCCACAGACTCAGAAGACAAAGCC		
PPARG Coactivator 1 alpha	PGC1α	F: TCTACCTAGGATGCATGG	93	XM_005608845.2
		R: GTGCAAGTAGAAACACTGC		
Tumor Necrosis Factor-Alpha	TNFα	F: AAGTGACAAGCCTGTAGCCC	254	XM_014831605.1
		R: GGTTGACCTTGGACGGGTAG		
Interleukin 1 Beta	IL-1β	F: TATGTGTGTGATGCAGCTGTG	352	XM_014852743.1
		R: ACTCAAATTCCACGTTGCCC		
B-Cell Lymphoma 2	BCL-2	F: TTCTTTGAGTTCGGTGGGGT	164	XM_014843802.1
		R: GGGCCGTACAGTTCCACAA		
BCL-2-Associated X protein	BAX	F: GCCAGCAAATTGGTGCTCAA	260	XM_014830923.1
		R: AGCAGTCACTTCCATGGCTC		
P53 tumor suppressor	p53	F: TTCCGCAAGAAGGAGGAACC	114	U37120.1
		R: TTTGGACAGAACTGCACCCT		
Fission, mitochondrial 1	FIS1	F: GGTGCGAAGCAAGTACAACG		
Myocyte Nuclear Factor		R: GTTGCCCACAGCCAGATAGA	118	XM_001504462.4
	MNF1	F: AAGTGGCATTTTTCGGCAGG	217	XM_001495170.5
		R: TCCATATGAAGGGCATGGGC		

Statistical analysis: The results used for statistical analysis were expressed as an arithmetic average of measurements obtained in subsequent repetitions. Difference between groups were determined using the nonparametric t-test. Statistical analysis was performed with GraphPad Prism 5 Software (La Jolla, USA). Differences were considered statistically significant for $p<0.05$.

RESULTS AND DISCUSSION

The effect of an exercise complemented with dietary nucleotides and anti-oxidant supplementation on muscle damage and inflammation: In order to determine the influence of the physical activity on muscle damage and inflammation, evaluation of muscle related enzyme activity such as CK and AST (Fig. 1) in serum was performed. It was distinguished that AST activity improved as a result of the implementation of the exercise which was expressed by the decreased concentration of kinases in the serum. The activity of CK, known as a regulator of energy metabolism was established to have similarly improved. Additionally, the downregulation of proinflammatory cytokine expression e.g., TNFα and IL-1β was observed. The SOD activity (Fig. 1 and 2) was significantly higher in both experimental groups when compared to the control.

The mRNA expression level of muscle-related PGC1 α (Fig. 3f) in horses supplemented with nucleotides. In turn, PDK4 transcript levels (Fig. 3f) were down regulated in both experimental groups, however, the control group compared to the nucleotide-fed group showed slight differences in mRNA expression levels.

The effect of dietary nucleotides combined with anti-oxidant supplementation on muscle autophagy: To determine whether apoptosis is correlated with muscle authophagy, expression levels of BCL-2 gene family members, known as regulators of cell survival were examined (Fig. 2). Upregulation of proapoptotic BAX expression level was observed as a result of nutrition. Comparison of BCL-2/BAX ratios between the control and

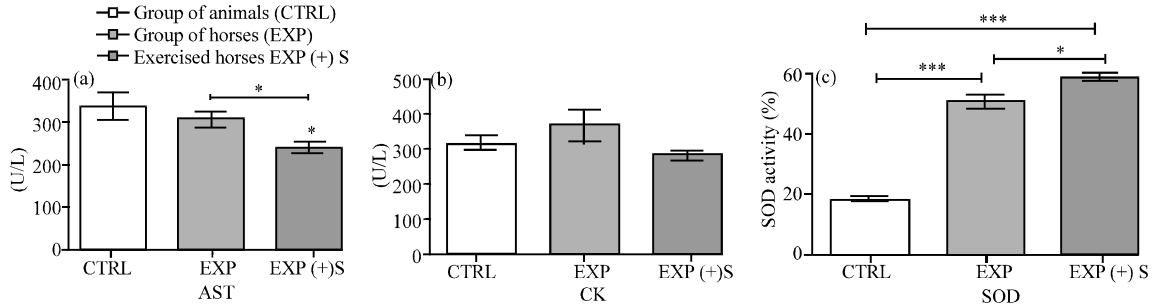

Fig. 1: a-c) The concentration of muscle-related enzymes and SOD activity in the serum. Results expressed as mean±S.D., *p<0.05, **p<0.01, ***p<0.001

