Animal Science Papers and Reports vol. 30 (2013) no. 1, 35-43
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

HMGR messenger RNA and protein expression in liver, kidney and muscle in pigs of two breeds*

Hai-chao Lin[1,2,3], Gui-fen Liu [1,2], Jin-lian Fu[1], Ai-guo Wang[1]**

[1] College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China

[2] Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, Jinan, 250100, China

[3] Shandong Key Lab of Animal Disease Control and Breeding, Jinan, China

(Received December 19, 2011; accepted November 27, 2012)

Distribution and expression of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) in different breeds and tissues of pigs were studied by means of immunohistochemical and RT-PCR methods. Three Huai and three Landrace pigs each were individually housed at the Fujian Shanghang Huai pig breeding farm under similar feeding conditions. Pigs were humanely slaughtered at saleable weight and samples of kidney, liver and skeletal muscle were collected for expression analyses. *HMGR* mRNA expressions in liver, muscle and kidney of Huai pigs both occurred higher than expression found in the Landrace. Positive staining of HMGR protein was revealed in the three tissues considered. The HMGR protein expression level was not significant statistically in liver, muscle and kidney across two breeds, but the amount of Huai pig HMGR protein expressed in three tissues was higher than that of the Landrace. The level of HMGR protein expression in liver was higher than in the muscle and renal tissues ($P<0.05$). The results demonstrate that *HMGR* expression in pigs may depend on tissue and species.

KEYWORDS: HMGR / immunohistochemistry / RT-PCR / pig

*This study was supported by National Natural Science Foundation of China (No.31172176) and National Scientific and Technical Supporting Programs of China (No. 2011BAD28B01)
Corresponding author:** Ai-guo Wang: agwang@cau.edu.cn

Cholesterol is a vital molecule, being a structural component of cell membranes and a precursor for steroid hormones and bile acids [Myant 1990]. The liver is the important site of lipid and carbohydrate homeostasis. Dysregulation of this homeostasis has been implicated in disease process, such as atherogenesis, insulin resistance, and hyper-metabolism [Gastaldelli *et al*. 2009].

Three-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is essential rate-limiting enzyme in the overall pathway of cholesterol biosynthesis [Jiang *et al.* 2006, Istvan and Deisenhofer 2000], about 97-kDa transmembrane glycoprotein, and its structure is highly conserved among species. The *HMGR* has been extensively studied in mammalian systems due to its critical role in cholesterol biosynthesis and has been widely cloned in many species, *i.e*. humans, chicken [Sato *et al*. 2003], rat (Sundaresan *et a*l. 1989, Duckworth *et al.* 1991, Simonet and Ness 1988, Rai *et al*. 2009]. The human *HMG-CoA* reductase gene has been localized to human chromosome 5q12 by *in situ* hybridization and contains 20 exons and 19 introns [Humphries *et al.* 1985, Luskey and Steven 1985]. In pigs, however, information regarding this gene is limited. To our knowledge, no study was conducted so far on HMGR protein expression and function in pig tissues.

The aim of this study was to determine the difference in *HMGR* expression in liver, muscle and kidney between Huai and Landrace pigs, and to prove up the expression pattern by RT-PCR and the protein location immunohistochemically in three tissues.

Material and methods

Animals and tissue collection

Three Chinese Huai and three introduced Landrace pigs were humanely slaughtered at saleable body weight (71 and 130 kg, respectively) by commercially acceptable practices. Sections of about 1.5 cm^3) from liver, *Longissimus dorsi* muscle and kidney of each pig were collected immediately, fixed in fresh paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 24 h, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin. Several sections of tissues were snap-frozen in liquid nitrogen, and stored at -80^{o}C for RNA extraction.

RNA isolation and RT-PCR analysis

The presence of *HMGR* mRNA was examined by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted by TRIzol Reagent (LIFE TECHNOLOGIES Inc., Gaithersburg, MD, USA) in accordance with manufacturer's instructions, and stored at -80°C. The purity and integrity of RNA was evaluated with electrophoresis and staining with ethidium bromide at optical density (OD) absorption ratio OD260/OD280 and rRNA (28S/18S) ratios. Total RNA (2 μg) was reverse transcribed in the presence of polythymidine oligonucleotide primers (Oligo-dT18) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT; Promega Corporation, USA) in 20 μl of reaction volume.

