



Molecular Characterization and Expression Analysis of Equine *Vascular Endothelial Growth Factor Alpha (VEGF α)* Gene in Horse (*Equus caballus*)

Ki-Duk Song^a, Hyun-Woo Cho^{1,a}, Hak-Kyo Lee*, and Byung Wook Cho^{1,*}

Genomic Informatics Center, Hankyong National University, Anseong 456-649, Korea

ABSTRACT: The objective of this study was to determine the molecular characteristics of the horse *vascular endothelial growth factor alpha* gene (*VEGF α*) by constructing a phylogenetic tree, and to investigate gene expression profiles in tissues and blood leukocytes after exercise for development of suitable biomarkers. Using published amino acid sequences of other vertebrate species (human, chimpanzee, mouse, rat, cow, pig, chicken and dog), we constructed a phylogenetic tree which showed that equine *VEGF α* belonged to the same clade of the pig *VEGF α* . Analysis for synonymous (Ks) and non-synonymous substitution ratios (Ka) revealed that the horse *VEGF α* underwent positive selection. RNA was extracted from blood samples before and after exercise and different tissue samples of three horses. Expression analyses using reverse transcription-polymerase chain reaction (RT-PCR) and quantitative-polymerase chain reaction (qPCR) showed ubiquitous expression of *VEGF α* mRNA in skeletal muscle, kidney, thyroid, lung, appendix, colon, spinal cord, and heart tissues. Analysis of differential expression of *VEGF α* gene in blood leukocytes after exercise indicated a unimodal pattern. These results will be useful in developing biomarkers that can predict the recovery capacity of racing horses. (**Key Words:** Differentially Expressed Gene, Exercise, Horse, Quantitative-Polymerase Chain Reaction, Vascular Endothelial Growth Factor)

INTRODUCTION

Racing horses, such as Thoroughbreds, have been selected for athletic performance traits by implementing systemic structural and functional adaptations. These processes have given racing horses a range of extreme physiological characteristics, including high aerobic and anaerobic metabolic capacities, a large lung volume, high maximum hemoglobin concentration and cardiac output as

well as a large muscle mass to body ratio, high skeletal muscle mitochondrial density and oxidative enzyme activity, and large stores of energy substrates (Essén-Gustavsson and Lindholm, 1985; Pösö et al., 1993; Hyypä, et al., 1997; Hinchcliff and Geor, 2008). Racing, as a sort of exercise, results in acute immune responses and inflammation in joints and muscle of horses (Auer et al., 1989; Bertone et al., 2001; Petersen et al., 2004; Firth et al., 2006). In horses, like human, intense physical activity induces subclinical tissue damage in muscle and joints, resulting in recruit of immune cells, i.e., neutrophils and monocytes. These cells trigger inflammation by up-regulating various cytokines, such as tumor necrosis factor alpha (TNF α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), and interleukin-10 (IL-10) (Streltsova et al., 2006; Lamprecht et al., 2008) and growth factors such as *VEGF α* in muscle (Breen et al., 1996; Roca et al., 1998; Richardson et al., 1999; Richardson et al., 2000; Brutsaert et al., 2002).

It is crucial for racing horses to recover from exercise as

* Corresponding Author: Byung Wook Cho. Tel: +82-55-350-5515, Fax: +82-51-581-2962, E-mail: bwcho@pusan.ac.kr / Hak-Kyo Lee. Tel: +82-31-670-5659, Fax: +82-31-670-5662, E-mail: breedlee@empas.com

¹ Department of Animal Science, College of Nature Resources and Life Science, Pusan National University, Miryang 627-706, Korea.

^a Ki-Duk Song and Hyun-Woo Cho equally contributed to this work.

