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Research Article

The mRNA Gene Expression Profiles for HSP60 and HSP70 in Various Aged Saanen Goats in Different Seasons [1]

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Abstract

In this study, the mRNA gene expression levels for HSP60 and HSP70 in various aged Saanen Goats in the spring were compared with the summer, fall and winter levels. The animal material was constituted by healthy 18 Saanen Goats at various ages. The animals were divided into three groups so that group I contained goats at 1-2 years old; group II 3-4 year- old-ones and group III 5-6 year-olds. In general, HSP60 and HSP70 mRNA expression levels were determined to have increased in all groups in the summer, fall and winter when compared with the levels in the spring which is known to be the most convenient season for goats in the region. The differences of mRNA expression levels for HSP60 and HSP70 among groups in the summer were found statistically insignificant. When the HSP60 expression levels in the winter in all groups were compared, the difference between Group I and the other two groups, Group II and III, was found statistically significant ($P<0.05$). The levels were seen to be higher in the young goats than in the old ones. This finding was that most significant aspect of this study. In other words, these levels decreased as the age increased. In resisting heat stres, to be able to define the roles and the functional mechanisms of mRNA gene expression levels of HSP60 and HSP70 in goats having high adaptation capacities to the environment particularly according to ages could be regarded as facilitators for the studies to be done on all species. In the following years, HSPs are believed to play significant roles in the selection of resistant animals particularly to the environmental conditions and to be one of the significant physiological parameters which will focus on farm animals.

Keywords: *Gene expression, mRNA, HSP 70, HSP 60, Saanen goats*

Farklı Yaşlardaki Saanen Keçilerinin Farkli Mevsimlerdeki HSP 60 ve HSP70 İçin mRNA Gen Ekspresyon Profilleri

Özet

Bu çalışmada, farklı yaşlardaki Saanen keçilerinin bahar mevsimine göre yaz, sonbahar ve kış mevsimlerindeki HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri karşılaştırılmıştır. Hayvan materyali olarak sağlıklı 18 baş Saanen keçisi kullanılmıştır. Hayvanlar yaşlarına göre üç gruba ayrılmıştır. Birinci grup hayvanlar; 1-2 yaşında, ikinci grup; 3-4 yaş, üçüncü grup hayvanlar; 5-6 yaşındadır. Genel olarak, bölgede keçiler için en uygun mevsim olan ilkbahardaki HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri yaz, sonbahar ve kış aylarında yaşlara göre karşılaştırılmıştır. Tüm gruplar için HSP60 ve HSP70 mRNA ekspresyon düzeyleri yaz için istatistiksel olarak önemsiz bulunmuştur. Kış mevsimde HSP60 mRNA gen ekspresyon düzeyleri gruplar arasında karşılaştırıldığında birinci grup ve diğer iki grup arasındaki fark istatistiki olarak önemli bulunmuştur ($P<0.05$). Gen ekspresyon düzeyleri genç olan birinci grupta, daha yaşlı olan diğer iki gruba göre daha yüksek bulunmuştur. Diğer bir değişle yaş artıkça ekspresyon düzeyi düşmektedir. Sıcaklık stresine karşı direnç bakımından adaptasyon yeteneği iyi olan keçilerde, özellikle yaşlara göre HSP60 ve HSP70 mRNA gen ekspresyon düzeyleri, rolleri ve fonksiyonel mekanizmaları üzerine yapılacak çalışma sonuçları tüm türlerde yapılacak benzer çalışmalar için önemli olabilir. Gelecek yıllarda çevre koşullarına dirençli hayvanların seçiminde, HSP'lerin önemli rol oynayacağına inanılmakta ve çiftlik hayvanları için üzerinde odaklanılacak önemli bir fizyolojik parametre olacağı düşünülmektedir.

