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Association of Hsp70 Gene Polymorphism and Bull Semen Quality in Winter and Summer Seasons

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Key words
HSP70, Polymorphism, Biochemical parameter, Hormonal profile, Semen quality

ABSTRACT:

Objectives of current study were to identify single nucleotide polymorphisms (SNP) in heat shock protein70 (*HSP70*) promoter gene of bulls (cattle and buffalo) and its association with semen quality traits and some enzymatic, hormonal and lipid parameters during winter and summer seasons. Semen samples of 37 animals (Cattle n=21 and buffalo n=16), were used in the study. Biochemical Parameter and hormonal profile were estimated. Qualities of some of fresh and frozen-thaw semen were evaluated. PCR-SSCP was used to detect SNPs and different patterns were sequenced. The study demonstrate all the measured biochemical parameters and hormonal profile of cattle and buffalo were significantly different ($P<0.05$) in winter as compared to summer. Results of PCR-SSCP and sequencing detected a new synonymous SNP 812 T>C transition was the same in both species and could clear two genotypes TT and TC. Association analysis revealed significance difference ($P<0.001$) with all the fresh and some of frozen-thawed semen traits in cattle population and T allele seemed to be the favorable allele. Regarding to buffalo population, different genotypes has significant difference in term of fresh motility, frozen motility at 1h and viability index. The results of association between biochemical and hormonal parameters there were no significance except for Aspartate-aminotransferase (AST) in cattle population and T_3 level in buffalo population and C allele appeared to be the favorable allele. This the first study concerned with the association of *HSP70* polymorphisms and semen quality, in winter and summer seasons in buffalo population. Conclusion: High temperature has negative impact on semen quality. Biochemical and hormonal profile can be used as good indicator for semen value. Polymorphisms in *HSP70* gene can affect semen quality. We suggest to be used in artificial insemination programs as marker assisted selection for anti-heat stress and good reproductive bulls.

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1. INTRODUCTION:

Bull fertility and semen quality are of vital importance to the bovine industry as sub-fertile bulls can cost producers a significant amount of money (Kastelic and Thundathil 2008). Among all climatic elements, temperature is the most important parameters affecting animal fertility (Kunavongkrit *et al*., 2005). The high ambient temperature increased the scrotal temperature in males and consequently a decline in semen quality (Taylor and Bogart 1988). Production and reproduction are impaired as a result of drastic changes in biological functions caused by heat stress (Marai *et al*., 1995).Seminal plasma, which is a complex mixture of secretions contain factors called heat shock proteins family which is one of the intrinsic molecular mechanisms in the cell protecting it from deleterious effects of heat and other stress (Pockley 2001; Rajoriya *et al*., 2014). The best known of the *HSP* families is the *HSP 70* family (Rynkowska *et al*., 2011) so *HSP70*, located in reproductive tissues, has critical roles in many events.
Artificial insemination (AI) is recognized as one of the most important techniques for the genetic improvement of semen quality traits in dairy cattle herds (Parmentier *et al.*, 1999 and Qing *et al.*, 2013).
Assuming that *HSP70* might be one of the candidate genes that can differentiate heat stress response in individual and the association of polymorphisms of the *HSP70* gene with fertility in cattle is still unclear (Rosenkrans *et al.*, 2010). So the objectives of this study were to identify polymorphisms located in the heat shock protein 70 (*HSP70*) promoter gene of bulls (cattle and buffalo) and its association with semen

quality traits and some enzymatic, hormonal and lipid traits of seminal plasma during seasons of extreme temperatures.

2.MATERIAL and METHODS:

2.1. Animals and semen collection: Twenty one bovine bull and sixteen buffalo bulls of known fertility, maintained on private farms in Giza and Alexandria governorates, were used in the present study. Early in the morning, using a pre-warmed artificial vagina, twice a day ejaculates were collected from each bull once per week for 3 consecutive weeks in winter and summer with daily mean temperature 16ºC and 38.5ºC respectively. Fresh semen samples were evaluated just after collection for motility, plasma membrane and acrosome integrities.

