Journal of Radiation Research and Applied Sciences

J. Rad. Res. Appl. Sci., Vol. 3, No. 4(B), pp. 1287 - 1303 (2010)

Heat-Induced Changes in Heat Shock Protein Genes Expression in Crossbred and Baladi Pregnant Cows and Their Offspring

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Received: 17 /10 /2010. Accepted: 24 /11 /2010.

ABSTRACT

The experiment was carried out during August (hot climate) on twelve pregnant cows of six crossbred (50% native Baladi and 50% Brown Swiss) and six native Baladi pregnant cows in the same age and the second parity during their mid-pregnancy as detected by rectal palpation. The experiment was repeated during December (mild climate) on similar twelve pregnant cows. Blood sample was obtained from each cow at the end of August (first group) and at the end of December (second group) to obtain heat shock protein genes expression (HSP72, HSP70.01, HSP70, HSP47, k Dalton and β-Actin) in pregnant cows under mild and hot climate to find out, which breed is more tolerant to heat stress and to estimate offspring birth weight and their growth performances during suckling period. Comparison was made between hot climate cows group and mild climate cows group to estimate heat- induced changes in both breeds in expression level of the Hsp genes and to compare with their neonate birth weight and growth performances during suckling period. The results revealed that expression level of the Hsp genes (Hsp72, Hsp70.1, Hsp70 and Hsp 47) was higher ($p<0.01$) in hot season compared to that of mild season. Expression level of the Hsp genes (Hsp70.1, Hsp70 and Hsp 47) was higher ($p<0.05$) in crossbred cows than in Baldi cows under summer hot season. This indicates that crossbred cows are less heat tolerant than Baladi cows under heat stress climate. Heat induced decrease ($p<0.01$) in offspring birth weight in Baladi and crossbred by 18.1% and 25%, respectively, in weaning weight by14.61% and 23.14%, respectively and in body weight gain by 14.61% and 21.18%, respectively.

Keywords: Heat Sock Protein, Heat Stress, Crossbred cows, Baladi cows, Pregnancy, offspring weight, weaning weight and body gain.

INTRODUCTION

Heat stress affects productivity of livestock, but little is known about how animals respond to heat at the cellular level. In this term, lymphocytes isolated from various animals were heat stressed *in-vitro* and responded by synthesizing heat stress proteins. This cellular response may be an important mechanism by which these animals respond to heat stress**.** All species examined showed increased synthesis of both HSP70 and HSBO, which implies a common response to heat stress**.** However, there were differences in the less dominant proteins, which indicate species uniqueness [(1)]. In this term, Krstensen *et al.*[(2)] revealed that cellular response to heat shock includes synthesis of proteins belonging to subgroup of molecular chaperones called heat-shock proteins (Hsp), and classified into 5 families according to their molecular weight (100,90,70,60, and small Hsp). Lindquist[(3)] added that the protective role of Hsp is usually confined to their chaperone function; that is, their capacity to bind denatured proteins and thus prevent their irreversible aggregation. Welch[(4)]and Kristensen *et al.*[(2)] reported that in the bovine, Hsp 72 is absent or expressed at low level under non-stress conditions and is referred to as inducible form of the Hsp 70 family. In addition, hyperthermia promotes oxidative stress in cells of laboratory animals, and that effect may be ascribed to different mechanisms, which include increased formation rate of reactive oxygen species [(5)]. Furthermore, Pahlavani and Harris [(6)] demonstrated that increased *in-vitro* generation of oxygen free radicals due to hyperthermia was associated with inhibition of proliferation (DNA synthesis) and IL- 2 gene expression in T cells from rats.

On the other hand, expression of HSP may vary in certain physiological conditions, such as pregnancy, besides being a response to stressful stimuli. In humans, some studies have shown changes in the circulating concentrations of HSP70 (in blood serum or plasma) during normal pregnancy, but their results are controversial. Fukushima *et al.* [(7)] did not find significant differences in serum HSP70 concentrations between the three trimesters of pregnancy. Conversely, Jirecek *et al.*[(8)] showed that serum HSP70 concentrations tended to decrease, whereas Bloshchinskaya and Davidovich [(9)] found that HSP70 concentrations in blood plasma tended to increase with advancing gestation. In a more recent and broader study on humans, serum HSP70 concentrations had a significant negative correlation with maternal age and a significant positive

correlation with gestational age [10].

It is worth to indicate that, crossing between native Baladi cows and European cattle is an effective and essential way for improving meat production of native cattle, because genotype has been reported to influence performance of cattle in many studies [11,12].