Anahtar sözcükler: *Gen Ekspresyonu, mRNA, HSP70, HSP60, Saanen keçileri*

INTRODUCTION

Heat stres, one of the environmental stresses, is the major constraint on animal productivity in tropical climatic conditions. Growth, productivity and reproductivity are impaired as a result of the drastic changes in biological functions caused by heat stress [1]. Determining the animal's thermotolerance ability is a must in order to

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measure the performance ability of a particular breed or animal against heat stress. Heat Shock Proteins (HSPs) are known as molecular chaperons and they maintain native structure of proteins and cell viability during stressful periods [2]. They are multigene families that range in molecular size from 10–150 kDa and are found in all major cellular compartments [3]. HSP60 is an important molecular chaperon under various stressful conditions [4]. These HSPs are known for their primary role as molecular chaperons that ensures the correct protein folding and apoptosis regulation during physiological stressful conditions. HSP60 is mostly found in the mitochondria. It helps in refolding of proteins and prevents aggregation of denatured proteins [3]. Another abundant and the best characterized HSP is the 70-kDafamily (HSP70) that consists of highly conserved stress proteins, expressed in response to stress, and plays crucial roles in environmental stress tolerance and adaptation [1]. HSP70 is mostly found in the cytosol and nucleus. Its functions are protein folding, cytoprotection, and as molecular chaperones [3].

Dairy breeds, especially the high-yielding animals, are typically more sensitive to heat stress as they generate more metabolic heat [5]. The heat stress effect becomes more detrimental when it is accompanied with high ambient humidity [6].

Goats adapted to a harsh environment perform better than other domesticated ruminants [7,8]. They are well adapted under different geographical and environmental conditions [1].

These animals have developed adaptive mechanisms that allow their survival at very high temperatures (45 to 50°C) as well as cold temperatures (–20 to –40°C). However, despite their extreme tolerance against temperature changes, the productivity of these animals softens the declines due to thermal stress [9]. Heat stress affects productivity of goats, but very little information is available about how they respond to heat at a cellular level. There is a strong correlation between the induction of HSPs and the induction of thermotolerance by preventing activation of stress kinases [10].

These proteins, called as Heat Shock Proteins (HSPs), have been determined to have increased not only due to high environmental or body temperatures but due to the stress factors, such as viral infections, heavy metals, chemical matterials, pesticides, oxygen and glucose deficiency as well [11,12].

Plenty of studies have recently been conducted on various diseases, particularly in humans, appearing with the aging due to dropping down HSP60 levels [13,14].

In this study, it was aimed to define the alterations in gene expression levels for HSP60 and HSP70 in various aged Saanen goat groups in different seasons. In resisting heat stress, to be able to define the roles and the functional mechanisms of mRNA gene expression levels of HSP60 and HSP70 in goats having high adaptation capacities to the environment particularly according to ages could be regarded as facilitators for the studies to be done on all species, including humans.

MATERIAL and METHODS

All experiments were done at the goat research unit in the Faculty of Agriculture and University of Adnan Menderes in AYDIN - Turkey whose location is (37° 45′ 03.31″ N and 27° 45′ 27.16″ E). It is 52 m above sea level. Experimental procedures were reviewed and approved by the Animal Ethics Committee of Adnan Menderes University, Aydin, Turkey (Approval No. B.30.2.ADU.2013/041). Mediterranean climate prevails in the region, namely summers are warm and dry and winters are rainy and warm. The annual average temperature is 17-18°C. Due to the north winds, the annual precipitation is between 580-1000 mm.

Animal Material

The animal material was constituted by healthy 18 Saanen Goats at various ages. The animals were divided into three groups. Group I contained at 1-2 years old goats; group II 3-4 year- old-ones and group III 5-6 year-olds. A constant and free feeding program containing energy and protein was applied throughout the year. For all of the goats in the groups, a constant and free feeding program in which the animals were free to reach their food freely 24 h in their pens or outside throughout the study period.

Recordings of Climatic Parameters

The experiments were carried out during four distinct phases coinciding with four seasons of the year, winter (mid-January), spring (mid -April), summer (mid-july) and autumn (mid-November). Blood sampling days were determined by tracking the meteorological agenda according to seasonal avareges for the spring and the falls and for the coldest day in the winter and the hottest day in the summer. All the animals were closely monitored and were provided similar managemental inputs during the experimental period.