2.2.1. Semen Processing: Semen samples were diluted according to (Reddy *et al.*, 2010) with tris-based extender at 37ºC to obtain a final concentration of 50 x 10^6 sperm cell/ml. Semen was loaded in straws (IMV, France) and stored in liquid nitrogen until thawing.

2.2.2. Evaluation of frozen-thawed semen: Motility estimations were done at hourly intervals for a period of 3 h. The viability index was calculated according to Milovanov (1962) it is equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd and 3rd h post-thawing. The procedure described by Jeyendran *et al.*, (1984) was used to determine the percentage of HOS positive sperm cells in each semen sample. Acrosome integrity was estimated using fast green stain (Wells and Awa, 1970).

2.3.1. Biochemical analysis: Fresh seminal plasma was harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. The separated seminal plasma was stored at -20°C until used. All biochemical measurement of the fresh seminal plasma was carried out using the spectrophotometer. Aspartate-aminotransferase (AST); alanine-aminotransferase (ALT) and alkaline phosphatase (ALP) enzymes concentration was assessed according to Tietz (1976). Total antioxidant capacity level (TAC) was measured at 532 nm according to Cortassa *et al.*, (2004). Determination of total cholesterol according to Allian *et al.*, (1974), phospholipids and Triglycerides according to McGowan *et al.*, (1983) and HDL in seminal plasma were performed using Bioscope commercial diagnostic kits at 520 nm wave length.LDL was calculated from the following equation: LDL (mg/dl) =total cholesterol-(trigycerides/5) –HDL.

2.3.2. Radioimmunoassay of triiodothyronine (T_3): Part of each semen sample are analysed for T_3 determination according to methods of Todini (2007) and was stored in aliquost at -20ºC until use.

2.4. Molecular aspect:

2.4.1. DNA extraction: Genomic DNA was extracted DNA was extracted from semen samples using Chelex-100® (Fluka, Sigma Aldrich, USA) as previously described by Walsh *et al*. (1991) and modified by Anju *et al*., (2010). Breifly, 50 µl of sample was added to 400 ml of 5% Chelex-100. Proteinase K (MBI fermentas, EU) at 0.1mg/ ml and 31 mM dithiothreitol (DTT) were added to 450 ml of semen and mixed thoroughly. After incubation at 56°C for 1 h, the mixture was boiled for 20 minutes. After vortexing the material was centrifuged at 10.000 rpm for 8 minutes. Then the supernatant was collected and stored at -20°C till using for PCR assay as template.

2.4.2. PCR amplification: Amplifications were performed in 25 µl reactions containing 5 ul of genomic DNA, 1X of master mix (Fermentas Life Science) and 25 pmol of each primer. Two sets of primers were used to amplify conserved region within the *HSP70* promotor **Table (1)** The PCR amplification cycling protocols of PCR amplification are summarized in **Table (2).** The PCR products were detected on 1.5 % ethidium bromide stained agarose gel

Table (1) Primers used in PCR amplification & sequencing of *HSP70* of cattle & Buffaloes

Target	Sequnce5'-3'	Amplicon size	Reference
Cattle HSP70 promotor	(5'-GCCAGGAAACCAGAGACAGA-3') (5'-CCTACGCAGGAGTAGGTGGT-3')	539bp	Rosenkrans et al., (2010)
Buffalo HSP70 promotor	(5'-GTTAGCCTCCGATCACTCTC-3') (5'-GAAGCTGCTCTCACGGACTA-3')	560bp	Sodhi et al., (2013)

Table (2) Cycling protocol of PCR amplification

Target	Amplicon size	Cycling condition			No. of cycle
		Step	Temp.	Time	
HSP70 promotor	539bp (Cattle)	Denaturation	94˚C	45 sec	35 cycles
		Anealing	55˚C	1min	
		Extension	72˚C	1min	
	560bp (buffalo)	Denaturation	95˚C	1min	30 cycles
		Anealing	59˚C	1min	
		Extension	72˚C	1 min	

The two PCR programs started with initial denaturation at 95°C for 2 minutes and were ended with final extension at 72°C for 10 minutes.