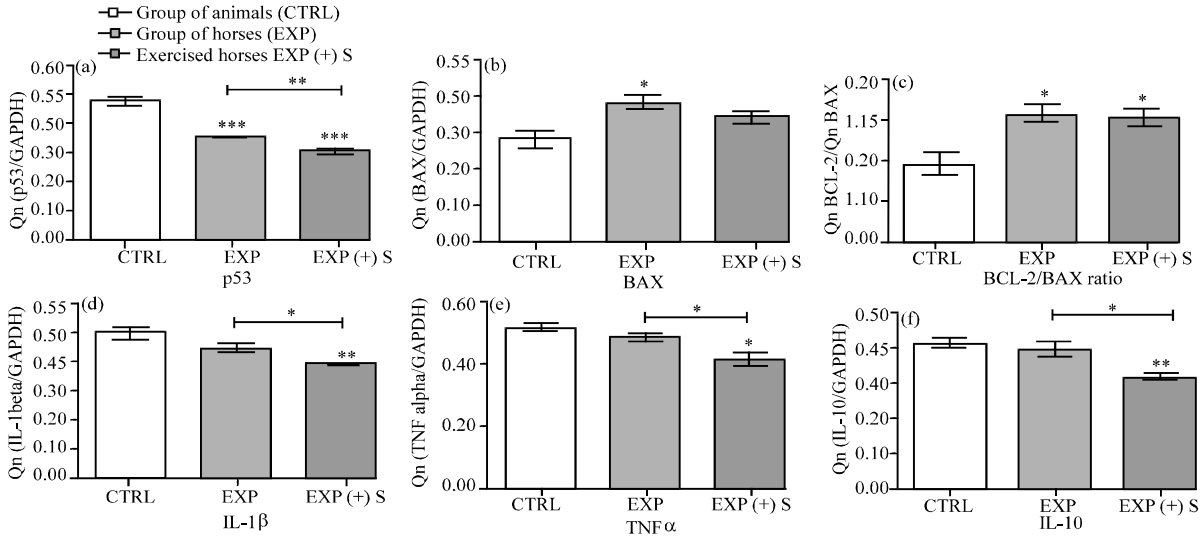

Fig. 2: a-f) The expression of apoptosis markers-p53, BAX, BCL-2 and BCL-2/BAX ratio and the expression levels of pro-inflammatory cytokines IL-1β, TNF α and IL-10. Results expressed as mean±S.D., *p<0.05, **p<0.01, ***p<0.001

experimental groups, calculated by Qn of BCL-2 and Qn of BAX, showed that the ratio for the experimental group was significantly higher than that of the control group.

Moreover, we have observed down regulation of transcription levels of p53, the positive regulator of autophagy which revealed to be significant (p<0.01). The analysis of other autophagic markers (Fig. 4) including Beclin, PARKIN, FOXO1 genes, displayed that the application of the physical activity was associated with a decrease in the expression of all the afore mentioned genes in muscle. The expression of autophagy-associated LAMP2 was upregulated as an effect of dietary nucleotide supplementation.

To evaluate the changes in endoplasmic reticulum functionality in muscles, the expression level of PERK and CHOP, known as ER stress-inducible genes was examined (Fig. 4). The levels of CHOP transcript were significantly lower in the two experimental groups. There were no significant differences in PERK mRNA levels between the control and experimental groups (p>0.05).

Analysis of mitochondria-related genes such as MNF1 and FIS1 (Fig. 3e) appeared to exhibit differences between the control and experimental groups, up-regulation of mRNA levels of MNF1 and downregulation of FIS1 in muscle was observed in horses fed with nucleotides.