The ambient temperature and humidity were continiously recorded all the year long with a hobo device installed a week before the first experiment day. Temperature Humidity Index (THI) was calculated to determine the effect of environmental conditions on animals. The following equation was used to calculate THI:

$$THI = T - (0.31-0.31*RH) * (T - 14.4)$$

where T is the dry bulb temperature (°C) and RH is the relative humidity (%) [6].

Blood Collection and RNA Extraction

On the experiment days, blood samples were taken from

the neck veins of the animals (vena jugularis) into lithium heparin-coated vacutainer tubes. As soon as the samples were taken, they were transported to the laboratory under refrigeration.

Total RNA was extracted from the blood with a commercial RNA isolation kit (High Pure RNA Isolation Kit, Roche, Version 12, 11828 665001) in accordance with the manufacturer's instructions. Supernatants were re-suspended by sterile DNase/RNase free water and the samples were stored at -80ºC until they were used. RNA concentrations were determined by optical density measurement at 260 and 280 nm. Purity was assesed by the 260/280 nm ratio.

Following RNA isolation, cDNA synthesis was performed using Light Cycler Nano Real Time PCR (Roche) for cDNA commercial kit (Transcriptor High Fidelity cDNA Synthese kit, Roche, Version 8, 05091284001).

Real time PCR analysis were performed using SYBR green (Roche, Fast Start Essential DNA Green Master), in acccordance with the manufacturer's procedure using a Light Cycler Nano Real Time PCR. All primers were synthesized by Genmar (İzmir/Turkey). The primer sequences as follows: HSP60-F 5'-ACTGGCTCCTCATCTCACTC -3'; HSP60-R 5'-TGTTCAATAATCACTGTCCTTCC-3', HSP70-F 5'-GACGAC GGCATCTTCAAG -3'; HSP70-R 5'-GACGACGGCATCTTCAAG -3', β actin-F 5'-AGTTCGCCATGGATGATGA-3'; β actin-R 5'-TGCCGGAGCCGTTGT-3'. β actin was also amplified in each assay as a control for using equal amounts of RNA in the RT-PCR reaction.

RT-PCR condition was an initial incubation at 40ºC for 10 minutes that was followed by a 10- minutes incubation at 95ºC then 45 cycles at 95ºC (10 s) , 60ºC (10 s) and 72ºC (15 s), and 20-second melting at 58ºC. The RT-PCR assay was repeated twice and the reproducibitiy was excellent with a correlation coefficient (r>0.95). The initial template was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction (Ct value). Relative gene expression was calculated using a $\Delta\Delta C_t$ method [15].

Statistical Analysis

Statistical analyses were preformed using the SPSS software v.22. The variables were investigated using Kolmogorov-Smirnov/Shapiro-Wilk's test to determine whether or not the parameters are normally distributed. As the measurements were not normally distributed the Kruskal-Wallis Analyse of Variance test were conducted to compare the parameters. The Mann-Whitney U test was performed to test the significance of pairwise differences. An overall 5% type-1 erroe level was used to infer statistical significance ($P<0.05$).

RESULTS

Climatic Data

The region where the study was conducted is under the effects of Mediterranean Climate. In other words, summers are dry and hot and winters are warm and rainy in the region. When the average values for the temperature and humidity data measured every single day of the year were evaluated, temperatures and THI values were seen to be the lowest in January and February and the highest in July and August.

According to the values given in *Table 1*, the average indoor temperature in the pen in July is 29.18ºC, and this value is in harmony with the long term average (28.4ºC for the last 10 years) for the province [16]. The daily temperature average measured in January was found as 8.63ºC and THI value as 9.09. On the other hand, the average values for the same parameteres in the summer were 29.18 and 26.67ºC respectively. The average temperatures in the spring and in the fall were found as 14.92 and 14.78ºC, respectively and the THI values were 14.26 and 14.74, respectively, all of which were close values to each other.

HSP60, HSP70 Gene Expression Levels

In this study, the spring season which has the best climatic comfort conditions for goats in this district was taken as the base. For this reason, the gene expression levels in the other seasons were compared with the values recorded in the spring.