2.4.3. PCR-SSCP analysis: The SSCP analysis was carried out according to **Han *et al.*, (2009)** using 12 % acrylamide: bisacrylamide gel (29:1). The different SSCP patterns of PCR products were sequenced.

2.4.4. Sequencing: PCR products were purified using GeneJET™ PCR Purification Kit (Thermo K0701) according to manufacture instructions. The purified PCR products were sequenced on GATC Company (Germany) by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technology. Nucleotide sequence alignments and comparisons were done using the Bio Edit version 7.2 software.

2.5. Statistical analysis: Two way analysis of variance and Duncan's multiple range tests. Data were analyzed using the 1984-version of Costat (Ecosoft, inc, USA), and the level of statistical significance was set at $P \leq 0.05$. The allelic frequency, genotypic and expected frequency and Hardy-Weinberg equilibrium assessed by the Chi-square (χ^2) test (at level of 5%) for *HSP70* promotor SNP were carried out on website: http://www.oege.org/software/hardy-weinberg.html.

3. RESULTS:

A 539bp fragment of the bovine HSP70 gene promoter (base positions 749-1288) was successfully amplified for bull **(Fig. 1A)** while 560bp fragment of predicted *HSP70* gene promoter region was amplified for buffalo bull **(Fig. 1B).** PCR-SSCP on polyacylamide gel revealed two different banding patterns (TT and TC) in cattle bulls **(Fig. 2)** and also within buffalo bull populations **(Fig.3).**

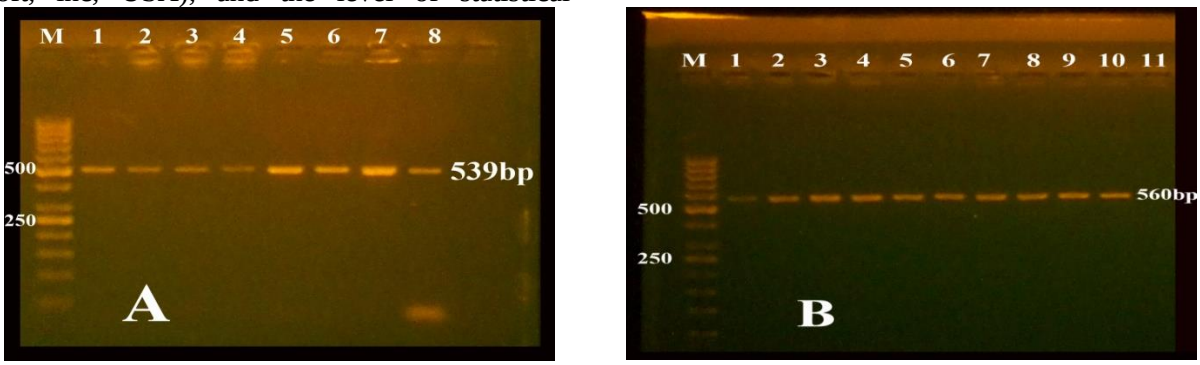

Fig (1) Showed ethidium bromide stained 1.5% agarose gel electrophoresis of PCR products of HSP70 of cattle (A) with predicted amplicon of 539bp and HSP70 of buffaloes (B) with predicted amplicon of 560bp (M) 50bp DNA ladder.