The polymerase chain reaction was performed using 1.5 μl cDNA template, 2.5 μl 10×PCR buffer (containing 100 mM Tris-HCl (pH8.0), 500 mM KCl, 10 mM of $MgCl_2$ and 0.1% glutin), 2.0 μl 10 mM dNTPs Mix, 0.6 μl forward and reverse primers (10 pmol/μl), 0.5 μl AmpliTaq DNA polymerase (5 U/μl), and 17.3 μl double distilled water (the reagents all from the National Laboratories for Agro-biotechnology, China Agricultural University, Beijing, China). Amplifying conditions of PCR were: 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, annealing for 30 s, 72°C for 40 s, then 72°C for 7 min. The annealing temperature for *GAPDH* and *HMGR* was 60°C. All samples were measured in triplicate. The sequences of PCR primers used in the amplification of *GAPDH*, *HMGR* cDNA fragments were: forward GTCCACTGGTGTCTTCACGA, reverse GCTGACGATCTTGAGGGAGT for *GAPDH* (154 bp, AF141959); forward CATTCCAGCCAAGGTTGTC, reverse TGTAGATGGCAGTCACGATGT for *HMGR* (150 bp DQ432054). The PCR products were observed on 2.0 % agarose gels, visualized by ethidium bromide staining, and analysed using an ALPHA INNOTECH (San Leandro, CA) imaging system.

Immunohistochemistry

A goat polyclonal antibody against the rabbit *HMGR* was purchased from UPSTATE Biotechnology Inc, Lake Placid, NY. *HMGR* immunohistochemistry were performed with the labeled streptavidin/peroxidase biotin method (ZYMED Laboratories, Inc., South San Francisco, CA). The tissue slides were cut at 4 μm thickness and mounted on silanized slides, dewaxed in xylene, and rehydrated in graded ethanol. Sections were treated with 3% H_2O_2 in PBS for 10 min to inactivate endogenous peroxidase activity. To eliminate nonspecific binding, 5% goat serum was added to the primary antibody in PBS for 20 min. After tapping the excess goat serum solution, sections were incubated overnight at 4°C with a goat anti-rabbit antibody (UPSTATE) diluted: *HMGR* 1:200 and then incubated for 20 min in biotinylated goat anti-rabbit IgG, followed by incubation with HRP-streptavidin (ZYMED) for 20 min. The antibody binding sites were visualized by incubating the tissue sections with DAB solution provided by a DAB kit (ZYMED). Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. For the negative controls, rabbit IgG (ZYMED) was used at the same concentration as primary antibodies. Images of the sections were captured using Olympus microscope BX51 and digital camera DP70 (OLYMPUS, Tokyo, Japan) and were quantified visually according to the intensity and density of stained cells.

Image analysis

Micro image analysis system (MIAS) is not only the basis of visualization of medical information, but also is useful for accuracy quantitative analysis in medicine and biology. With the help of image analysis system, the study of HMGR protein expression can be made. This system uses the ratio of total positive immunoreactivity area and total statistic area to reflect the quantity of protein expression. The final value is an average of five times ratio.

Statistical

All the data were subjected to procedures using the one-way ANOVA of the SAS programme (SAS Institute, Inc., Cary, NC, USA, version 8.02). Results were given as means ±SE. Significance of difference was evaluated by Duncan's multiple range tests using SAS statistical package.

Results and discussion

Expression of *HMGR* mRNA in three tissues of pigs

As shown in Figure 1, RT-PCR primers specific for the *HMGR* were used to investigate the expression difference of *HMGR* in liver, kidney and muscle tissues between Landrace and Huai pig. *HMGR* expressions in Huai pig liver, muscle and kidney were higher than those in the Landrace. Regardless of breed, strong *HMGR* expression was observed in liver tissue, moderate in kidney, and weak in the muscle. The amount of RNA used in RT-PCR was normalized by *GAPDH* (higher band).