Comparison of transmission electron micrographs did not show any difference in myofibrillar structure and sarcomere arrangement between the control and experimental groups (Fig. 3a-c). The length of sarcomeres was different within groups and reached the highest value in the group of horses fed with the nucleotides (Fig. 3d). It was found that in the sarcoplasm there were numerous

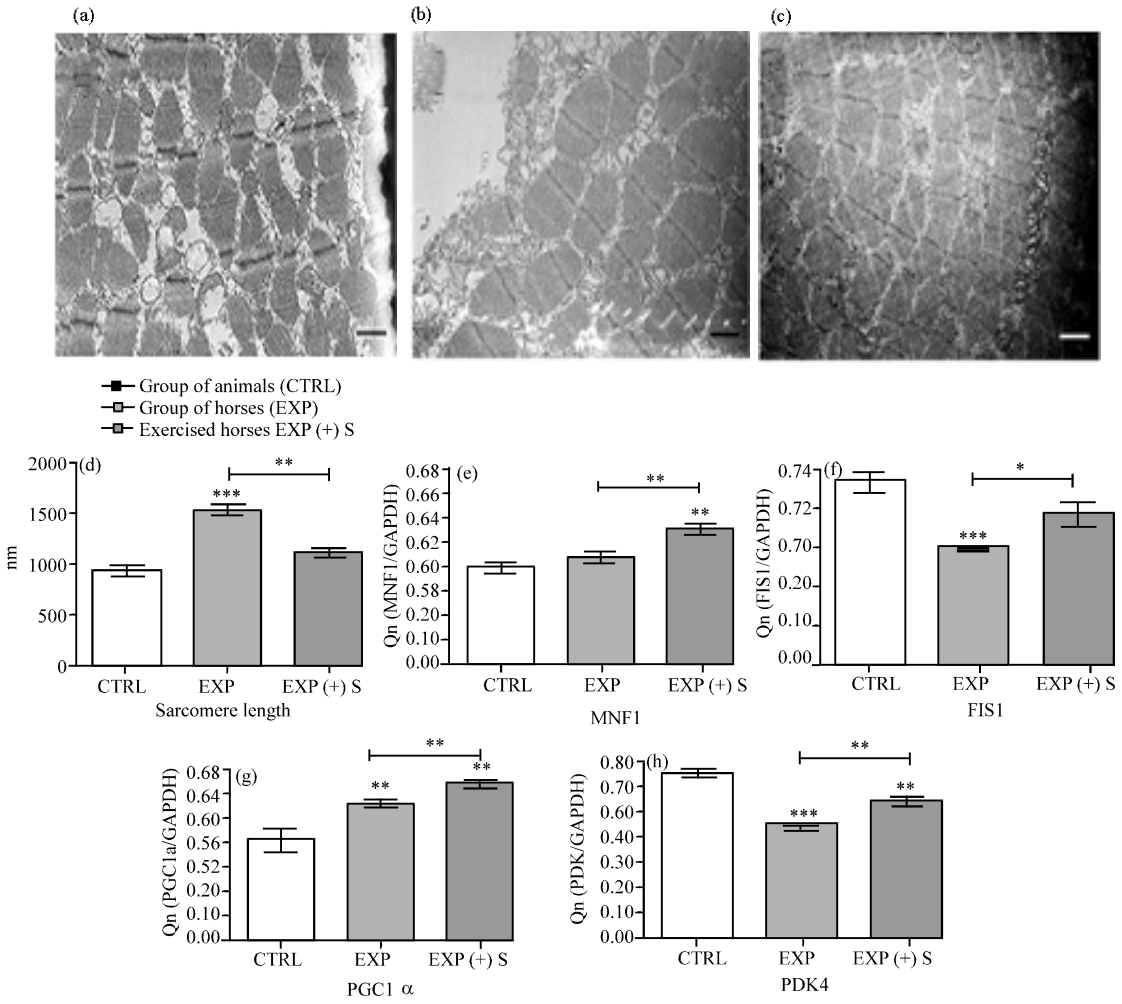

Fig. 3: The expression levels of muscle-related PGC-1α, PDK4 genes and mitochondria-related FIS1 and MNF1 genes established using a qRT-PCR. Comparison of myofibrillar structure and sarcomere arrangement between the control; a) Experimental groups; b) Horses received feed; c) Horses received feed and additive; d) Sarcomere length; e) MNF1; f) FIS1; g) PGC1 α and h) PDK4. Results expressed as mean±S.D., *p<0.05, **p<0.01, ***p<0.001. TEM images-magnification: 10000×, scale bar: 1 μm

mitochondria whose ultrastructure differed significantly between groups. It was found that in sarcoplasm of experimental horses ultrastructural changes of mitochondria were observed. In horses fed with nucleotides damaged double mitochondrial membranes as well as disturbed mitochondria cristae were noticed (Fig. 4a-c).