Submitted Dec. 16, 2013; Revised Mar. 2, 2014; Accepted Mar. 18, 2014

soon as possible. This recovery process might be mediated by soluble factors such as cytokines and growth factors. Nonetheless, there is no biomarker based on cytokines and/or growth factors that may reflect the degree of recovery from exercise in racing horses. In a previous study, we identified differentially expressed genes (DEG) in muscle and blood from Thoroughbred horses in response to exercise (Park et al., 2012). Furthermore, the findings provides a chance to develop molecular markers that may be related to economic traits of racing horses. The identified DEGs can be used to develop biomarkers that might be useful for monitoring the health of a horse after racing. We found that VEGF α expression was significantly increased in muscle, indicating that VEGF α may play an important role in recovery from exercise. In spite of the importance of VEGF α in various biological processes, such as angiogenesis, so far few studies have been conducted on this gene at the molecular level in horses. Therefore, in this study, we investigated the molecular structure of horse VEGF α gene, constructed a phylogenetic tree, and examined the expression profiles in various horse tissue samples. In addition, since there is a lack of suitable biomarkers to monitor recovery in horses after exercise, we explored the possibility of using VEGF α as a biomarker for exercise and recovery in blood leukocytes.

MATERIALS AND METHODS

Blood and tissue sample in horse

Blood samples were obtained from three male Thoroughbred horses (2 through 4 year-old; two horses: 2 year-old, one horse: 4 year-old) maintained at Ham-An racing Horse Resort and Training Center. Horses were placed on a treadmill and the treadmill exercises were performed at the speed of 10 to 15 km/h. The blood samples were taken before exercise and every 30 min after exercise up to 120 min. The National Institute of Subtropical Agriculture, Rural Development Administration, provided three male Jeju horses (4 year-old) that were used for tissue sampling. Skeletal muscle, kidney, thyroid, lung, appendix, colon, spinal cord and heart tissues were obtained from each horse and kept in liquid N₂ until RNA extraction.

Sequence data analysis

The VEGF α DNA and amino acid sequences of ten species (cattle, chicken, chimpanzee, dog, horse, human, pig, mouse, rat, sheep) were retrieved from Ensembl (<http://www.ensembl.org/>), The Ensembl ID for the sequences are as follows: horse- ENSECAG00000009402, human- ENSG00000112715, chimpanzee- ENSPTRG00000001080, mouse-ENSMUSG00000023951, rat- ENSRNOG00000019598, cow- ENSBTAG00000005339, pig: ENSSSCG00000001695, chicken- ENSGALG00000010290,

dog- ENSCAFG00000001938, cat- ENSFCAG00000026462.

The retrieved sequences were aligned in BioEdit with the CLUSTERW option. Phylogenetic analysis was conducted using MEGA version 5.0 (Arizona State University, AZ, USA) (Tamura et al., 2011) The Neighbor Joining method (Saitou et al., 1987) was used with the following options: pairwise-deletion, 1,000 bootstrap replications and Kimura 2-parameter. Pairwise deletion was chosen to retain all sites initially, then excluding them as necessary in the pairwise distance estimation. Substitutions of nucleotides were obtained by using the Kimura 2-parameter model. We also showed that synonymous substitutions per site (Ks) and non-synonymous substitutions per site (Ka) in VEGF α genes for individual species. In addition, pairwise distance was calculated by analyzing the nucleotide and amino acid similarity.

RNA extraction and cDNA synthesis

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the tissue samples (skeletal muscle, kidney, thyroid, lung, appendix, colon, spinal cord, and heart) and leukocytes after exercise according to the Invitrogen manual. In order to prevent contamination of genomic DNA, RNase-free DNase kit (Qiagen) was used according to the manufacturer's manual. Total RNA quantification was performed using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNAs were synthesized in a reaction with oligo-dT primers, moloney-murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA), RNase inhibitor (Promega) and RNase-free ddH₂O, which was incubated at 37°C for 4 h.

