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CHANGES IN THE ABUNDANCE OF HEPATIC GENES INVOLVED IN SOME METABOLIC REGULATION AMONG DIFFERENT CATTLE GENOTYPE SLAUGHTERED AT BASRA SLAUGHTER HOUSE

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ABSTRACT

The current study was conducted at the laboratories of Agriculture College, University of Basra from 3/12/2015 till 13/10/2016. Liver samples were obtained from 100 cows divided into Brahman (24), cross (Friesian x Jenubi, 28), Jenubi (local breed, 26) and Roman (22) from Basra Slaughter House. Liver samples were analyzed for mRNA abundance of genes related to gluconeogenesis (mitochondrial phosphoenolpyruvate carboxykinase, *PEPCKm*), fatty acid oxidation (carnitine palmitoyl transferase 1A, *CPT 1A*), cholesterol biosynthesis (3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, *HMGCS1*), ketogenesis (3-hydroxy-3-methylglutaryl-coenzyme A synthase 2, *HMGCS2*), triglyceride synthesis (Acetyl- CoA-Carboxylase, *ACoC*) and urea cycle related parameters (ornithine transcarbamylase, *OCT*) as well as housekeeping (reference) gene (Ribosomal protein S9, *RPS9*). The purpose of this study was to investigate the mRNA abundance variations of 6 different hepatic candidate genes responsible for some regulation of body metabolism among four different genotypes, sex and age. In addition, studying the differences in body live and carcass weight as well as dressing percentages among four studied genotypes, sex and age. The findings of the current study shows that the mRNA gene abundance related to cholesterol synthesis were observed to be significantly higher in all studied genotypes relative to Jenubi. Roman breed got two genes (*OTC and CPT1A*) with significantly down regulated mRNA gene abundance relative to Jenubi (-1.957 and -1.587 respectively). Brahman breed got one significant down regulated gene (CPT1A, -2.293). Females showed clear increased of mRNA abundance in almost all studied genes except *OTC* and *CPT1A* relative to male. The hepatic mRNA gene abundance of *HMGCS1*, *PEPCKm*, and *CPT1A* increased with animals' age, while the mRNA abundance of *OTC*, *ACOC* and *HMGCS2* decreased relative to 2 years old.

KEYWORDS : hepatic gene, breeds, age, sex, adaptation.

Introduction

The abundance of hepatic genes are essentially controlled by transcription factors which respond to environmental, autocrine, or paracrine signals (Costa *et al*., 2003). Interestingly, it has been noticed that even under the same conditions and similar production levels, the achievement of adaptation differs greatly between animals (Van Dorland *et al*., 2009). Number of studies have reported that the individual cows are vary in the physiological and endocrine adaptation to support milk production (Kessel *et al.,* 2008; Van Dornald *et al*., 2009). Animals with less health problems and best adaptive performance would be more chance to select for breeding programs aimed to gain high metabolic robustness. Therefore, the differences among individuals to adapt successfully to high production may have a genetic base (Drackley *et al*., 2005, Ha *et al*., 2015).

The hypothesis of this study was that genetic adaptation and hepatic regulation of metabolism are different among studied genotypes as well as among the individuals for the same genotype at the time points related to age.

The present study aims to estimate the gene abundance (mRNA abundance) of target genes which are responsible for metabolic processes in the liver and their possible relationships with body and carcass weights for possible use in the genetic improvement of local livestock programs.

Materials and methods

Animals

Different sex and genotype of 100 cattle's were chosen randomly from a slaughter house in Basra. All liver samples were immediately taken after slaughter. Animals were classified into genotypes, gender and age (Table, 1). Body and carcass weight were recorded at the slaughter house. Animals' ages were estimated depending on the breeders' information and dentition of cattle. This experiment was held at Basra University (Basra governor, Iraq) from December 2015 to September 2016.

Table (1) Number of samples of different genotypes, sex and age

Breed	Number	Gender		Age/year		
		Male	Female	1	2	3
Brahman	24	16	8	7	8	9
Cross	28	18	10	9	9	10
Jenubi	26	18	8	7	10	9
Roman	22	14	8	7	7	8
Total	100	66	34	30	34	36

Liver samples collections

Liver samples were taken from 100 experimental animals at slaughter house. Samples of 200 gm of liver tissues were straightaway put into an RNA stabilization reagent (RNAlater_ from Ambion, Applied Biosystems Business, Austin, TX, USA) and kept at +5 °C for 24 h, and thereafter stored at – 80 °C until analyzed as described by Dorland *et al.*(2014a).

RNA Isolation from liver

Total RNA was isolated from liver samples with Phenol based solution type RNA Extraction Kit – Tissue RNA PrepMate™ (BIONEER), Korea, ISO certificate 9001, according to the manufacture instructions.