High ambient temperatures compromise reproductive efficiency of farm animals in both sexes and hence affect milk and meat production and the results of animal selection. In these situations the calving interval is longer, the birth rate is lower and farm milk yield per year can be reduced [13]. Heat stress during pregnancy slows down growth of the fetus, although active mechanisms attenuate excursions in fetal body temperatures when mothers are thermally stressed [14].

Therefore, the objective of this study was to determine the diversity in the gene expression patterns of HSP-72, HSP-70.1, HSP-70 and HSP-47 genes of pregnant Crossbred and Baladi cows as a new technique for heat tolerance indices to predict heat tolerant pregnant cows, to establish indices for selection based on temperament in relation to genotype. Establishment of selection criteria and indices of animal temperament in relation to genotype could provide an additional tool for selection of animals for optimal growth and reproductive performance in relation to their production-management environment, secondly, to compare their neonate birth weight and growth performances during suckling period and to compare, which breed is more tolerant to heat stress under Egyptian desert summer conditions.

MATERIALS AND METHODS

Animals, climatic conditions and feeding

Six crossbred (50% Brown Swiss and 50% native Baladi) and six native Baladi cows in their mid pregnancy period (as detected by rectal palpation) were used during August 2009 (hot summer conditions), where average ambient temperature (38.22 ± 0.17ºC) and average relative humidity (50.5 ± 1.04%) at mid day and temperature humidity index equaled (89THI). Other crossbred (n = 6) and Baladi cows (n = 6) in their mid pregnancy period were used during December 2009 (mild conditions), where average ambient temperature (22.4± 0.58°C) and average relative humidity (65.8 ± 0.4 %) at mid day and temperature humidity index equaled (72THI). All data which had been collected

during hot summer and mild conditions were used to make comparison between crossbred and Baladi pregnant cows in Hsp, genes expression and offspring performances.

The animals were kept outdoors under two open reinforced sheds in two yards surrounded with special wires during day and night .All animals were belonging to Bovine Farm Project at the Atomic Energy Authority, at Inshas area, which is a semi-arid desert area near the Nile valley.

Ambient temperature and relative humidity percentage were measured (under shade) twice a week at 12.00 and 16.00 hr by sensitive digital thermo-psychometric and temperature humidity index (THI) was obtained according that described by Armstrong.[15] .Feeds were offered for the group once daily at 9.00 am according to the animals´ body weights at a rate of 2.25 kg per 100 kg b.wt of a commercial concentrate mixture. Wheat straw was offered at the rate of 1.5 kg per 100 kg b.wt daily according to NRC [16]. Each 100 kg concentrates were supplemented with 1 kg sodium chloride, 1 kg di-calcium phosphate and 0.1 kg minerals and vitamins mixture. Animals were allowed to drink fresh water ad libitum.

Blood sampling

Blood samples were withdrawn from the jugular vein from Crossbred and native Baladi (Egyptian breeds) at 16:00 hour at the end of August 2009 (HS), and at the end of December 2009 (thermonutral). Blood samples were stored immediately on ice and transferred to the laboratory in 2 h after collection for HSP determination.

Semi-quantitative RT-PCR

Synthesis for the first cDNA strand using total RNA extracted from blood samples

Total RNA was isolated from 300 to 500 µl of blood samples collected from the Crossbred and Baladi (Egyptian breeds) cattle. The blood samples were centrifuged for 5 min at 2000 g, then the pellet of the cells was used to extract the total RNA by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µl of diethyl pyrocarbonate (DEPC)-treated water. In order to remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase and removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol.

The complete Poly (A)- RNA isolated from Crossbred and Baladi cattle samples were reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo(dT) primer. According to Ali *et al.*[17], the composition of the reaction mixture (MM), consisted of 50 mM $MgCl_2$, 200 U/µl reverse transcriptase (RNase H free), 10x reverse transcription (RT) buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3 Perkin-Elmer), 10 mM of each dNTP (Amersham, Brunswick, Germany), and 50 µM of oligo (dT) primer. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through polymerase chain reaction (PCR).