Skeletal muscle exhibits enormous plasticity when exposed to a variety of stress factors including exercise which induced a broad range of mechanisms for functionality maintenance. Muscles adapt to exercise via sequential gene expression that orchestrates complex protein synthesis and degradation pathways for specific fibre type composition and metabolic profiles in addition to satellite cell activation and myogenic differentiation. During training, muscles exposed to fibre degradation, oxidative stress factor or proinflammatory cytokine accumulation are subjected to strenuous conditions which could be reduced with dietary complements. Anti-oxidant supplements including vitamin E and C, together with nucleotides has been reported to possess anti-inflammatory activity (Jang *et al*., 2014). Our study found that an exercise combined with dietary nucleotides and anti-oxidant supplementation reduces proinflammatory cytokine expression in skeletal muscle cells on the mRNA level with simultaneous up-regulation of anti-oxidant defense mechanisms coming from Superoxide Dismutase activity (SOD) in peripheral blood.

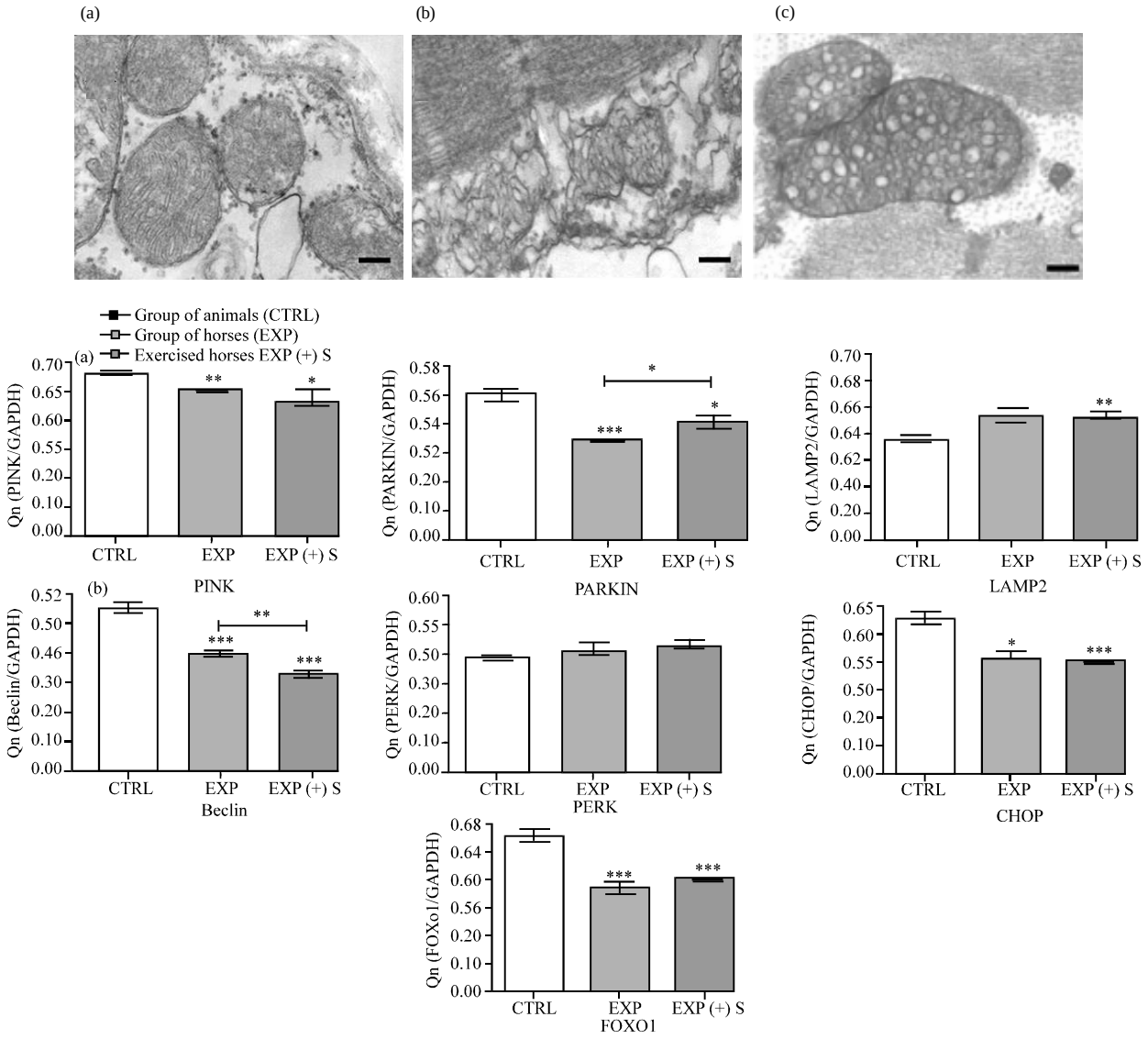

Fig. 4: a-j) The evaluation of endoplasmic reticulum functionality by qRT-PCR analysis of CHOP and P3ERK expression levels and expression of autophagy-associated LAMP2, Beclin, PARKIN, PINK and FOXO1 genes. Ultrastructure of mitochondria in control group; a) Experimental group; b) Group of experimental horses received an additive that contained the anti-oxidant; c) Nucleotide supplements; d) PINK; e) PARKIN; f) LAMP; g) Beclin; h) PERK; i) CHOP and j) FOXO1. Results expressed as mean±S.D., *p<0.05, **p<0.01, ***p<0.001. TEM images-magnification: 30000x, scale bar: 200 nm

It was showed significant suppression of TNFα, IL-1β and IL-10 (interleukin 10) on the mRNA level in muscles of the group horses fed with the nucleotides. It was unveiled that several anti-oxidants including vitamin E and C, besides their antioxidative properties also suppress muscle inflammation. In this study, a combination of Vitamin E and C in daily ratios of 1300 and 5000 mg, respectively were used, since, this has been reported as an effective dosage to reduce muscle inflammation during endurance training (Ciocoiu *et al*., 2007). However, Silva *et al*. (2010) showed that vitamin E in humans reduces muscle inflammation. Furthermore, Taghiyar *et al*. (2013) determined that the combination of Vitamin E and C supplemented with protects against muscle damage in female athletes. Along with anti-inflammatory effects as well as anti-apoptotic effects on the mRNA level in experimental horses were also observed. The experimental horses which received tested additive exhibited

suppression of p53 transcript as well as BAX mRNA expression. Obtained data, stand in good agreement with Nunes and colleagues findings which also showed that Vitamin E prevents cell apoptosis induced by mild oxidative stress (Nunes *et al*., 2005).

It is well known that both creatine kinase and aspartate transaminase activities are induced as an answer to exercise. Freestone *et al*. (1989) indicated that intensively exercised thoroughbred exhibit increased CK and AST activity. In the research the exercise and supplementation decreased CK and AST in horses. It was observed significant reduction of CK in experimental, exercised horses fed with tested additives in comparison to the control group. This could be due to the nucleotide supplements, since, in other studies animals exposed to treadmill exercise complemented with Cytosine Monophos Phate (CMP) and Uridine Monophos Phate (UMP) supplements exhibit lower CK and AST (Mizushima *et al*., 2008). It was proposed that the