RT-PCR analysis of equine VEGF α

The horse VEGF α expression level was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) amplification. The primers were designed using the PRIMER3 software (MIT, Cambridge, MA, USA, <http://frodo.wi.mit.edu/primer3/>) with an expected product size of 184 bp. To detect equine VEGF α mRNA, following primers were used: forward 5'-CTA CCT CCA CCA TGC CAA GT-3' and reverse 5'-CAC ACA GGA TGG CTT GAA GA-3'. The RT-PCR conditions were as follows: an initial step of 94°C for 10 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final step of 72°C for 10 min. RT-PCR products were analyzed with gel electrophoresis on a 2.0% SeaKem LE agarose gel (Lonza, NJ, USA). Equine GAPDH gene was used as a normalizer for RT-PCR.

Quantitative PCR analysis of equine VEGF α expression and statistical analysis

The cDNA was analyzed by quantitative-PCR (qPCR)

on BioRad CFX-96 (Bio-Rad, Hercules, CA, USA). Each reaction was performed in a total volume of 25 μ L with 14 μ L SYBR green master mix, 2 μ L (5 pmol) VEGF α forward primer, 2 μ L (5 pmol) reverse primer, 5 μ L distilled water and 2 μ L (50 ng/ μ L) of the cDNA. The PCR conditions were as follows: pre-denaturation step at 94°C for 3 min, 39 cycles at 94°C for 10 s and at 60°C for 30 s, 72°C for 30 s, followed by 72°C for 10 min as a final step. Dissociation was accomplished in a condition at temperature increase from 55°C to 95°C over 25 min. Dissociation temperature increased in 0.5°C each. All samples were measured in triplicate to ensure reproducibility, and C_t value was calculated using the 2^{- $\Delta\Delta$ C_t} method (Livak et al., 2001). Statistical significance was calculated by *t*-test. A p values of less than 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Molecular structure and distribution of the equine VEGF α gene

In a previous study, we performed RNA-seq analyses to identify DEGs in response to exercise. Among the identified DEGs, we found that the transcript of VEGF α was expressed up to 2.59 folds higher after exercise (Park et al.,

2012). As not much is known about the horse VEGF α gene, we retrieved horse VEGF α sequences from the Ensembl database and analyzed its molecular structure. We found that the gene had eight exons and was 696 bp long in horses (Figure 1A). When we examined the expression of VEGF α transcripts by RT-PCR in eight tissues, we found that all tissues ubiquitously expressed VEGF α transcripts. However, thyroid and lung tissue expressed the highest levels of VEGF α transcript whereas the skeletal muscle and appendix tissue expressed relatively low levels of VEGF α transcript (Figure 3).

Evolutionary and phylogenetic analysis of VEGF α gene

Phylogenetic analysis of the VEGF α gene by neighbor-joining method revealed that equine VEGF α belongs to the same clade as the porcine VEGF α gene (Figure 2).

To better understand the evolution of the VEGF α gene, we analyzed synonymous (Ks) and non-synonymous (Ka) substitutions of VEGF α amino acids from ten species (Table 1). Pairwise comparison showed that Ks ranged from 0.168 to 2.112, whereas Ka ranged from 0.191 to 1.468. It is noted that Ks of equine VEGF α was the lowest with the pig gene (0.188) and the highest with the dog gene (2.112). The lowest Ks values were obtained between mouse and chimpanzee (0.092). The average of Ka and Ks were 0.839