Polymerase chain reaction (PCR)

The first strand cDNA of cattle samples was used as templates for RT-PCR with the specific primers of several Heat shock protein genes. The sequences of specific primer of the genes used (Chandolia *et al.*[18]; Knijn *et al.* [19]; Lacetera *et al.*[20]) and product sizes are listed in Table (1). β-Actin was used as a housekeeping gene for normalizing mRNA levels of the target genes. According to Ali *et al* [17], the reaction mixture for RT-PCR was consisted of 10 mM dNTP's, 50 mM $MgCl_2$, 10x PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.3, Gibco BRL, Eggenstein, Germany), and autoclaved water. RT-PCR amplification with Hsp72, Hsp70.1, Hsp70 and Hsp47 gene-specific primers was performed for 45 (Hsp72), 36 (Hsp70.1), 42 (Hsp70) and 40 (Hsp47) cycles. The PCR cycling parameters were one cycle of 94°C for 5 min, 36-45 cycles of 95°C for 10 s, 55°C to 61°C (Table 1) for 30 s, 72 °C for 40 s, and a final cycle of 72 °C for 7 min. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different fish samples. The PCR reaction was repeated at least five times within each group.

Statistical analysis

A-The binomial data for semi-quantitative RT-PCR analysis showed normal distribution. Heat shock proteins data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (SAS) [21] followed by Scheffé-onati and Tiberio,[22] test to assess significant differences between treatments. All statements of significant were based on probability level of $P < 0.05$.

B-The effect of heat stress on neonate birth weight and neonate body weight gain in this study was tested statistically by Student "t" test (Snedecor and Cochran) [23] using the unpaired variants. The percentage change to heat stress was calculated as follows: (hot-mild) x100/mild).

Table (1). Primers sequences used for RT-PCR

Gene	Primer sequence (5′–3′)[a]	Product length (bp)	Annealing Tm (°C)
Hsp72	F: 5′-AACATGAAGAGCGCCGTGGAGG-3′	169	61
	R: 5′-GTTACACACCTGCTCCAGCTCC-3′		
Hsp 70.1	F: AAGGTGCTGGACAAGTGCC AGGAGGTGATT	488	59
	R: ACTTGGAAGTAAACAGAAA CGGGTGAAAAA		
Hsp70	F: GTCATCAACGACGGAGACAA	555	59.4
	R: GGTGCTGGACGACAAGGT		
Hsp47	F: CCA GGA AAT GGC ACA TGT AT	290	60
	R: TAT AAG CAT GCT GTC GGG TC		
β-Actin	F: TGG GGC AGT ATG GCT TGT ATG	165	55
	R: CTC TGG CAC CCT AAT CAC CTC T		

[a] **F: forward primer; R: reverse primer. Tm: temperature.**

RESULTS AND DISCUSSION

Genetic alterations study

Expression of heat-shock proteins genes (Hsp72, Hsp70.1, Hsp70 and Hsp47) in the blood samples of Crossbred and Baladi cattle is summarized in Table (2) and Figures (1-4). The results revealed that expression level of the Hsp genes (Hsp72, Hsp70.1, Hsp70 and Hsp47) was higher in the hot compared to that level of mild season (Figures 1-4). Nearly the same results were obtained when the PCR assay was performed for each individual sample within each group (6 animals). The expression of Hsp72 gene was slightly higher in Crossbred than Baladi cattle in both mild and hot seasons, separately. However, the expression level was not significantly different between both breeds with each season (Fig. 1). Moreover, the expression of Hsp72 was lower than the other genes in the both breeds and at the two seasons. In agreement with theses findings, Kristensen *et al.*[2] reported that bovine Hsp72 is absent or expressed at a low level under non-stress conditions and is referred to as the inducible form of the Hsps family [4].

Hsp70.1 and Hsp70 genes expressed in Crossbred and Baladi cattle showed that their expression level was higher in Crossbred than Baladi cattle in both hot and mild seasons (Fig. 2 and 3). This level did not differ between both breeds in the mild season. However, the level of the expression was significantly higher in Crossbred than Baladi cattle in the hot season (Figures 2 and 3). In the same trend, the expression level of Hsp47 in mild season was

relatively similar in both Crossbred and Baladi cattle (Fig. 4). However, the expression level was significantly over expressed in Crossbred compared to Baladi cattle in the hot season (Fig. 4).