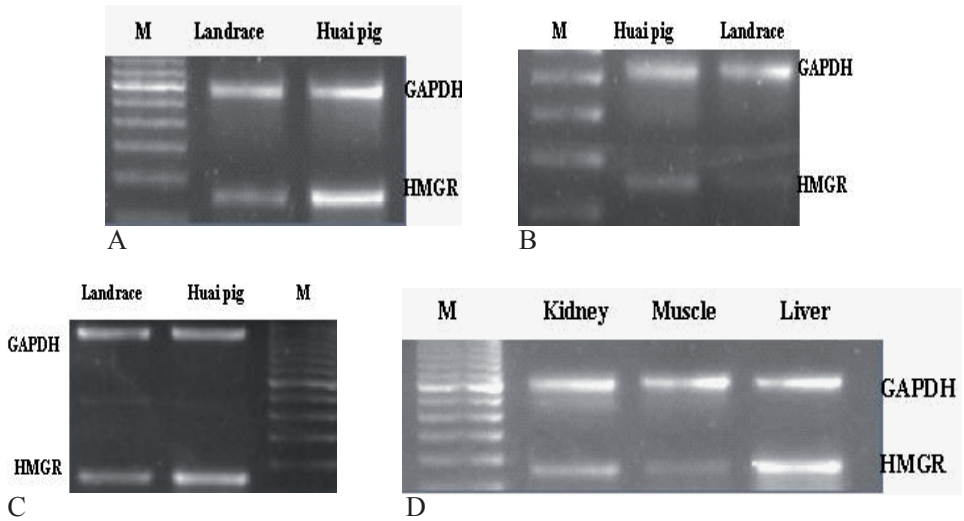
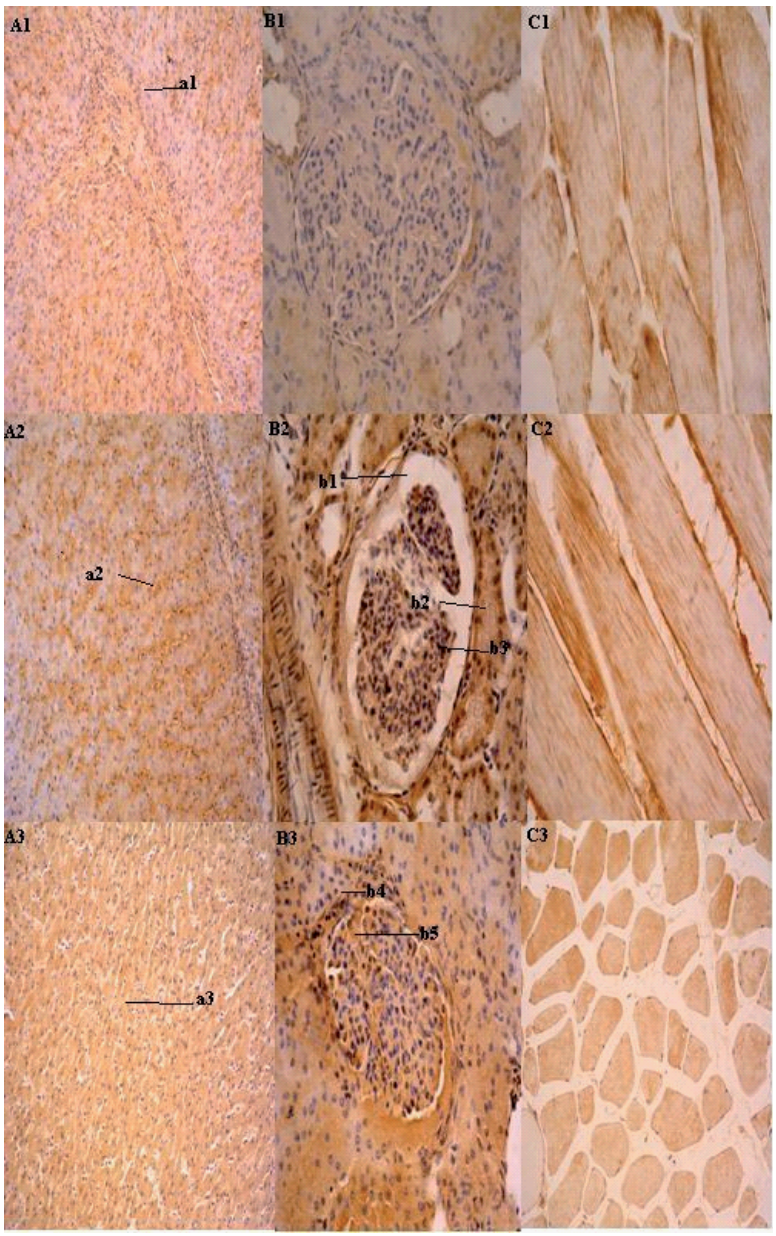


Fig. 1. Detection of *HMGR* mRNA expression by RT-PCR. M — 100 bp Marker. A — liver; B — muscle; C — kidney, D — expression level of three tissues.