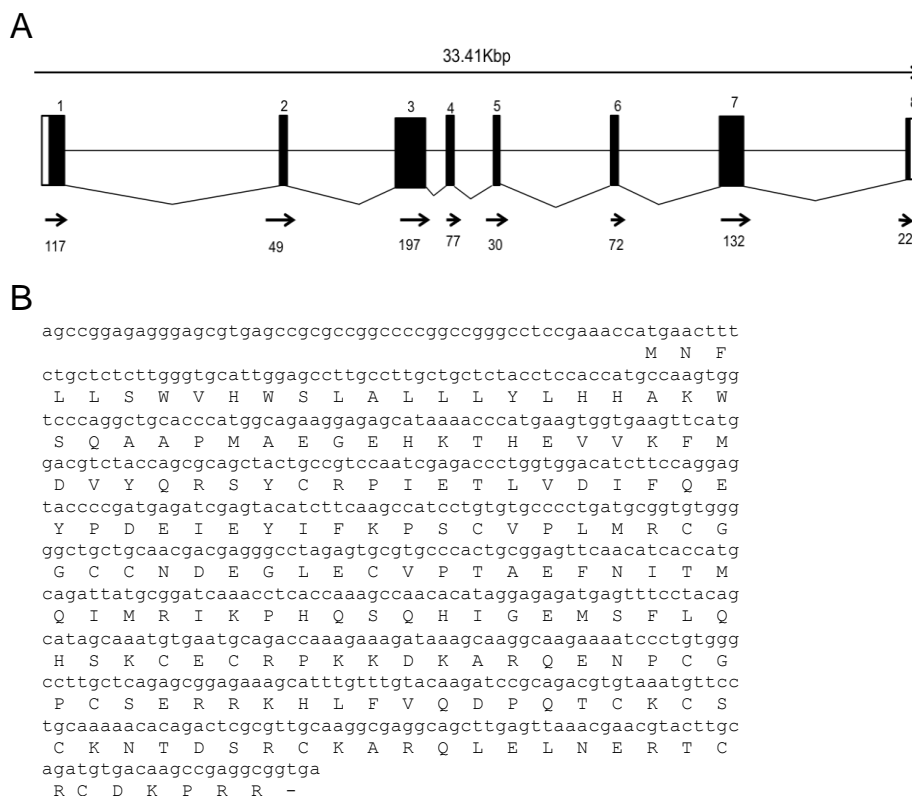


Figure 1. Gene structure and deduced amino acids sequences of equine VEGF α gene. (A) Structure of equine VEGF α gene. (B) Deduced amino acid sequences of equine VEGF α from mRNA sequences.

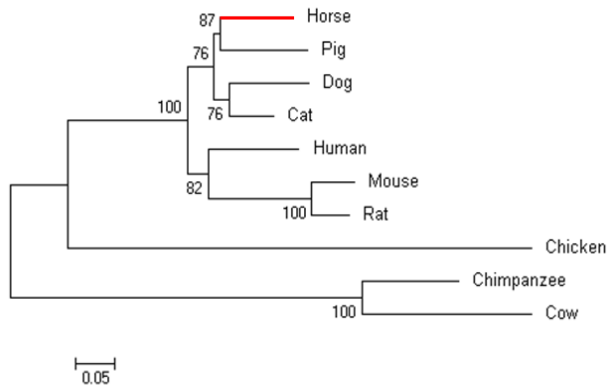


Figure 2. Phylogenetic tree of the *VEGFα* gene from ten different species using the neighbor-joining method.

and 0.689, respectively, resulting in a Ka/Ks ratio of greater than 1. This means that non-synonymous substitutions occurred more rapidly than synonymous substitutions. These results are based on the assumption that the protein-coding regions in the human genome have been under positive selection during evolution (Bustamante et al., 2005). These data indicated that positive selection occurred in the *VEGFα* genes during the evolutionary history of the horse.

Differential expression of equine *VEGFα* transcripts during recovery post exercise

To investigate the relationship between *VEGFα* gene expression level and time length after exercise, we performed qPCR analysis using blood leukocyte samples obtained before and after exercise (30 min, 60 min, 90 min, 120 min) from three horses (Figure 4A and B). Interestingly, the expression level of *VEGFα* was the highest after 60 min of exercise and then gradually decreased after upto 120 min. But the expression level of 120 min was slightly higher than that of 0 min (before exercise, $p > 0.05$).