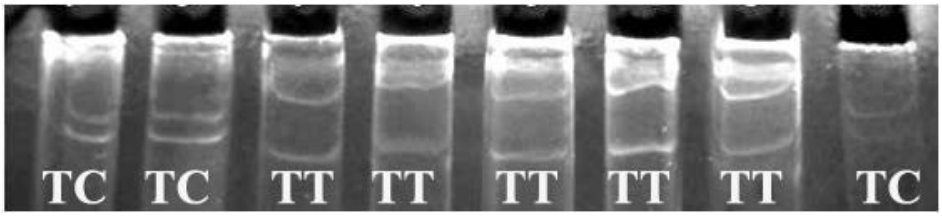

Fig (2) PCR-SSCP products for cattle bulls stained with ethidium bromide

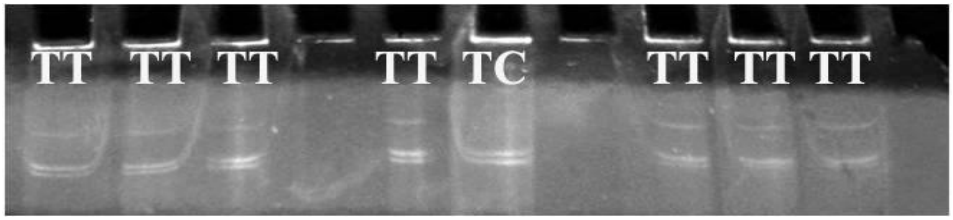

Fig (3) PCR-SSCP products for buffalo bulls stained with ethidium bromide

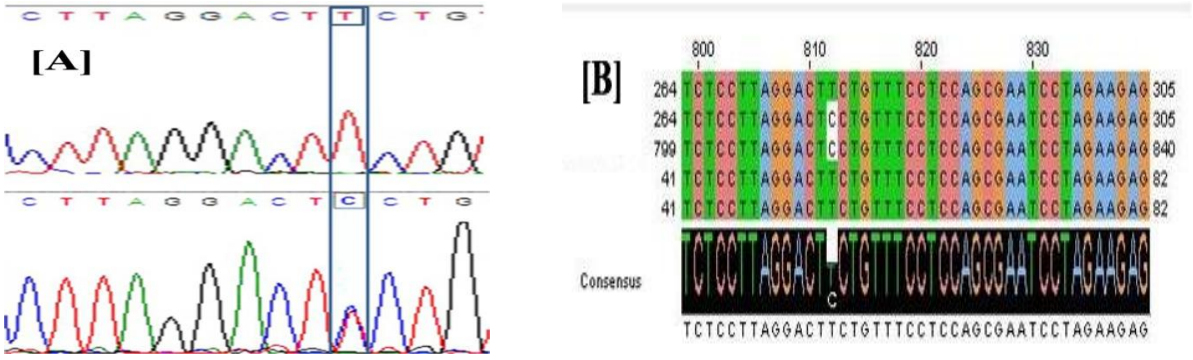

Fig (4): [A] Sequence electropherogram show T > C transition at nucleotide 812 **[B]** The alignment of the sequences of the two genotypes using free tool on line (ClustalW2< Multiple Sequnce Alignment<EMBL-EBI) WWW.ebi.ac.uk/Tool/msa/clustalw2/

Table (3): Allele frequency and observed and expected genotype frequency for HSP70 promotor SNP and chi-square (χ2) values for Hardy-Weinberg equilibrium test (P<0·05).

Species	Cattle (X^2)=1.16		Buffalo (X^2)=0.17	
Allele frequency	Allele T	Allele C	Allele T	Allele C
	0.81	0.19	0.91	0.09
Genotype frequency	TT	TC	TT	TC
Observed	13	8	13	3
Expected	13.76	6.48	13.14	2.72

χ^2 at 1 degree of freedom and Chi-square = 3.84 at 95 % probability level

Results of sequencing and the multiple sequence alignment revealed a new synonymous SNP T>C transition at nucleotide 812 of bovine HSP70 promotor gene (GenBank accession number M98823). The detected SNP was the same in both species and could clear two genotypes TC (n=8 and n=3 in cattle and buffaloes respectively) and TT (n=13 in both species) **(Fig. 4)**.

As shown in **table (3)** The T allele showed higher frequencies in both bulls. The allele frequencies were 0.81 and 0.19 for allele T and allele C respectively in bull while the frequencies were 0.91 and 0.09 for allele T and allele C respectively in buffalo bulls. X^2 test showed that the population was at Hardy-Weinberg equilibrium in both species.