administration of CMP/UMP favours the uptake of glucose in muscles and maintains the level of hepatic glycogen constant during exercise. Also, reduction in muscle autophagy in experimental horses was observed in comparison to control horses. The Beclin-1 gene expression is important for localization of autophagic proteins to a Pre-Autophagosomal Structure (PAS), depending on interactions with the Class 3 type Phosphoinositide 3-Kinases (PI3KC3)/Vps34 and Beclin-1 gene expression was significantly downregulated in trained horses when compared to control horses. Beclin-1 was found to be involved in induction of a correct autophagic flux in muscles during fasting and exercise (Jamart *et al*., 2012). It was observed reduced expression of Beclin-1, that is strongly correlated with observed reduced apoptosis in horses fed with supplements. In the case of excessive autophagy, the reduced activity of Beclin-1 and p53 mRNA levels together with no statistical differences between CHOP and PERK expression might suggest that anti-oxidants and nucleotide supplements might serve a protective role. Also, excessive autophagy in muscles leads to induction of several pathologic processes including muscle atrophy. However, mitophagy and mitochondrial biogenesis seem to be crucial processes in the progression of muscle development and resistance in young horses. Here, it was found simultaneous upregulation of FOXO1 and PGC1 α mRNA levels in supplemented horses versus control horses. PGC1 α is a pivotal regulator of mitochondrial biogenesis, it also interacts with and stimulates the expression of transcription factors that function in control of nuclear-mitochondrial interactions such as nuclear respiratory factors NRF1 and NRF2. It was demonstrated that up-regulation of PCG1 α transcript in cultured muscle cells and adult skeletal muscle induced mitochondrial biogenesis (Wu *et al*., 1999). Moreover, observed FOXO1 up-regulation in the experimental horses suggests that anti-oxidants and dietary nucleotides have regulatory effect on muscle energy homeostasis during catabolic conditions. It was also demonstrated that FOXO1 reduces carbohydrate catabolism during fasting by increasing the expression of the Pyruvate Dehydrogenase Kinase 4 (PDK4), resulting in the conservation of glucose and gluconeogenic substrates (lactate, pyruvate and alanine) and a decrease in glycolytic flux by the inactivation of the pyruvate dehydrogenase complex (Lin *et al*., 2002). It was found that PDK4 was up regulated in experimental horses in comparison to the control individuals. Additionally, simultaneous up-regulation of Parkin muscle expression in experimental horses confirms the abundance of mitophagy and mitochondrial biogenesis. It was demonstrated that Parkin in knockout mice exhibited a 30% reduction in state 3 (active) mitochondrial respiration (Taghiyar *et al*., 2013). Likewise, upregulation of both: Mitofusin 1 (MFN1) and Fission 1 (FIS1) mRNA levels in skeletal muscles that regulate mitochondrial dynamics and maintain their proper function, occurred. Thus, the combination of exercise and supplementation of anti-oxidants as well as dietary nucleotides seems to not only increase the number of mitochondria but also to improve their function and efficiency. It was shown that training appears to regulate both mitochondrial fusion and fission processes. In 7 sessions of high intensity interval training, it was examined to progressively increase the protein content of MFN1 and FIS1 (Perry *et al*., 2010). Ding *et al*. (2010) further showed that a single bout of treadmill running in rats induced increased MFN1 and MFN2 mRNA levels in 24 h post-exercise while both MFN1 and MFN2 proteins remained at baseline.