When the mRNA gene expression levels for HSP60 in Group I (1 or 2 years old goats) were analized, the level measured in the winter was seen to be approximately 1.72 times more than the level measured in the spring. On the other hand, the difference between the other HSP60 expression levels for Group I determined in the summer and in the fall was found statistically insignificant. The

Table 1. *Temperature, Humidity and THI values belonging to experiment months which represent the four seasons lived in the region best*

Seasons	Month	Average Temperature (ºC)	Minimum Temperature (ºC)	Maximum Temperature (ºC)	Average Humidity (%)	THI
Winter	January	8.63	-6.12	22.52	74.2	9.09
Spring	April	14.92	2.94	29.85	57.57	14.26
Summer	July	29.18	18.61	40.53	46.00	26.67
Fall	November	14.78	5.96	25.12	62.08	14.74

expression level in the summer was found to be slightly more (1.2 times) than the spring level.

When the mRNA gene expression levels for HSP70 in Group I were examined, the level measured in the winter was seen to be approximately 1.68 times more than the level measured in the spring. The difference between the expression levels in the summer and in the fall was found statistically insignificant.

The seasonal differences among the mRNA gene expression levels for HSP60 in Group II (3 or 4 years old goats) were found statictically insignificant. The difference between the HSP60 expression levels in the summer and in the fall was found statistically insignificant.

When the HSP70 expression levels for Group II were analized, the level in the winter was seen to be 1.5 times more than the level in the spring.

The HSP60 and HSP70 expression levels in all seasons were compared in Group III and the differences were found statistically insignificant.

When the HSP60 expression levels in the winter in all groups were compared, the differences between Group I and the other two groups, Group II and III were found statistically significant. The difference between the HSP70 expression levels in Group I and II were found statistically insignificant. However, mRNA gene HSP70 expression level in Group I was approximately 1.25 times more than Group III, which was a significant finding.

When mRNA gene expression levels of the groups in

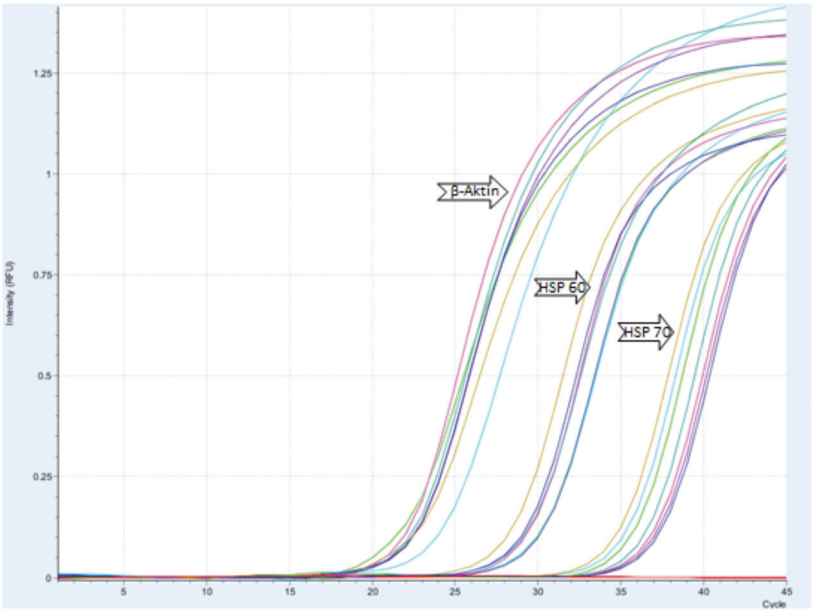
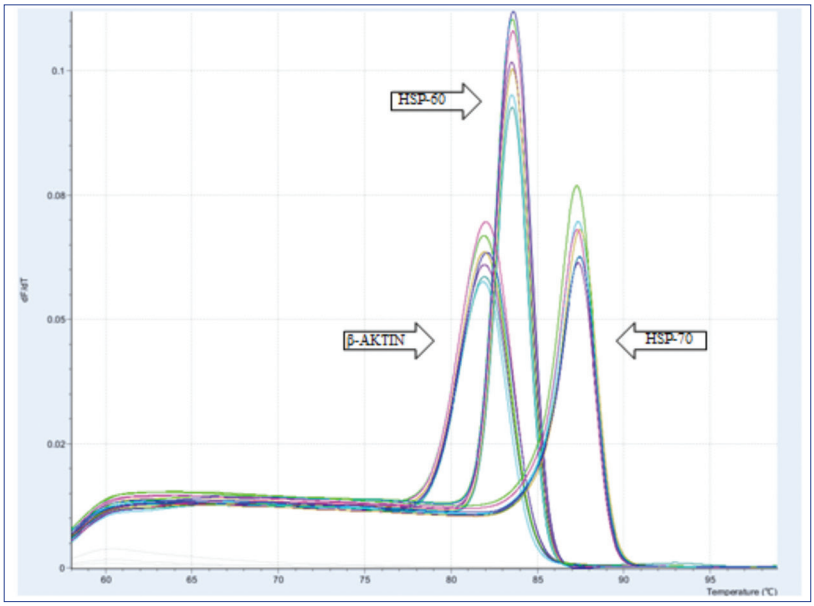