Previous studies performed on bovine or other species evaluated mRNA levels or synthesis of Hsp72 following short-term exposure to heat-shock conditions. Kamwanja *et al.*[24] found higher cellular resistance to elevated temperatures in lymphocytes from Brahman and Senepol cattle with respect to Angus cattle. They found a tendency for a lower amount of Hsp70 in the 2 thermo tolerant breeds. Hansen[25] speculated that the reduced Hsp70 expression in heat stressed Brahman and Senepol cattle might be indicative of reduced protein denaturation (one of the signals for Hsp70 synthesis). Accordingly, others indicated that activation of Hsp genes is primarily related to the defense against cell damage consequent to heat shock (Schiaffonati and Tiberio) [22], or that Hsp70 expression is positively related with increase of cell injury score [26]. Recently, Kristensen *et al.* [2] and Lacetera *et al.*[20] suggested that increase of Hsp72 expression might function as a biological indicator for changes in the stress level. These observations may be of help to explain the lower amount of Hsp72 mRNA found in Crossbred and Baladi cattle in our study compared to other Hsp genes.

Table (2): Heat-induced blood heat shock proteins genes expression in pregnant crossbred and Baladi cows.

Heat shock proteins genes expression		Pregnant crossbred			Pregnant Baldi		
		Mild	Hot	change%	Mild	Hot	change%
Hsp72/B-actin	$\bar{X}$	0.53	0.86**	62.26	0.45	0.79**	75.56
	± SE	0.01	0.01	0.93	0.01	0.01	2.537
Hsp70.1/B-actin	$\bar{X}$	0.58	1.13** b	94.83	0.46	0.79** b	67.35
	± SE	0.02	0.02	2.02	0.02	0.01	7.44
Hsp70 B-actin	$\bar{X}$	0.67	1.37** b	104.48	0.480	0.84** b	75.00
	± SE	0.03	0.03	2.59	0.01	0.02	6.13
Hsp47/B-actin	$\bar{X}$	0.63	1.42** b	125.40	0.55	0.83** b	50.91
	± SE	0.01	0.01	0.28	0.01	0.01	0.85

****Significant at p<0.01 (b) Significant at p<0.01 between breed**
*** Significant at p<0.05.**

To our knowledge, measurement of HSP70.1, HSP70 and HSP47 mRNA levels in blood cells of Crossbred and Baladi cattle exposed to high temperature regimens during the hot season in Inshas area, which is a semi-arid desert area near the Nile valley, Egypt has not been conducted before. On the other hand, Lindquist [3] indicated that the heat-shock response is accompanied

by a reduction in protein synthesis, favoring induction of the heat shock response over the ongoing gene program. These observations are consistent with our finding that the expression of HSP70.1, HSP70 and HSP47 genes were at their highest level during the hot season and may be attributed to low protein level.

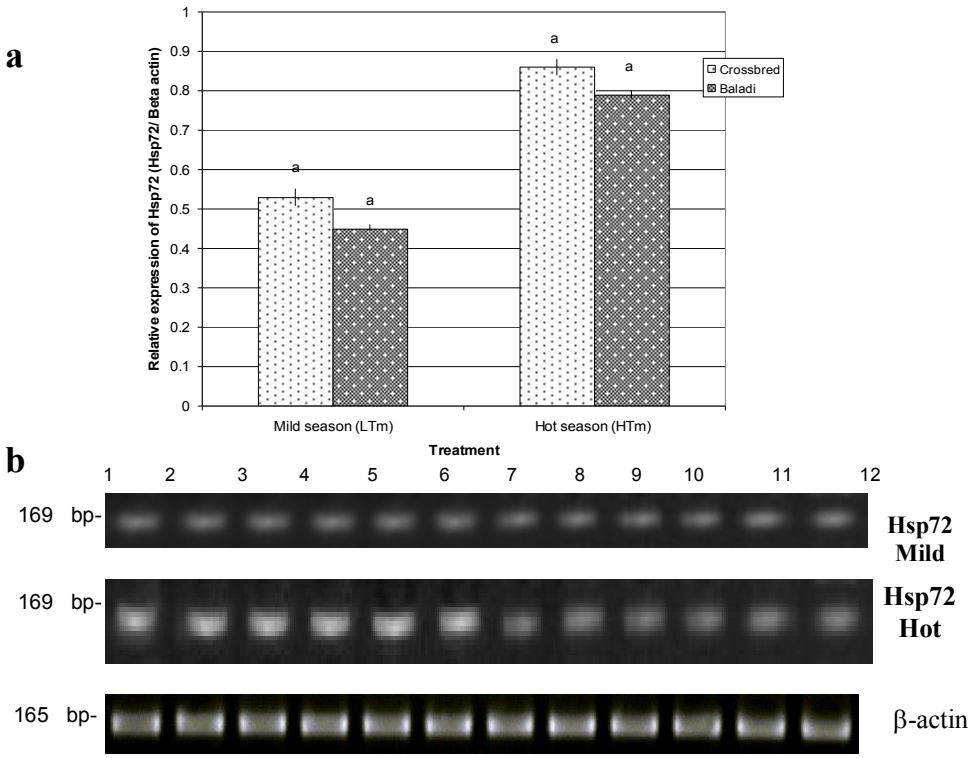

Figure (1): Expression of Hsp72 gene in the blood of Crossbred (lanes 1-6) and Baladi (lanes 7-12) cattle determined by semi-quantitative RT-PCR (a&b). The RNA recovery rate was estimated as the ratio between the intensity of Hsp72 gene and the β-actin gene. Within each column, means superscripts with different letters are significantly different ($P \leq 0.05$).