CONCLUSION

The obtained data suggests that anti-oxidant and dietary nucleotide supplementation may significantly reduce apoptosis and inflammation in skeletal muscles of young trained horses. Over and above, it was determined that the proposed combination of nutrients enhanced mitochondrial biogenesis as well as, selective mitophagy and mitochondrial dynamics with induction of antioxidative defense. Consequently, exercise combined with dietary nucleotides and anti-oxidant supplementation could be a prospective and effective non-invasive dietary strategy in muscle protection for young horse performers.

REFERENCES

Agudelo, L.Z., T. Femenia, F. Orhan, M. Porsmyr-Palmertz and M. Goiny *et al*., 2014. Skeletal muscle PGC-1a1 modulates kynurenine metabolism and mediates resilience to stress-induced depression. Cell, 159: 33-45.

Alessio, H., A.H. Goldfarb and R.G. Cutler, 1988. MDA content increases in fast-and slow-twitch skeletal muscle with intensity of exercise in a rat. Am. J. Physiol. Cell Physiol., 255: C874-C877.

Booth, F.W., C.K. Roberts and M.J. Laye, 2012. Lack of exercise is a major cause of chronic diseases. Compr. Physiol., 2: 1143-1211.

Carver, J.D. and W.A. Walker, 1995. The role of nucleotides in human nutrition. J. Nutr. Biochem., 6: 58-72.

Chomczynski, P. and N. Sacchi, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159.

Ciocoiu, M., M. Badescu and I. Paduraru, 2007. Protecting antioxidative effects of vitamins E and C in experimental physical stress. J. Physiol. Biochem., 63: 187-194.

Davies, K.J., A.T. Quintanilha, G.A. Brooks and L. Packer, 1982. Free radicals and tissue damage produced by exercise. Biochem. Biophys. Res. Commun., 107: 1198-1205.

Dillard, C.J., R.E. Litov, W.M. Savin, E.E. Dumelin and A.L. Tappel, 1978. Effects of exercise, vitamin E and Ozone on pulmonary function and lipid peroxidation. J. Appl. Physiol., 45: 927-932.

Ding, H., N. Jiang, H. Liu, X. Liu and D. Liu *et al*., 2010. Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle. Biochim. Biophys. Acta (BBA)-Gen. Subjects, 1800: 250-256.

Freestone, J.F., S.G. Kamerling, G. Church, C. Bagwell and J. Hamra, 1989. Exercise induced changes in creatine kinase and aspartate aminotransferase activities in the horse: Effects of conditioning, exercise tests and acepromazine. J. Equine Vet. Sci., 9: 275-280.