Fig 1. Synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR (The amplification curve of randomized spring blood sampling of the goats in Group II and III)

Fig 2. Melting point (Tm) graphics of β actin , HSP60, HSP70 mRNA expression



the spring for HSP60 were compared, Group I was seen to have 1.5 times higher expression levels than Group II and Group III, which was found statistically significant. The difference between Group II and Group III was found statistically non significant.

The differences of the mRNA genes HSP60 and HSP70 expression levels among the groups in the summer and in the fall were found statistically non-significant.

Fig. 1 is a Synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR (The amplification curve of randomized spring blood sampling of the goats in Group II and III).

Melting point (Tm) analysis was done in order to determine the purity of the samples expressed, whether there were interfering matters, whether the same gene region was analized in distinct samples and whether the primer dimer was created.

Fig. 2 contains Melting point (Tm) graphics of β actin, HSP60 and HSP70 mRNA expressions.

The synchronous expression image of the amplification of HSP60, HSP70 and β actin belonging to some goats provided by qRT-PCR was shown in *Fig. 1*. As seen in *Fig. 2*, peakings at the same spot indicated that there were no interference and no primer dimer in the samples. The same case was valid for β actin as well which is used as reference gene and any primer dimer was the case in the reference gene as seen in *Fig. 2*.

DISCUSSION

In this study, HSP60 and HSP70 expression levels of various aged Saanen Goats in the spring were compared with the summer, fall and winter levels. In general, HSP60 and HSP70 mRNA expression levels were determined to have increased in all groups in the summer, fall and winter when compared with the levels in the spring which is known to be the most convenient season for goats in the region. With regard to the age groups, the greatest increase was recorded in the youngest group, Group I (1-2 years old). The values of the second age group, Group II (3-4 years old) were less than this increase and the lowest increase was measured for the oldest group, Group III (5-6 years old).

HSP60 and HSP70 expression levels were found higher in Group III than in the other groups in the winter. This increase in the winter in Group III, which contained older goats, was thought to be due to the pregnancy of the goats.

In this study, the differences of mRNA expression levels for HSP60 and HSP70 among groups in the summer were found statistically insignificant. In various studies comparing summer and winter values, the following results were determined. During summer seasons, HSP60 mRNA expression was found significantly ($P<0.05$) higher in all age groups in tropical and temperate region goats in comparison to winter season [3]. In a study, Increase of HSP70 gene expression during summer was observed to be higher in cold-adapted goat breeds [1].

In our study, the mRNA expression level for HSP60 in the fall was observed to have fallen as the age increased, and the the differences among the mRNA expression levels for HSP70in the three groups were found statistically non-significant.

The young, Group I, were observed to have expression levels three times more than the other groups and the levels decreased with aging. Similarly, the levels for HSP70 were high in the young group, and it also decreased with aging.