Detection and quantification of mRNA

The quantity and purity of RNA were measured using Thermo Scientific Nano Drop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Fisher Scientific, Loughborough, UK). The absorbance of 1µl of RNA at 260nm and 280nm was determined. The purity of RNA was assessed by (A260/A280) ratio, which was above 1.81 for all samples. All measurements were made with respect to a blank consisting of the nuclease free water in which the RNA was suspended.

Synthesis of cDNA (Reverse Transcription)

For reverse transcription, 1 µg of extracted total RNA was reversely transcribed to complementary DNA (cDNA) using 200 U RocketScript Reverse Transcriptase kit according to the manufacturer's instructions (Bioneer Corporation, Korea).

Total RNA, DEPC-water and primer was thawed before use. Then 1 µL of template RNA, 1 µL of random Hexamer primers 100 pmol

(Invitrogen, Leek, The Netherlands) and 18 µL of DEPC- water into the AccuPower RocketScript RT PreMix tubes.

The cDNA synthesis was performed in a reaction volume of 20 µl. All reaction mixtures were prepared with ice. The samples were then placed in a 96 Well Thermal Cycler, and cycled at the following conditions: primer annealing 30ºC for 10 min., cDNA synthesis 60ºC for 1 hr. followed by 95ºC for 5 minutes for heat inactivation. The converted cDNA was stored at -20°C and used as a template for PCR amplification of HMGCS1, HMGCS2, ACOC, CPT1A, PEPCKm, and OTC genes. The obtained cDNA was diluted to a final concentration of 25 ng/µl.

Real Time Quantitative RT–qPCR

AccuPower® 2X GreenstarTM qPCR Master Mix kit from Bioneer Company, Korea, was used in the real time PCR experiment to confirm the abundance of target genes (HMGCS1, HMGCS2, ACOC, CPT1A, PEPCKm, and OTC) as well as the housekeeping gene (RPS9) which work as a referencing gene. The reaction volume was performed in a 25 µl, the master mixture components and their volume are explained in the manufacture instructions. The primer information for all target genes, including the housekeeping gene were shown in Table (2) as described by Graber et al., (2010). The RT-qPCR reaction condition was run on a program as described by Dorland et al. (2014a). Denaturation for 10 min at 95 °C then amplification, 40 cycles each consisting of 15 sec at 95 °C. The primer-specific annealing temperature were different according to the primers of target genes for 40 sec. Extension at 72 °C for 20 sec. Finally a melting curve program (60–95 °C).

The cycle threshold (CT) determinations were automatically performed by the instrument using default parameters.

Table 2. RT qPCR primer information, the annealing temperature, and the PCR product length.

Gene	Sequence 5'-3'	Gene Bank accession no.	Annealing temp. (°C)	Length
ACOC for ACOC rev	CTCTTCCGACAGGTTCAAGC ACCATCCTGGCAAGTTTCAC	AJ_132890	61	248
CPT1A for CPT1A rev	CAAAACCATGTTGTACAGCTTCCA GCTTCCTTCATCAGAGGCTTCA	BF039285	54	111
HMGCS1 for HMGCS1 rev	TGTACGGCTCCCTGGCTTCTG CATGTTCCTTCGAAGAGGGAATC	BC_102850	60	313
HMGCS2 for HMGCS2 rev	TCTGGCCCATCACTCTGCC AGTGGGGAGCCTGGAGAAGC	NM_001045883	60	126
OTC for OTC rev	AGGCTTTCCAAGGTTACCAG GGATACCATGACAGCCATG	AF_134841	61	185
PEPCKm for PEPCKm rev	TACGAGGCCTTCAACTGGCGT AGATCCAAGGCGCCTTCCTTA	XM_583200	60	365
RPS9 for RPS9 rev	AAGCTGATCGGCGAGTATG GCATTACCTTCGAACAGACG	NM_001101152	58	140

ACoC, Acetyl- CoA-Carboxylase; CPT1A, carnitine palmitoyl transferase 1A; HMGCS1, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; HMGCS2, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; OCT, ornithine transcarbamylase; PEPCKm, mitochondrial phosphoenolpyruvate carboxykinase; RPS9, Ribosomal protein S9.