CONCLUSION

In horse, as there are few reliable biomarkers for

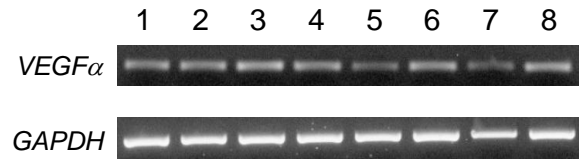


Figure 3. Expression levels of equine *VEGFα* gene by RT-PCR analysis in various horse tissues. RT-PCR products were visualized by gel electrophoresis. 1, skeletal muscle; 2, kidney; 3, thyroid; 4, lung; 5, appendix; 6, colon; 7, spinal cord; 8, heart. This is a representative result of three animals ($n = 3$). *GAPDH* gene was used as a control.

recovery after exercise except creatine kinase (Linder et al., 2006) and lactate (Lindner et al., 2009), it is necessary to identify and evaluate markers for post exercise recovery. In this sense, RNA-seq based transcriptome analysis may be useful. In a previous study, horse *VEGFα* transcript increased significantly in response to exercise (Park et al., 2012) similar to humans suggesting a conserved mechanism for the regulation of *VEGFα* transcription. Therefore, we selected the equine *VEGFα* gene for further evaluation at the molecular level. A phylogenetic analysis showed that the horse *VEGFα* gene is grouped into the same clade as the pig gene and have undergone positive selection. In terms of expression, horse *VEGFα* was found to be ubiquitously expressed but regulated by exercise in blood leukocytes. Especially, expression kinetics obtained in this study showed that the transcript level gradual increased up to 60 min ($p < 0.05$), and then remained at a slightly higher level than at time 0, which is not statistically significant ($p > 0.05$). It is not certain how *VEGFα* expression is upregulated and reached the highest level after 60 min of exercise. One explanation would be that acute exercise may induce the hypoxia condition in leukocytes, which triggers hypoxia-inducible factor-1 α (HIF-1 α) activation. Activated HIF-1 α will induce the transcriptional activation of *VEGFα* by binding to HIF-responsive elements which are located in the promoter region of *VEGFα* (Jiang et al., 1996). Therefore, it is worth examining equine HIF-1 α expression

Table 1. Synonymous (Ks) and non-synonymous substitutions (Ka) per amino acid site in the *VEGFα* gene of ten species

Ks	Ka									
	1	2	3	4	5	6	7	8	9	10
1. Horse	-	0.255	0.374	0.359	0.242	0.191	0.188	0.291	1.468	1.111
2. Human	0.239	-	0.303	0.300	0.299	0.259	0.272	0.411	1.515	1.161
3. Chimpanzee	0.349	0.312	-	0.103	0.436	0.364	0.384	0.545	1.492	1.253
4. Mouse	0.350	0.314	0.092	-	0.432	0.352	0.375	0.521	1.466	1.210
5. Rat	0.203	0.280	0.400	0.401	-	0.241	0.279	0.246	1.354	1.045
6. Cow	0.168	0.264	0.361	0.362	0.185	-	0.212	0.343	1.53	1.144
7. Pig	0.188	0.284	0.375	0.372	0.247	0.215	-	0.324	1.496	1.150
8. Chicken	0.281	0.416	0.539	0.529	0.221	0.344	0.328	-	1.515	1.140
9. Dog	2.112	2.188	2.308	2.206	1.722	2.158	2.168	2.238	-	1.065
10. Cat	1.287	1.412	1.482	1.443	1.110	1.291	1.339	1.411	1.275	-