Gil, A., 2002. Modulation of the immune response mediated by dietary nucleotides. Eur. J. Clin. Nutr., 56: S1-S4.

Jamart, C., M. Francaux, G.Y. Millet, L. Deldicque and D. Frere *et al*., 2012. Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. J. Appl. Physiol., 112: 1529-1537.

Jang, I.S., Y.H. Ko, Y.S. Moon and S.H. Sohn, 2014. Effects of vitamin C or E on the pro-inflammatory cytokines, heat shock protein 70 and antioxidant status in broiler chicks under summer conditions. Asian Australas. J. Anim. Sci., 27: 749-756.

Lin, J., H. Wu, P.T. Tarr, C.Y. Zhang and Z. Wu *et al*., 2002. Transcriptional co-activator PGC-1a drives the formation of slow-twitch muscle fibres. Nat., 418: 797-801.

Mammucari, C., G. Milan, V. Romanello, E. Masiero and R. Rudolf *et al*., 2007. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab., 6: 458-471.

Marycz, K., K. Kornicka, J. Grzesiak, A. Smieszek and J. Szlapka, 2016. Macroautophagy and selective mitophagy ameliorate chondrogenic differentiation potential in adipose stem cells of equine metabolic syndrome: New findings in the field of progenitor cells differentiation. Oxid. Med. Cell. Longevity, 2016: 1-18.

Masiero, E., L. Agatea, C. Mammucari, B. Blaauw and E. Loro *et al*., 2009. Autophagy is required to maintain muscle mass. Cell Metab., 10: 507-515.

Mizushima, N., B. Levine, A.M. Cuervo and D.J. Klionsky, 2008. Autophagy fights disease through cellular self-digestion. Nat., 451: 1069-1075.

Nascimbeni, A.C., M. Fanin, E. Masiero, C. Angelini and M. Sandri, 2012. Impaired autophagy contributes to muscle atrophy in glycogen storage disease type II patients. Autophagy, 8: 1697-1700.

Neel, B.A., Y. Lin and J.E. Pessin, 2013. Skeletal muscle autophagy: A new metabolic regulator. Trends Endocrinol. Metab., 24: 635-643.

Nunes, V.A., A.J. Gozzo, I. Cruz-Silva, M.A. Juliano and T.A. Viel *et al*., 2005. Vitamin E prevents cell death induced by mild oxidative stress in chicken skeletal muscle cells. Comp. Biochem. Physiol. Part C. Toxicol. Pharmacol., 141: 225-240.

Penna, F., D. Costamagna, F. Pin, A. Camperi and A. Fanzani *et al*., 2013. Autophagic degradation contributes to muscle wasting in cancer cachexia. Am. J. Pathol., 182: 1367-1378.

Perry, C.G., J. Lally, G.P. Holloway, G.J. Heigenhauser and A. Bonen *et al*., 2010. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J. Physiol., 588: 4795-4810.

Rogers, M.A. and W.J. Evans, 1993. Changes in skeletal muscle with aging: Effects of exercise training. Exercise Sport Sci. Rev., 21: 65-102.

Sandri, M., 2010. Autophagy in skeletal muscle. FEBS Lett., 584: 1411-1416.

Silva, L.A., C.A. Pinho, P.C. Silveira, T. Tuon and C.T.D. Souza *et al*., 2010. Vitamin E supplementation decreases muscular and oxidative damage but not inflammatory response induced by eccentric contraction. J. Physiol. Sci., 60: 51-57.

Sun, Q., W. Fan, K. Chen, X. Ding and S. Chen *et al*., 2008. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc. Nat. Acad. Sci., 105: 19211-19216.

Taghiyar, M., L. Darvishi, G. Askari, A. Feizi and M. Hariri *et al*., 2013. The effect of vitamin C and E supplementation on muscle damage and oxidative stress in female athletes: A clinical trial. Intl. J. Preventive Med., 4: S16-S23.

Twig, G., B. Hyde and O.S. Shirihai, 2008. Mitochondrial fusion, fission and autophagy as a quality control axis: The bioenergetic view. Biochim. Biophys. Acta BBA. Bioenerg., 1777: 1092-1097.

Wu, Z., P. Puigserver, U. Andersson, C. Zhang and G. Adelmant *et al*., 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGG-1. Cell, 98: 115-124.