As displayed in **table (4).**The association analysis with the fresh semen quality traits showed bovine

bulls with the TT genotype had significantly higher (P<0.05) individual motility, membrane integrity and percentage of spermatozoa with normal acrosome than bulls with TC genotype during summer season. The table also show a high significant (P<0.001) interaction between genotypes and seasons on all of the parameter analyzed of fresh semen. Concerning to the frozen-thawed semen characteristics there was no significant difference with any of the parameter analyzed between different genotypes except for viability indexes that were significantly higher (P<0.001) in bulls carrying TT genotype than those carrying TC genotype in summer season (118.46 versus 82.85 respectively). The interaction between genotype and season was significantly higher (P<0.05) in case of viability index trait only. Regardless to genotype there was significant differences (P<0.05) in nearly all of the parameter analyzed (fresh and frozen-thawed semen) between ejaculates collected in winter than those collected in summer except that of sperm membrane integrity swollen spermatozoa (HOS +ve%) of frozen-thawed semen.

As illustrated in **table (5)** different genotypes has significant difference in term of fresh motility, frozen motility at 1h and viability index during winter and summer seasons. The table also clarify significant interaction (P<0.01) between genotype and season in term of fresh individual motility, (P<0.001) in term of 1st h, (P<0.01) in term of 2h and (P<0.05) in terms of 3h post-thaw motility and the viability index. Irrespective to genotype there was significant difference (P<0.05) between ejaculates collected in winter and those collected in summer in nearly all fresh and post-thaw semen traits analyzed.

As presented in **Table (6)**, all the measured lipids in seminal plasma of bulls and buffalo bulls (cholesterol, phospholipids and triglycerides) were significantly higher (P<0.05) in winter semen samples as compared to those of summer, on the other hand the cholesterol/phospholipids ratio was significantly higher (P<0.05) in samples collected during summer. The metabolic enzymes (AST,ALT and ALP) were significantly higher (P<0.05) in bulls and buffalo bulls summer samples as compared with those of winter samples. Total anti oxidants, T3 hormone and fructose levels in seminal plasma of bulls and buffalo bulls of winter samples were more or less two-fold higher than those of summer samples.

Table (4) Association of 812 T>C SNP in *HSP70* promotor gene with some fresh and frozen semen traits of bull during winter and summer seasons

Semen Traits \ Genotype			TT (n=13)		TC (n=8)		(I)
			Winter	Summer	Winter	Summer	
Fresh semen		Mot. (%)	68.84 ± 1.40^{a,b}	65.76 ± 1.48^{b}	72.5 ± 1.63^{a}	55 ± 1.34^{c}	***
		acrosome(%)	90.07 ± 0.32^{a}	84 ± 1.39^{b}	90.13±0.39^{a}	72.87± 1.44^{c}	***
		(HOS +ve %)	82.54 ± 0.69^{a}	81.92 ± 0.71^{a}	83.62 ± 0.70^{a}	61.42 ± 2.04^{b}	***
Post-thaw semen	Mot.	0 h	53.84 ± 1.4^{a}	54.23 ± 3.57^{a}	57.5 ± 1.63^{a}	50 ± 5.35^{a}	ns
		1 h	47.69 ± 1.21^{a}	41.53 ± 3.55^{a}	48.75 ± 1.87^{a}	38.57 ± 5.56^{a}	ns
		2h	39.61 ±2.07^{a}	34.61 ± 3.69^{a,b}	38.75±1.57^{a,b}	27.5 ± 3.67^{b}	ns
		3h	31.15 ± 1.40^{a}	25.38 ± 3.32^{a}	30 ±0 .94^{a}	21.42 ± 4.75^{a}	ns
	Viability Index		158.07 ± 3.36^{a}	118.46 ± 6.81^{b}	142.5±3.28^{a}	82.85 ± 7.69^{c}	*
	acrosome (%)		74.07 ± 0.32^{a}	67.69 ± 0.86^{b}	74.125 ± 0.39^{a}	65.42 ± 1.78^{b}	ns
	(Hos +ve %)		63.53 ± 0.69^{a}	63.46 ± 0.66^{a}	64.62 ± 0.70^{a}	62.5 ± 0.84^{a}	ns

Mean ± SE with different superscripts in the same raw were significantly (P<0.05) different. (I) = the interaction between (Genotype & seasons), *** significant at P<0.001, ** P<0.01, * P<0.05, ns= non significant, Mot. = Motility, HOS = swollen spermatozoa.