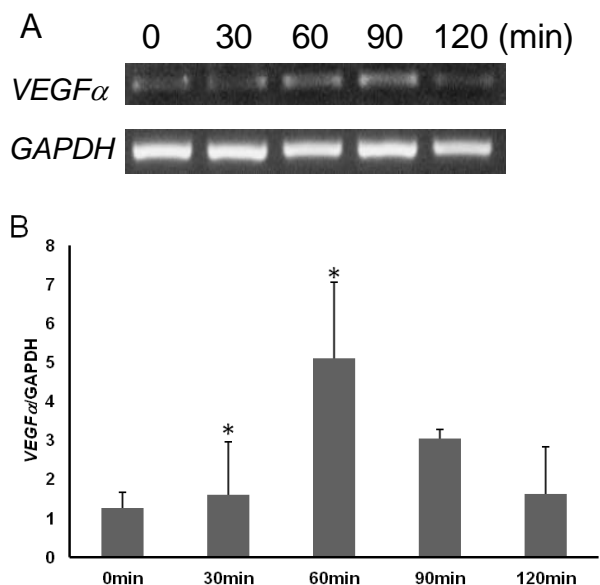


Figure 4. Differential expression of *VEGFα* gene in horse blood leukocytes after exercise. (A) RT-PCR of horse *VEGFα* gene in leukocytes of horse before exercise to post exercise at 120 min (a representative result of three horses). (B) Expression analysis of the *VEGFα* gene used to qPCR. Expression was calculated by $2^{-\Delta\Delta CT}$ method. 0 min, before exercise; 30 min, 30 min after exercise; 60 min, 60 min after exercise; 90 min, 90 min after exercise; 120 min, 120 min after exercise. This is a representative result of three animals ($n = 3$). *GAPDH* gene was used as a control. * $p < 0.05$.

in blood leukocytes after exercise to reveal the causal relationship between HIF-1 α and VEGF α expression.

Nonetheless, the role of VEGF α in response to exercise and recovery is not clear in blood. It is likely that the circulating VEGF α facilitates mobilization of monocytes into injured areas after exercise, and promotes wound healing by stimulating endothelial cell proliferation and extracellular matrix changes as process of recovery (Rocha et al., 1988; Richardson et al., 1999). Additionally, vascular distribution and density increase in response to exercise in skeletal muscle as an adaptation, which accompanies increase VEGF α expression (Brodal et al., 1977). Further study is warranted to explore the relationship between expression levels of VEGF α in muscle and blood with physiological changes.

In summary, we analyzed molecular structure of equine *VEGFα* gene and performed phylogenetic analysis with homologues of other species, which revealed that *VEGFα* gene of horse belongs to the same clade with that of pig. Gene expression analysis using various horse tissues showed the ubiquitous expression of horse *VEGFα* gene. Also, it is of note that VEGF α expression in blood leukocytes is regulated in response to exercise, suggesting that horse VEGF α expression in leukocytes can be used as

a biomarker for recovery after exercise.

ACKNOWLEDGMENTS

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008106, PJ008196), Rural Development Administration, Republic of Korea.