Expression of HMGR protein in three tissues of pigs

The cellular localization of HMGR in the tissues was evaluated immunohistochemically. Strong staining of HMGR was observed in liver blood corpuscles and hepatic sinusoid, and weak staining in some cytoplast of liver cell (Fig. 2). In kidney tissue, the staining on the renal tuber and capillary vessel were strong and the staining on podocyte of renal capsule and *macula densa* of juxtaglomerular complex

were weak. There was no apparent regularity in muscle tissue staining of HMGR (Fig. 2). It varied with the tissues of HMGR protein expression. Thus, HMGR protein expression level depends on tissue and its substructure elements.



(I)

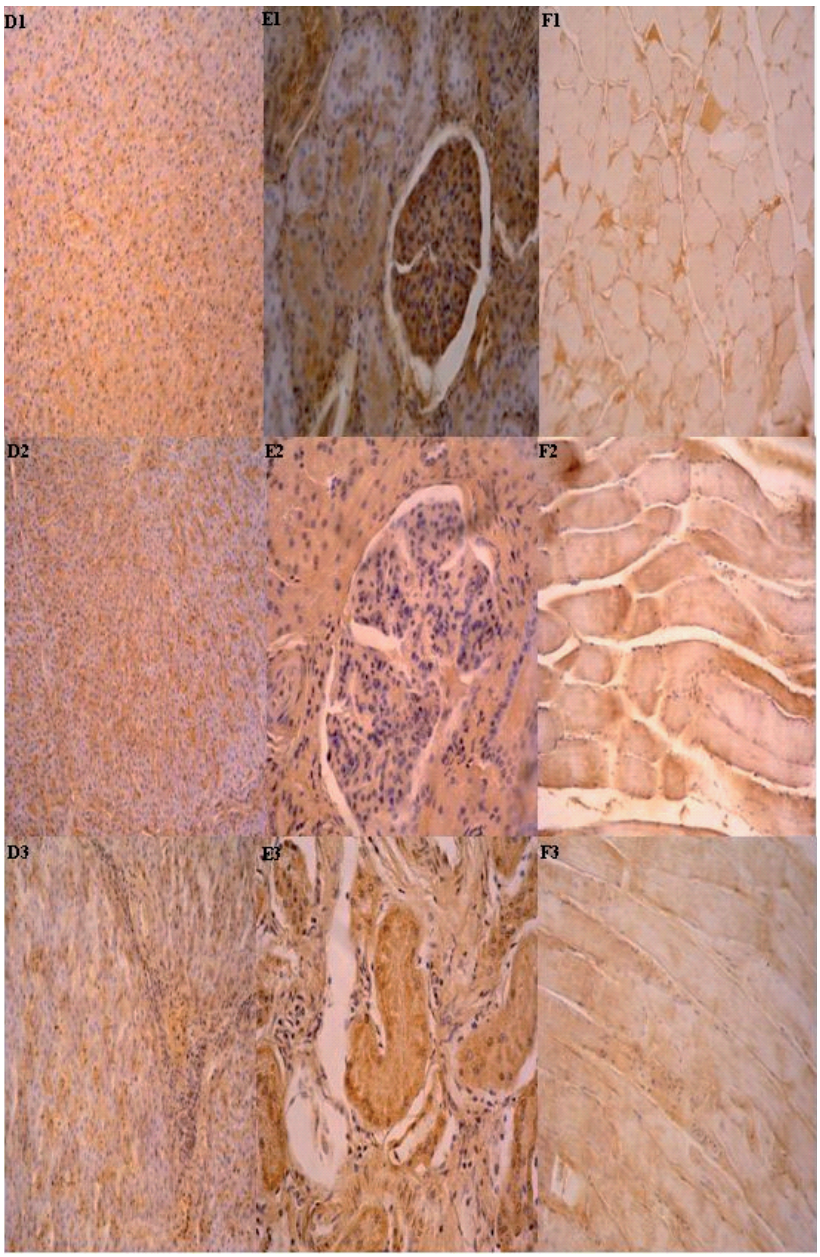


Fig. 2. Immunohistochemical staining of porcine liver, kidney and muscle with HMGR polyclonal antibody in Landrace and Huai pig. (I) – Landrace; (II) – Huai pig. A and D – liver; B and E – kidney, C and F – muscle. a1-cytoplast, a2-liver sinusoid, a3-blood corpuscle; b1-renal capsule antrum, b2-renal tuble, b3-podocyte, b4-macula densa, b5-capillary vessel.