In addition, previous studies indicated that elevated Hsp mRNA levels are associated with enhanced heat tolerance [27]. The current results suggest that further research is needed to verify whether mechanisms different from Hsp may exist in thermo-tolerance bovine. Finally, our study did not indicate whether the different steady-state mRNA levels of Hsp genes in the Crossbred and Baladi breeds were dependent on transcriptional or posttranscriptional regulation of gene expression. However, previous studies indicated that mRNA

levels of Hsp72 in cells exposed to stressful stimuli may depend either on transcriptional [22] or on posttranscriptional regulation [28] of gene expression. Additionally, they demonstrated that among the different mechanisms through which translational regulation may occur (pre-mRNA splicing, mRNA transport, mRNA stability), posttranscriptional regulation of Hsp70 gene expression occurs mainly by mRNA stabilization.

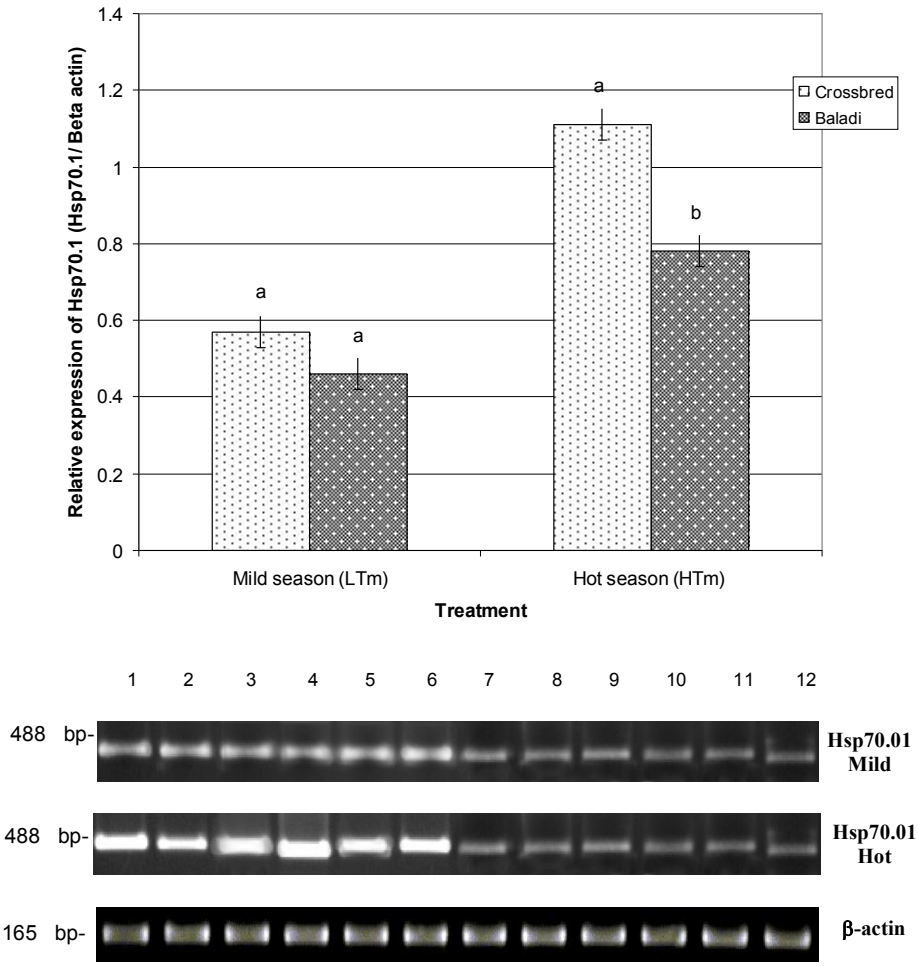

Figure (2): Expression of Hsp70.1 gene in the blood of Crossbred (lanes 1-6) and Baladi (lanes 7-12) cattle determined by semi-quantitative RT-PCR (a&b). The RNA recovery rate was estimated as the ratio between the intensity of Hsp72 gene and the β-actin gene. Within each column, means superscripts with different letters are significantly different ($P \leq 0.05$).