REFERENCES

- Auer, D. E., J. C. Ng, J. Hrdlicka, and A. A. Seawright. 1989. The elimination of injected superoxide dismutase from synovial fluid of the horse. *Aust. Vet. J.* 66:117-119.
- Bertone, A. L., J. L. Palmer, and J. Jones. 2001. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet. Surg.* 30:528-538.
- Breen, E. C., E. C. Johnson, H. Wagner, H. M. Tseng, L. A. Sung, and P. D. Wagner. 1996. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J. Appl. Physiol.* 81:355-361.
- Brodal, P., F. Ingjer, and L. Hermansen. 1977. Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. *Am. J. Physiol. Heart Circ. Physiol.* 232:H705-H712.
- Brutsaert, T. D., T. P. Gavin, Z. Fu, E. C. Breen, K. Tang, O. Mathieu-Costello, and P. D. Wagner. 2002. Regional differences in expression of VEGF mRNA in rat gastrocnemius following 1 hr exercise or electrical stimulation. *BMC Physiol.* 2:8
- Bustamante, C. D., A. Fledel-Alon, S. Williamson, R. Nielsen, M. T. Hubisz, S. Glanowski, D. M. Tanenbaum, T. J. White, J. J. Sninsky, R. D. Hernandez, D. Civello, M. D. Adams, M. Cargill, and A. G. Clark. 2005. Natural selection on protein-coding genes in the human genome. *Nature* 437:1153-1157.
- Essén-Gustavsson, B. and A. Lindholm. 1985. Muscle fibre characteristics of active and inactive standard bred horses. *Equine Vet. J.* 17:434-438.
- Firth, E. C. 2006. The response of bone, articular cartilage and tendon to exercise in the horse. *J. Anat.* 208:513-526.
- Hinchcliff, K. W. and R. J. Geor. 2008. *The Horse as an Athlete: A Physiological Overview. Equine exercise physiology: The science of exercise in the athletic horse.* Saunders/Elsevier, Edinburgh, UK, New York, USA. pp ix, 463.
- Hyypä, S., L. A. Räsänen, and A. R. Pösö. 1997. Resynthesis of glycogen in skeletal muscle from standardbred trotters after repeated bouts of exercise. *Am. J. Vet. Res.* 58:162-166.
- Jiang, B. H., E. Rue, G. L. Wang, R. Roe, and G. L. Semenza. 1996. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.* 271:17771-17778.
- Lamprecht, E. D., C. A. Bagnell, and C. A. Williams. 2008. Inflammatory responses to three modes of intense exercise in Standardbred mares-A pilot study. *Comp. Exer. Physiol.* 5:115-125.
- Lindner, A., R. Signorini, L. Brero, E. Arn, R. Mancini, and A. Enrique. 2006. Effect of conditioning horses with short intervals at high speed on biochemical variables in blood. *Equine Vet. J.* 38(Suppl. 36):88-92.

- Lindner, A., H. Mosen, S. Kissenbeck, H. Fuhrmann, and H. P. Sallmann. 2009. Effect of blood lactate-guided conditioning of horses with exercises of differing durations and intensities on heart rate and biochemical blood variables. *J. Anim. Sci.* 87:3211-3217.
- Livak, K. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402-408.
- Park, K. D., J. S. Park, J. S. Ko, B. C. Kim, H. S. Kim, K. Ann, K. T. Do, H. S. Choi, H. M. Kim, S. H. Song, S. W. Lee, H. S. Kong, Y. M. Yang, B. H. Jhun, C. H. Kim, T. H. Kim, S. W. Hwang, J. Bhak, J. K. Lee, and B. W. Cho. 2012. Whole transcriptome analyses of six thoroughbred horses before and after exercise using RNA-Seq. *BMC Genomics* 13:473.
- Petersen, H. H., J. P. Nielsen, and P. M. Heegaard. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. *Vet. Res.* 35:163-187.
- Pösö, A. R., B. Essén-Gustavsson, and S. G. Persson. 1993. Metabolic response to standardbred trotters with red-cell hypervolemia. *Equine Vet. J.* 25:527-531.
- Richardson, R. S., H. Wagner, S. R. Mudaliar, R. Henry, E. A. Noyszewski, and P. D. Wagner. 1999. Human *VEGF* gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. *Am. J. Physiol. Heart Circ. Physiol.* 277:H2247-2252.
- Richardson, R. S., H. Wagner, S. R. D. Mudaliar, E. Saucedo, R. Henry, and P. D. Wagner. 2000. Exercise adaptation attenuates *VEGF* gene expression in human skeletal muscle. *Am. J. Physiol. Heart Circ. Physiol.* 279:H772-H778.
- Roca, J., T. P. Gavin, M. Jordan, N. Siafakas, H. Wagner, H. Benoit, E. Breen, and P. D. Wagner. 1998. Angiogenic growth factor mRNA responses to passive and contraction-induced hyperperfusion in skeletal muscle. *J. Appl. Physiol.* 85:1142-1149.
- Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
- Streltsova, J. M., K. H. McKeever, N. R. Liburt, M. E. Gordon, H. M. Filhoa, D. W. Horohova, R. T. Rosena, and W. Frankeet. 2006. Effect of orange peel and black tea extracts on markers of performance and cytokine markers of inflammation. *Equine Comp. Exerc. Physiol.* 3:121-130.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.