Table (5) Association of 812 T>C SNP in *HSP70* promotor gene with some fresh and frozen semen traits of buffalo bull during winter and summer seasons

Semen Traits \ Genotype			TT (n=13)		TC (n=3)		(I)
			Winter	Summer	Winter	Summer	
Fresh semen		Mot. (%)	80.76 ± 2.03^{b}	65.38 ± 1.83^{c}	90 ± 2.94^{a}	53.33 ± 3.33^{d}	**
		acrosome(%)	91.16 ± 0.32^{a}	89.66 ± 0.72^{a}	90.33 ± 1.45^{a}	90.23 ± 1.45^{a}	ns
		(HOS +ve %)	83.30 ± 0.66^{a}	78.15 ±1.36^{b}	82 ± 1.52^{a,b}	79 ± 4.91^{a,b}	ns
Post-thaw semen	Motility	0 h	70.76 ± 2.03^{b}	58.46 ±1.54^{c}	80 ± 2.9^{a}	56.66 ± 3.33^{c}	ns
		1 h	59.23±1.77^{b}	53.07±1.33^{c}	66.6±3.33^{a}	38.33 ± 1.66^{d}	***
		2h	48.46 ± 1.91^{b}	37.69 ± 1.66^{c}	60 ± 0^{a}	33.33 ± 3.29^{c}	**
		3h	37.69 ± 2.01^{a}	31.15 ± 1.97^{b}	45 ± 2.89^{a}	23.33 ± 3.33^{b}	*
	Viability Index		177.62 ± 5.06^{a}	148.46 ± 5.56^{b}	189.16 ± 10.84^{a}	118.33 ± 6.01^{c}	*
	acrosome (%)		91.23 ± 0.34^{a}	87.69 ± 0.82^{a}	90.33 ± 1.45^{a}	87.33 ± 2.89^{a}	ns
	(Hos +ve %)		65.30 ± 0.66^{a}	60.15 ± 1.36^{b}	64 ± 1.52^{a,b}	61 ± 4.93^{a,b}	ns

Mean ± SE with different superscripts in the same raw were significantly (P<0.05) different. (I) = the interaction between (Genotype & seasons), *** significant at P<0.001, ** P<0.01, * P<0.05, ns= non significant, Mot. = Motility, HOS = swollen spermatozoa

Table (6): Enzymatic, hormonal and lipid profile in fresh seminal plasma of bulls and buffalo bulls during winter and summer seasons

Animal	Bulls		Buffalo bulls	
Season	Winter	Summer	Winter	Summer
Cholesterol (mg/dL)	93.85 ± 4.30^{a}	78.03 ± 1.54^{b}	118.89 ± 6.11^{a}	96.73 ± 3.42^{b}
Phospholipids (mg/dL)	195.54 ± 1.97^{a}	112.52 ± 1.33^{b}	204.37 ± 3.85^{a}	157.37 ± 3.03^{b}
Chol./Phosphol. ratio	0.58 ± 0.03^{b}	0.69 ± 0.01^{a}	0.58 ± 0.25^{b}	0.61 ± 0.01^{a}
Triglycerides (mg/dL)	63.45 ± 4.44^{a}	54.80 ± 2.96^{b}	58.32 ± 0.78^{a}	50.79 ± 0.81^{b}
AST (U/L)	16.22 ± 0.68^{b}	18.61 ± 0.96^{a}	19.70 ± 3.29^{a}	18.15 ± 7.27^{a}
ALT (U/L)	13.91 ± 1.01^{b}	17.15 ± 0.86^{b}	13.28 ± 1.67^{b}	16.25 ±1.39^{a}
ALP (U/L)	45.56 ± 1.89^{b}	58. 22 ± 1.36^{a}	41.10 ± 1.46^{b}	46.53 ± 4.89^{a}
Total antioxidants (mMol/ml)	0.52 ± 0.02^{a}	0.22 ± 0.01^{b}	0.61 ± 0.0.02^{a}	0.24 ± 0.03^{b}
T_3 (ng/ml)	0.44 ± 0.01^{a}	0.23 ± 0.08^{b}	0.52 ± 0.01^{a}	0.30 ± 0.14^{b}
Fructose (mg/dL)	230.02 ± 2.70^{a}	126.24 ± 2.45^{b}	238.90 ± 5.78^{a}	111.00 ± 4.94^{b}

Means with different superscripts (a ,b) within row are significantly different at P < 0.05.