Differences in HMGR protein level in three tissues across breeds

The expression of HMGR protein was analysed by MIAS software. No difference was identified in HMGR protein level in three tissues between two breeds (Fig. 3a). However, intensive expression in liver, moderate in kidney and weak in muscle were found both in Landrace and Huai pig. The HMGR protein expression was highest in liver and lowest in the muscle (Fig. 3b). There was significant difference between liver and muscle, and kidney and muscle ($P<0.05$). However, no difference was identified between liver and kidney.

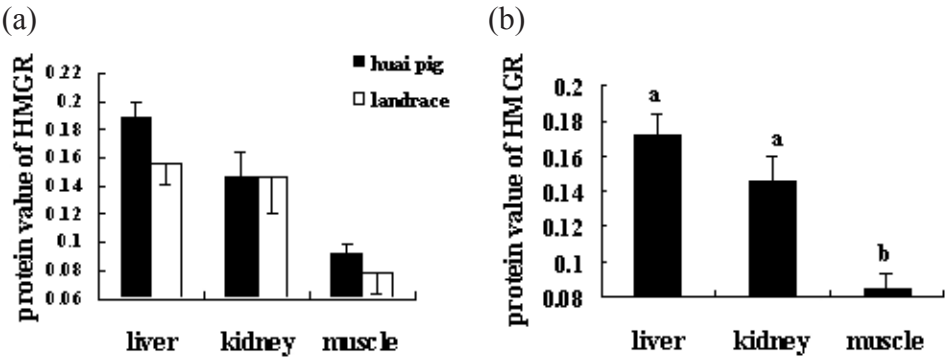


Fig. 3. Detection of HMGR protein expression by MIAS. (a) – expression level of HMGR protein in three tissues across breeds; (b) – expression level of HMGR protein in three tissues of pigs, regardless of breed. Means bearing different letters are significantly different at $P<0.05$.

The mRNA of *HMGR* was expressed at a high level in most tissues by RT-PCR. However, the highest levels were found in liver and spleen [Liu *et al.* 2008]. In this study, intensive immunoreactivity was found in liver blood corpuscle and liver sinusoid. In kidney tissues, intensive HMGR immunostaining was seen in the renal tuber and capillary vessel, no remarkable HMGR stating was found in podocyte of renal capsule and *macula densa* of juxtaglomerular complex. Intertissue differences were observed in HMGR protein expression level in liver, kidney and muscle and the *HMGR* mRNA expression in these tissues was coherent with HMGR protein expression. Jiang *et al.* [2006] reported the tissue expression pattern showing the *HMGR* strongly expressed in the leaves and stems whereas it is only poorly expressed in the roots suggesting that *HMGR* might be a constitutively expressing gene.

HMGR expressions in Huai pig tissues considered in this study were higher than those found in Landrace. It is interesting that the difference was noticed in *HMGR* expression between the two breeds. The liver is an important site for cholesterol synthesis, controlled by the microsomal enzyme *HMGR*. HMGR is encoded by a single gene, and participates in the production of sterol and many isoprenoids, which function in numerous aspects of organism growth, development, reproduction and disease [Bach 1995, Denbow *et al.* 1996, Goldwasser *et al*. 2010].

The Huai pig is considered a highly desirable Chinese local breed because it is miniature and produces high quality pork. Harris *et al.* [2003] reported the genetic selection for high or low plasma cholesterol to be not a proper criterium of cholesterol or fat accretion, yet argued that the source of dietary fat may influence total fat content in muscle tissues. According to Dorado *et al*. [1999], there is a high correlation between cholesterol content and fat content of the body *(r*=0.88, *P*<0.05). Furthermore, the Huai pigs are considered a lard-type breed because the fat content of their ccarcass is considerably higher than that of Landrace. Thus, the difference in expression of both *HMGR* mRNA and protein noticed between the two breeds may be related to overall fat content.

In summary, Huai pigs showed higher expression of *HMGR* than did Landrace pigs in liver, kidney and muscle. Apart from breeds, the mRNA and protein expression of *HMGR* gene in three tissues had consistent pattern, and the expression level was liver > kidney > muscle (*P*<0.05). However, the functional role of *HMGR* in the pig tissues needs further study.