In a study they investigated HSP gene expression profiles belonging to goats in different seasons, ıt was found an increase in the mRNA gene expression level for HSP60 together with the age increase in the summer while they found the same levels in different age groups in winter statistically non-significant [3]. In a similar way, the HSP60 expression was indicated to have increased with aging in male Fischer 344xBrown Norway rats. Because of all these findings, it was concluded that apoptosis had increased and also the HSP60 increased, and for this reason, HSP60 increase could be possible with aging [17]. In a study he conducted, took blood samples from two different age groups (1-8 months old and 4-6 years old) of healthy Saanen goats subjected to the same management conditions. The mRNA expression levels for HSP60 and HSP70 was measured quantitatively using qRT-PCR SYBR Green Method. When the mRNA levels between the two groups were compared, the HSP60 expression levels were seen to be approximately 2 times lower in the old group than in the young group [18]. And similarly, the HSP70 expression levels were approximately 1.7 times lower, and it was observed that the older the goats, the lower HSP60 and HSP70 expression levels.

It was determined in various studies as well as in the ones conducted on humans that the mRNA expression levels for HSP60 and HSP70 decreased as the age increased. It was also determined that HSP60 and HSP70 levels diminished depending on the increase in the age in their study in which they searched the serum HSP60 and HSP70 levels in the individuals between 20 and 96 years old [19]. So they concluded that in the resisting ability against stres, there was a reduction related with the increasing age. Similarly, it was reported that mRNA expression levels for HSP70 in aged T lymphocytes [20]. It was found in a study they conducted on humans in various age groups that HSP70 level diminished depending on aging [21]. In another study, they conducted on young (5 months old) and old (24 months old) male Winstar rats that HSP70 expression was decreased in the shin and lung cells after a heat stres exposure [22]. That's why; they concluded that

HSP induction was disrupted with age. In a study they conducted on young (4-7 months old) and old (22-28 months old rats, in which they exposed all rats to 42.5°C for 30 min, found HSP70 expression in old rats 40-50% lower than young rats [23]. Holstein-Friesian milk cows was divided into 5 groups that Group 1 contained cows younger than 235 days old, Group 2 between 235-305 days old, Group 3 between 305-565 days old, Group 4 early lactation and Group 5 late lactation. HSP72 expression levels were found the highest in old cow milk group (305-565 days old). While HSP72 levels were low in late lactation group, they were found higher in early lactation group [24]. Similar studies in humans, it was found that HSP60 and HSP70 expression levels decreased with age and that a reduction happened in the ability to resist heat stress [19]. In another study, they conducted that HSP70 expression level was the best determiner with regard to the resistance to heat stress and that there was a logarhitmic relationship between HSP70 and resistance to heat stress in winged species. Due to their findings, they claimed that the HSP70 expression level in the cells was the indicator of the heat resistance power of the cells [25,26]. In a study, it was concluded that cyclical higher incubation temperatures at embryonic ages from 12 to 14 could increase liver HSP70 gene expression with no effect on body and liver weights, plasma T3, T4 and TG levels at day-old chicks [27]. In another study, it was demonstrated that the HSP70 expression significantly increased with the age in rainbow trout [28].

In this study, quantitative RT-PCR analyses of the HSP60 and HSP70 mRNA expressions according to seasons in various age groups of Saanen goats and gene expression amounts were compared among the groups composed that were defined numerically.

In conclusion, when the THI values were taken into account in the region where the study was conducted, severe heat stress was seen to have been effective in July and August, and heat stres was the case in June and July as well. In accordance with the other studies conducted, in the goats that were exposed to heat stress, mRNA expression levels for HSP60 and HSP70 were seen to be higher in the young goats than in the old ones. In other words, these levels decreased as the age increased. However, in the experiment done in the winter in this study, HSP60 value was seen to have increased as well although the age increased. It was thought that the pregnancy state of the goats could have been effective in these results, and HSP60 levels were found higher in the aged goats than younger ones.

In the study, HSPs were determined to be a significant mechanism against heat stress in humans like in farm animals. It is of great importance to breed resistant animals to particularly environmental conditions and to diseases and parasites on farms beside their productivity. Recently, researches on HSPs have focused on the development of cancer treatment and tumor vaccine in humans. In the following years, HSPs are believed to play significant roles in the selection of resistant animals particularly to the environmental conditions and to be one of the significant physiological parameters which will focus on farm animals.

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