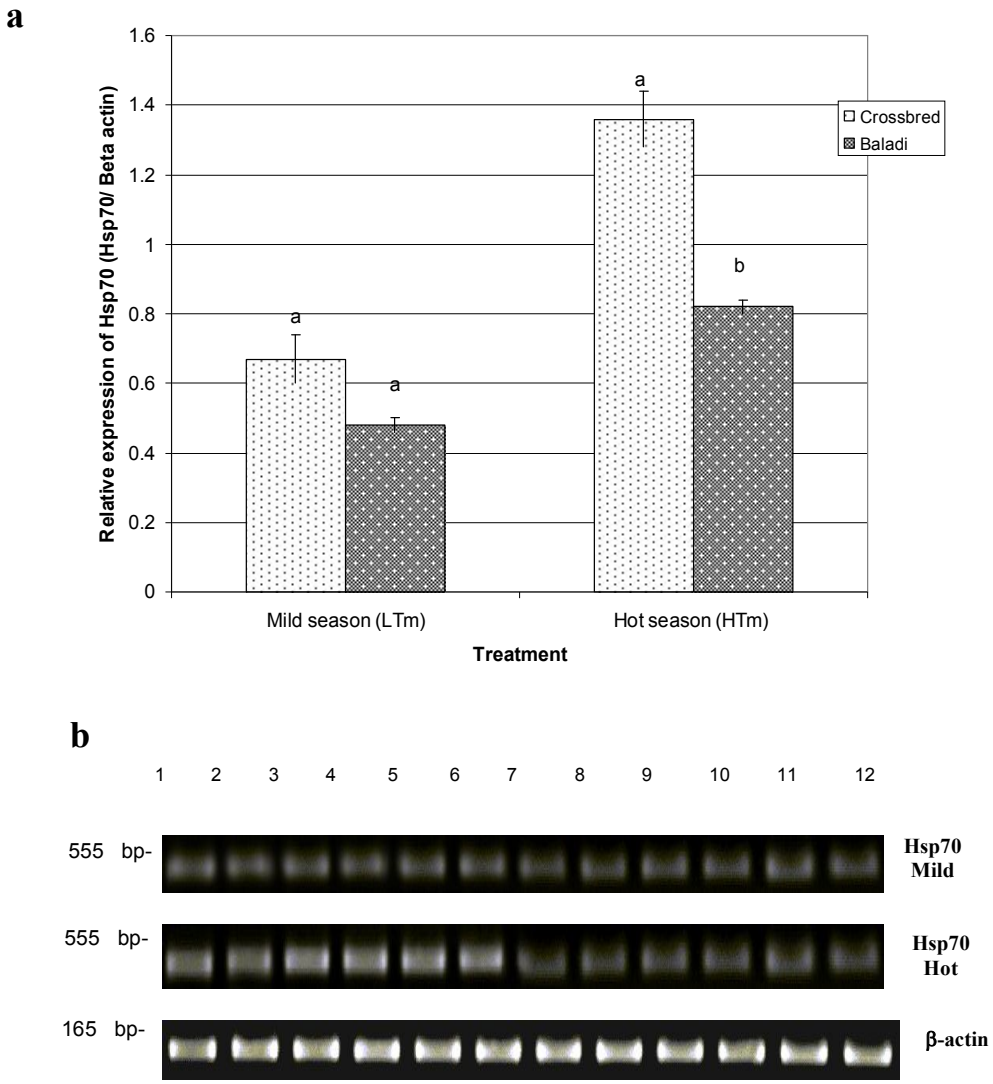

Figure (3): Expression of Hsp70 gene in the blood of Crossbred (lanes 1-6) and Baladi (lanes 7-12) cattle determined by semi-quantitative RT-PCR (a&b). The RNA recovery rate was estimated as the ratio between the intensity of Hsp72 gene and the β-actin gene. Within each column, means superscripts with different letters are significantly different ($P \leq 0.05$).