Acknowledgement*. The author greatly appreciates the Fujian Shanghang Huai pig breeding farm for collecting samples.*

REFERENCES

1. BACH T.J. 1995 – Some new aspects of isoprenoid biosynthesis in plants – A review. ***Lipids***. 30, 191-202.
2. DENBOW C.J., SAARA L., CAROLE L.C., 1996 – The N-terminal domain of tomato 3-hydroxy-3-methylglutaryl-CoA reductase. ***Journal of Biological Chemistry*** 271(16), 9710-9715.
3. DORADO M., MARTIN GOMEZ E.M., JIMENEZ-COLMENERO F., MASOUDB T.A., 1999 – Cholesterol and fat contents of Spanish commercial pork cuts. ***Meat Science*** 51, 321-323.
4. DUCKWORTH P.F., VLAHCEVIC Z.R., STUDER E.J., 1991 – Effect of hydrophobic bile acid on 3-hydroxy-3-methyl- glutaryl-CoA reductase activity and mRNA levels in the rat. ***Journal of Biological Chemistry*** 266, 9413-9418.
5. GASTALDELLI A, KOZAKOVA M, HOJLUND K, FLYVBJERG A, FAVUZZI A, MITRAKOU A, BALKAU B; RISC Investigators 2009 –. Fatty liver is associated with insulin resistance, risk of coronary heart disease, and early atherosclerosis in a large European population. ***Hepatology*** 49(5), 1537-1544.
6. GOLDWASSER J., COHEN P.Y., YANG E., BALAGUER P., YARMUSH M.L. & NAHMISA YAAKOV., 2010 – Transcriptional regulation of human and rat hepatic lipid metabolism by the grapefruit flavonoid natingenin: role of PPARα, PPARγand LXRα. ***PLoS ONE*** 5(8), e12399.
7. HARRIS K.B., POND W.G., MERSMANN H.J., SMITH E.O., CROSSA H.R.., SAVELLA J.W., 2003. – Evaluation of fat sources on cholesterol and lipoproteins using pigs selected for high or low serum cholesterol. ***Meat Science*** 66, 55-61.
8. HUMPHRIES S.E., TATA F., HENRY I., 1985 – The isolation, characterization, and chromosomal assignment of the gene for human hydroxy3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). ***Human Genetics*** 71, 254-258.
9. Istvan E.S., Deisenhofer J., 2000 – The structure of the catalytic portion of human HMG-CoA reductase. ***Biochimica et Biophysica Acta*** 1529(1-3), 9-18.

10. JIANG J.H., KAI G..Y., CAO X.Y., CHEN F.M., HE D.N., LIU Q., 2006 –Molecular cloning of a HMG-CoA reductase gene from Eucommia ulmoides Oliver. ***Bioscience Reports*** 26, 171-181.
11. LIU G.F., WANG A.G., FU J.L., 2008 – Study on the Characteristics of Tissue Expression of 3-hydroxy-3-methylglutaryl Coenzyme A in Different Pig Breeds. ***Acta Ecologiae Animalis Domastici*** 29(3): 16-19.
12. LUSKEY K.L., STEVENS B., 1985 – Human 3-hydroxy-3-methylglutaryl coenzyme A reductase. Conserved domains responsible for catalytic activity and sterol-regulated degradation. ***Journal of Biological Chemistry*** 260, 10271-10277.
13. MYANT N.B. 1990 – Cholesterol metabolism, LDL, and the LDL receptor. Academic Press, San Diego.
14. RAI S.K., SHARMA M., TIWARI M., 2009 – Inhibitory effect of novel diallyldisulfide analogs on HMG-CoA reductase expression in hypercholesterolemic rats, CREB as a potential upstream target. ***Life Science*** 85(5-6), 211-219.
15. SATO K., OHUCHI A., SOOK S.H., TOOMIZU M., AKIBA Y., 2003 – Changes in mRNA expression of 3-hydroxy- 3-methylglutaryl coenzyme A reductase and cholesterol 7 alpha-hydroxylase in chickens. ***Biochimica et Biophysica Acta*** 1630, 96-102.
16. Simonet W.S., Ness G..C., 1988 – Transcriptional and post-transcriptional regulation of rat hepatic 3-hydroxy-3-methylglutaryl-CoA reductase by thyroid hormones. ***Journal of Biological Chemistry*** 263, 12448-12453.
17. SUNDARESAN S., YANG-FENG T.L., FRANCKE U., 1989 – Genes for HMG-CoA reductase and serotonin 1a receptor are on mouse chromosome 13. ***Somatic Cells and Molecular Genetics*** 15, 465-469.