Data displayed in **table (7)** revealed significant difference (P<0.05) in term of AST between bovine bulls carrying TT genotype and those carrying TC genotype where C allele appeared better than T allele in summer season. Concerning to buffalo bulls there were significant difference (P<0.05) in terms of T_3 and cholesterol/phospholipid ratio between buffalo bulls carrying TT genotype and those carrying TC genotype in summer and winter seasons as TC genotype seem to be more favorable genotype for hormonal T_3 level even in summer season. Regarding the interaction between genotype and season there was significant interaction (P<0.05) in terms of T_3 and cholesterol/phospholipid ratio.

Table (7) Association of SNP of *HSP70* promotor gene with enzymatic, hormonal and lipid profile of bulls and buffalo bulls during winter and summer seasons

Animal	Cattle Bull				Buffalo Bull			
Genotype / Enzymatic & lipid profile	TT (n=13)		TC (n=8)		TT (n = 13)		TC (n=3)	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
Cholesterol (mg/dL)	96.15 ± 3.02^{a}	77.18 ± 1.04^{c}	92.13 ± 1.53^{a,b}	83.01 ± 4.72^{b,c}	117.84 ± 2.15^{a}	96.24 ± 1.38^{b}	121.55 ± 7.4^{a}	97.5 ± 3.51^{b}
Phospholipid (mg/dL)	159.48 ± 0.68^{a}	112.81 ± 0.64^{b}	159.34 ± 1.5^{a}	112.15 ± 1.01^{b}	205.96 ± 1.61^{a}	157.39 ± 1.61^{b}	200.4 ± 0.49^{a}	157.31 ± 1.24^{b}
Triglyceride (mg/dL)	60.76 ± 2.9^{a,b}	55.37 ± 2.25^{b}	65.48 ± 1.6^{a}	54.38 ± 1.31^{b}	58.15 ± 0.39^{a}	50.53 ± 0.40^{b}	58.80 ± 0.30^{a}	50.76 ± 0.54^{b}
AST (U/L)	16.23 ± 0.36^{c}	19.36 ± 0.31^{a}	16.21 ± 0.4^{c}	18.05 ± 0.20^{b}	20.43 ± 1.64^{a}	20.14 ± 0.54^{a}	17.84 ± 0.92^{a}	22.55 ± 0.95^{a}
ALT (U/L)	13.30 ± 0.28^{b}	17.33 ± 0.63^{a}	14.37 ± 0.55^{b}	17.01 ± 0.35^{a}	13.83 ± 0.57^{b}	16.56 ± 0.50^{a}	11.89 ± 0.85^{b}	15.46 ± 1.55^{a,b}
ALP (U/L)	45.16 ± 0.60^{b}	57.99 ± 0.87^{a}	45.86 ± 1.24^{b}	58.65 ± 0.6^{a}	40.81 ± 0.32^{a}	46.63 ± 2.5^{a}	41.94 ± 2.07^{a}	46.29 ± 2.7^{a}
Total antioxidants (mMol/ml)	0.51 ± 0.00^{a}	0.21 ± 0.01^{b}	53. ± 0.03^{a}	0.23 ± 0.01^{b}	0.61 ± 0.01^{a}	0.24 ± 0.02^{b}	0.62 ± 0.0^{a}	0.24 ± 0.01^{b}
T_3 (ng/ml)	0.45 ± 0.00^{a}	0.24 ± 0.05^{b}	0.43 ± 0.02^{a}	0.22 ± 0.35^{b}	0.51 ± 0.01^{a}	0.22 ± 0.04^{b}	0.52 ± 0.01^{a}	0.42 ± 0.0^{a}
Fructose (mg/dL)	231.27 ± 0.73^{a}	125.30 ± 0.05^{b}	229.29 ± 1.68^{a}	126.94 ± 1.61^{b}	241.05 ± 2.2^{a}	126.84 ± 1.56^{b}	233.57 ± 3.49^{a}	125.54 ± 3.45^{b}
Chol/Phospol. %	0.58 ± 0.01^{b}	0.68 ± 0.01^{a}	0.58 ± 0.01^{b}	0.69 ± 0.01^{a}	0.57 ± 0.01^{b}	0.61 ± 0.01^{a}	0.61 ± 0.01^{a}	0.62 ± 0.0^{a}

Mean ± SE with different superscripts in the same raw were significantly (P<0.05) different.