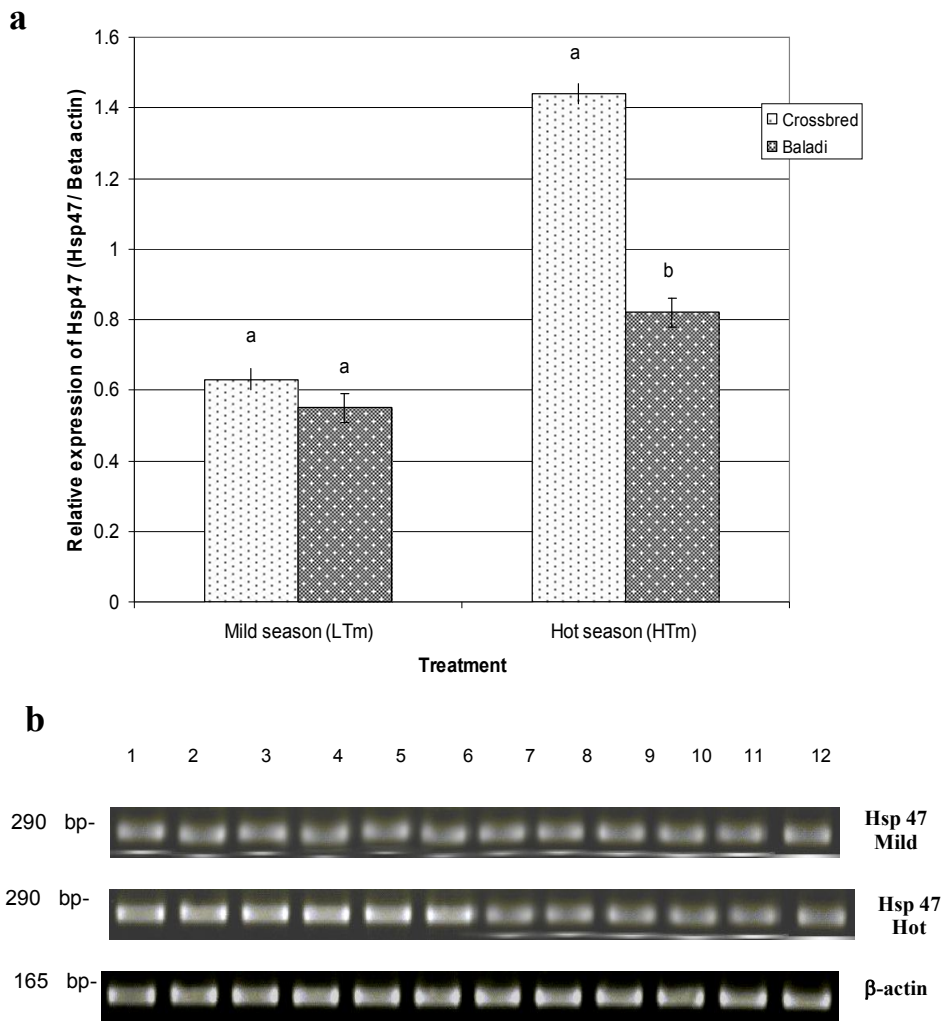

Figure (4): Expression of Hsp47 gene in the blood of Crossbred (lanes 1-6) and Baladi (lanes 7-12) cattle determined by semi-quantitative RT-PCR (a&b). The RNA recovery rate was estimated as the ratio between the intensity of Hsp72 gene and the β-actin gene. Within each column, means superscripts with different letters are significantly different ($P \leq 0.05$).

Crossbred and Native Baladi cows neonate growth performance

Data presented in Table (3) display that hot summer condition caused a significant ($p<0.01$) decrease in live body weight at birth by 18.14% and 24.97% in Baladi and crossbred, respectively, and at weaning by 14.61% and 21.18 %, respectively. Moreover, hot summer condition caused a significant ($p<0.01$) decrease in each of Baladi and crossbred calves daily body gain by 14.61 % and 21.17%, respectively. It can be noted also that calves birth weights, weaning weights and daily body gain weights were higher in crossbred than in native Baladi.

Table (3): Live body weight at birth, at weaning and daily body weight gain in Baladi and croosbred calves under winter and summer conditions.