Statistical analyzes

Significant differences among CT due to different genotypes, sex and age were determined by using analysis of variance, GLM method within SPSS statistical program (version, 21). Gene abundance differences were considered as a significant difference at p-value (< 0.05) by using one sample t-test as described by (Kwekel et al., 2010)

Results

Gene abundance of different genotypes

The results found in Table (3) showed the differences of hepatic mRNA genes abundance (log_2) among three types of genotypes relative to Jenubi. One sample t-test revealed the significant differences of gene abundance from zero was ± 1.5 fold. Based on t-test analysis, data showed cross breed had only one positive significant (*P<0.05*) differences of *HMGCS1* mRNA gene abundance relative to Jenubi breeds (1.515 fold). Whereas, Brahman breed had two significant (*P<0.05*) mRNA gene abundance of upregulated *HMGCS1* and downregulated *CPT1A* (1.872 and -2.293 folds respectively). As well as the mRNA gene abundance of Roman breed had three significant (*P<0.05*) differences of *HMGCS1*, *OTC* and *CPT1A* (3.460, -1.957 and -1.587 folds respectively) relative to Jenubi. Therefore, from the mRNA gene abundance of *HMGCS1* can be indicated that all breeds (including cross) show high significant (*P<0.05*) differences relative to Jenubi, reflecting an increasing in cholesterol synthesis. This may explain that metabolic pathways of Jenubi breed differ significantly than those of other genotype.

Among all investigated genotypes, cross and Jenubi had quite similar mRNA gene abundance of *ACOC* and *PEPCKm* (-0.018 and 0.018 respectively) which nearly zero differences except that of *HMGCS1*. This may explain that cross is closer genotype to Jenubi as Jenubi contribute in a different level of genotype into cross animals (nonsystematic crossing).

Table 3. Relative mRNA gene abundance (ΔCT, log2) of hepatic studied genes in different cattle genotypes relative to Jenubi

Breed	HMGCS1	OTC	ACOC	PEPCKm	CPT1A	HMGCS2
Cross	1.515*	0.455	-0.018	0.018	-0.798	-1.309
Brahman	1.872*	-0.419	-0.209	-0.614	-2.293*	-0.403
Roman	3.460*	-1.957*	0.928	1.047	-1.587*	0.675
Over all	1.696*	0.187	0.034	0.032	-1.007	-1.069

***HMGCS1*= cholesterol biosynthesis, *OTC*= urea cycle, *ACOC*= fatty acid & triglyceride synthesis, *PEPCKm*= gluconeogenesis, *CPTA*= fatty acid oxidation, *HMGCS2*= ketone bodies production. * mean difference significantly (*P<0.05*).**

Gene's abundance of different sex and age

The female showed a clear increased of mRNA abundance in almost all studied genes except *OTC* and *CPT1A* relative to male (Table 4). Hepatic mRNA abundance of *HMGCS1* gene involved in cholesterol synthesis reached the highest (*P<0.05*) value among targeted genes of 1.647 fold compared to male. While, the lowest (*P<0.05*) mRNA abundance was -1.967 fold in *OTC* involved in urea cycle relative to male.

Table 4. Relative mRNA female gens abundance (Δ CT, log2) of hepatic studied genes relative to male and among different ages (year) relative to 2 years

Gender	HMGCS1	OTC	ACOC	PEPCKm	CPT1A	HMGCS2
Female	1.647*	-1.967*	1.180	0.438	-1.274	1.301
Age						
1	-0.031	-1.934*	-1.041	-0.247	-0.901	-0.752
3	0.200	-0.185	-0.710	0.342	0.537	-3.494*

*** mean difference significantly (*P<0.05*).**

The data in Table (4) also revealed that the hepatic mRNA gene abundance of *HMGCS1*, *PEPCKm*, and *CPT1A* increased with animals' age, while the mRNA abundance of *OTC*, *ACOC* and *HMGCS2* decreased relative to 2 years old. A slight decline had been seen in the mRNA abundance of *OTC* gene at age 3 by about -0.185 fold compare to animals at age 2. However, the mRNA abundance of *HMGCS2* gene decreased significantly (*P<0.05*) in animals at age 3 by about -3.494 fold relative to age 2. Only the mRNA abundance of *OTC* and *HMGCS2* genes were downregulated significantly (*P<0.05*) when animal ages were 1 years and 3 years respectively.

Discussions

Increasing importing and exporting live animals around the world may create check in metabolism activities of these animal. Since liver is well-known as a principle place for entire-body homeostatic and homeorhetic controlling of metabolic processes and consequently for the maintenance of adaptation performance (Swanson, 2008). Hepatic mRNA gene abundance data are increasingly utilized to investigate genes mechanisms at molecular level to understand metabolic adaptation and physiological processes (Graber, 2010, Dorland *et al.*, 2014b, Ha *et al.,* 2015)

It was proved that animals have different genetic ability for adaptation performance. The mRNA abundance of hepatic genes related to cholesterol synthesis, fatty acid oxidation, triglyceride, urea cycle and glycogenesis were measured as an indicator of metabolic adaptation levels at animal's liver (Graber, 2010, Dorland *et al.*, 2014b). The present results agreed with the previous studies (Van Dorland *et al*., 2009, Graber, 2010, Schlegel *et al.*, 2012, Dorland *et al.,* 2014b). The variation in hepatic mRNA genes abundance between animals may therefore be explained by the variation that exist among animals at a molecular level in liver. This may reflect the variation of animal's ability to a successful adaptation performance among individuals. This results supported by findings of Drackley *et al.*, (2005) and Ha *et al.*, (2015) who reported that the variation in animals performance to adapt successfully may have a genetic base. Therefore, the present variation among animals are due to how these animals success metabolically and physiologically adapted (Kessel et al., 2008). Some animals of a breed are able to deal with metabolic challenge more successfully than other under similar condition.