Items	Bladi calves			Crossbred calves		
	Winter	Summer	Change %	Winter	Summer	Change %
Live body weight at birth (Kg) ± SE	28.71 1.69	23.50** 2.1	-18.14	37.6 1.56	28.21** 1.68	-24.97
Live body weight at weaning (Kg) ± SE	86.83 4.11	74.14** 3.82	-14.61	121.6 6.89	93.46** 3.44	-23.14
Daily body weight gain (g) ± SE	723.6 3.61	617.88 5.14	-14.61	988.19 8.79	778.9** 0.65	-21.18

*** Significant at p< 0.05** **** Significant at p< 0.01**

Concerning the comparison between Baladi and crossbred, EL-Fouly *et al* [29] found that the crossing between Broun Swiss bull and Baladi cow resulted in significant improvement in body weight at birth and at weaning (four months of age) Also, the study of Ruvuna *et al.* [12] showed that body weight of crossbred were superior at eleven months postpartum .In addition, Nasser *et al.*[30] and Marai[31] reported that higher values in live body weight at birth and at weaning were shown by grading up Baladi native cows with Freisian or Broun Swiss bulls that superiority mainly due to heterosis in growth rate of the offsping . Above 30°C adverse effects are recorded in daily weight gain. Under high ambient air temperature and solar radiation, daily dry matter intake decreased, hence average daily gain and carcass weight fall down, fat thickness drops [32] and an increase in disease incidence can occur. In addition, El-masry and Marai[33]and El-fouly *et al.* [29] found that the productivity and most blood constituent were different in Baladi compared to crossbred under both winter or summer conditions. The difference was more apparent in cross bred than in Baladi calves, which proposed that Baldi calves are more tolerant than

crossbred calves under hot environmental conditions.

It can be noted that Hsp genes expression in pregnant cows under hot summer conditions exhibited a reverse relationship with neonate body weight at birth, but this trend was not significant between Baladi and crossbred cows and their neonates.

CONCLUSION,

The present results revealed that expression level of the Hsp genes (Hsp70.1, Hsp70 and Hsp 47) was significantly higher in crossbred cows than in Baldi cows under summer hot season. This finding indicates that crossbred cows are less heat tolerant than Baladi cows under heat stress climate. This study demonstrated that the Hsp genes represent precious biomarkers to detect variations of thermo-tolerance bovine conditions.

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مجلة البحوث الإشعاعية
والعلوم التطبيقية

مجلد 3 عدد 4(ب) ص ص 1287– 1303 (2010)

التغيرات الناشئه عن العبء الحرارى فى التعبير الجينى لبروتينات الصدمة الحرارية فى الأبقار الخليط والبلدى الحوامل ونتاجها.

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اجريت هذة التجربة خلال شهر اغسطس (جو حار) على إثنى عشرة بقره حوامل فى الموسم الثانى للحمل وفى منتصف فترة الحمل حيث تم الفحص عن طريق الجس من خلال الشرج.

يشمل مجموع الحيوانات عدد ستة بقرات خليط (براون50%وبلدى 50%) وستة بقرات بلدى. تم تكرارهذه التجربة على مجموعة مماثلة فى شهر ديسمبر (جو معتدل).تم سحب عينات الدم فى نهاية اغسطس للمجموعه الاولى وفى نهاية ديسمبر للمجموعة الثانية.تم عمل مقارنه بين مجموعة الجو الحار ومجموعة الجو المعتدل لمعرفة تأثير الجو الحار على التغير فى التعبير الجينى لبروتينات الصدمة الحرارية وكذلك التغير فى اوزان النتاج عند الميلاد وعند الفطام ومعدل النمو فى هذه الفتره.

اوضحت النتائج:

وجود فارق معنوى عند مستوى اعلى من ($p<0.05$) فى زيادة بروتينات الصدمة الحراريه بالدم فى الابقار المعرضة للجو الحار عن الابقار المعرضة للجو المعتدل فى كل من الابقار الخليط والبلدى.

ادى الجو الحار الى إنخفاض معنوى ($p<0.01$) فى وزن العجول عند الميلاد بمقدار18.1% فى الخليط و25.5% فى البلدى.وعند الفطام بمقدار14.6% فى الخليط و23.1% فى البلدى. كما ادى الجو الحار الى انخفاض معنوى($p<0.01$) فى معدل النمو خلال فترة الرضاعه بمقدار14.6% فى الخليط و21.2% فى البلدى.

اوضحت الدراسه:

إرتفاع معنوى فى مستوى التعبير الجينى لبروتينات الصدمة الحرارية(70.1و 70 و47) فى الأبقار الخليط عن الأبقار البلدى تحت ظروف الجو الحار صيفا وهذة النتائج توضح ان الأبقار الخليط اقل تحملاً للعبىء الحرارى من الأبقار البلدى . وهذا يوضح امكانية استخدام التعبير الجينى لبروتينات الصدمة الحراريه للكشف عن الأبقار ذو التحمل للعبء الحرارى تحت ظروف الجو الحار.