The present study illustrates that the *HMGCS1* gene abundance involved in cholesterol synthesis were all upregulated in tested genotypes relative to Jenubi. This findings is in agreement with Janovick-Guretzky *et al.*, (2007) who found that pathway analysis of *HMGCS1* gene upregulated in steers during early feedback mechanism period and exhibited an extra body gain and feed efficiency compare with controls. Suggesting that tested animals in the present study are in stable metabolic rate and generally adapted to the quality and quantity of local feed during the first 3-6 months from importing. They do able to meet their body energy demands for body maintenance compare with Jenubi genotype.

As well as, previous study by Loor *et al.*, (2007) document that feed restriction altered the network expression of hepatic genes. They were also observed that the down regulated of *HMGCS1* gene, which involved with cholesterol synthesis were associated with starvation and ketosis as the case of Jenubi breeds. This results consider as a biological sign for negative energy balance in transition period cows (Allen, 2014). To cope with energy shortage animals increases their metabolic of body fat and convert short chain fatty acid (SCFA) to the ketone bodies which can be used as a sources of oxidative fuels in the different tissues (Naeem *et al.*, 2012, Allen, 2014). The results of cross, Brahman and Roman in the present study are in agreement with Graber (2010) who found that mRNA abundance of HMGCS1 gene involved with cholesterol biosynthesis were high at week 13 postpartum (positive energy balance) relative to week three pre partum and week four postpartum when there is in negative energy balance. In addition, several other studies (Viturro *et al*., 2009, Van Dorland *et al*., 2009, Kessler *et al*., 2014), have also reported that the *HMGCS1* gene was markedly downregulated during transition period as a consequences of considerable increased in energy demands.

The measurement of liver genes abundance alterations at key developmental periods of the animal's life, additionally, at the sex most commonly focused in metabolic adaptation, as this study do. The liver plays an important roles in different physiological function involved in cholesterol catabolism to bile acid, glycogen storage and drug metabolism (Si-Tayeb *et al*., 2010). Several studies have also mention that, approximately over 1000 hepatic genes are expressed differently between males and females and mainly controlled by pituitary gland patterns to the secretion of growth hormone (Mode and Gustafsson, 2006, Waxman and Holloway, 2009). Changes in hepatic gene expression are primarily related to sex differences and regulation of estrogen and testosterone (Chowen *et al.,* 2004).

In addition, it was proved that hepatic genes abundance pattern are different between sexes based on stage of age. Kwekel *et al*., (2010) have assess the whole liver genes expression in rat and divided them into two clusters based on sex and age, they found that females had higher liver gene expression than males in adulthood and late age due to the differences in biosynthesis pathways and levels of steroid and metabolic hormones.

Androgen expressed in liver tissues of both male and female in mammals (Shen and Shi, 2015). In addition to androgen consider as a specific sex differences, Chatterjee *et al*., (1996) found that the mRNA expression of androgen in adult male rat was approximately 20 fold higher than female. A recent study by Seo *et al.,* (2016) who identified that there were a significant relationship among genes related to sex shared between cattle and rat (two mammal species) by using qRT-PCR.

Several studies have proved that liver genes expressions altered their action pattern relative to animal's age (Kwekel *et al*., 2010, Naeem, 2012, Takeo *et al.*, 2013, Fiore *et al.,* 2016). Down regulation of all studied genes of animals aged 1 years relative to animals aged 2 years. Animals 3 years old exhibited up-regulation to genes related to cholesterol syntheses, glycogenesis and fatty acid oxidation. Naeem, (2012) demonstrated that there was an increases in blood urea associated with up-regulation of expression of urea transporter gene and increase in age (10 week) in rumen epithelium tissue. Similar phenomenon revealed in this study in the case of up-regulation of gene expression relative to cholesterol, glycogenesis and fatty acid oxidation with down-regulation in OTC gene related to urea cycle even blood urea concentration was similar of age 2 and 3 years old. Addition supported by Hayashi *et al.,* (2006) who reported that the recycling of urea increases a long with age due to the high use of urea breakdown by rumen microbes which led to increases plasma urea concentration.

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