As shown from **table (8)** there were strong positive correlation (P<0.01) between cholesterol, phospholipids, triglycerides, total antioxidants and T_3 seminal plasma levels, in one sides and individual motility of bulls ("r" ranged from 0.630 to 0.790) and buffalo bulls spermatozoa ("r" ranged from 0.560 to 0.850). On the other hand there was negative correlation between cholesterol/phospholipids ratio AST, ALT and ALP in one side and individual motility of bull (r ranged from -0.690 to -0.822) and buffalo bull spermatozoa ("r" ranged from 0.450 to 0.790). The percentage of buffalo bulls spermatozoa with intact cell membrane correlates positively with cholesterol, phospholipids, total anti-oxidants, T_3 , fructose and triglycerides ("r" ranged from 0.430 to 0.620) and negatively with cholesterol/phospholipids ratio, ALT and ALP ("r"range from -0.620 to -0.760). On the other hand, there was no such correlation in case of bulls. The cholesterol/phospholipids ratio correlates negatively with the percentage of spermatozoa with intact acrosomes of bulls ("r" = -0.740) and buffalo bulls ("r"= -0.520).

As presented in **table (9)**, the post-thaw bulls and buffalo bulls sperm motility at 0,1,2 and 3h as well as viability indexes correlated positively with cholesterol, phospholipids, triglycerides, total antioxidants and T_3 ("r" rang from0.350 to 0.850) and negatively with cholesterol/phospholipids ratio, AST, ALT and ALP ("r" range from -0.420 to -0.820). Fructose levels in seminal plasma were correlated well ("r" range from0.700 to 0.800) with post-thaw in 0h, 1h, and 2h,3h motility and viability index of buffalo bull spermatozoa. Such significant correlations were not found in case of bulls.

Concerning the percentage of spermatozoa with intact cell membranes in buffalo bulls, it has been found to correlate positively with cholesterol, phospholipids, triglycerides, total antioxidants, T_3 and fructose ("r"= 0.430 to 0.620) and negatively with cholesterol/phospholipid ratio, ALT and ALP ('r" range from -0.620 to -0.760) such significant correlations were not found in case of bulls.

Table (8): Correlation coefficients between fresh semen characteristics and enzymatic, hormonal and lipids profile of the seminal plasma of bulls

Parameters	Individual motility		Swollen spermatozoa (HOS +ve %)		Normal acrosome (%)	
Animal	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls
Cholesterol	0.775**	0.850**	0.025	0.430*	0.650**	0.090
Phospholipids	0.790**	0.640**	0.100	0.550**	0.730**	0.240
Chol./Phosph. ratio	-0.690**	-0.530**	-0.200	-0.620**	-0.740**	-0.520**
Triglycerides	0.756**	0.730**	0.280	0.620**	0.720**	0.260
AST	-0.822**	-0.450**	0.180	0.30	-0.580**	-0.390*
ALT	-0.707**	-0.490**	0.110	-0.760**	-0.790**	-0.400*
ALP	-0.741**	-0.790**	-0.077	-0.660**	-0.683**	0.110
Total antioxidants	0.760**	0.700**	0.080	0.590**	0.740**	0.350*
T3	0.630**	0.560**	0.240	0.500**	0.690**	0.490**
Fructose	0.120	0.760**	0.270	0.460**	0.280	0.260

*** P < 0.05 ** P < 0.01**

4. DISCUSSION:

Seasonal factors can impact semen production resulting in decreased semen quality due to variations of semen characteristics (Mathevon *et al.,* 1998).

In the present study results of sequencing and PCR-SSCP detected a new synonymous SNP T>C transition at nucleotide 812 was the same in both species and could clear two genotypes TT and TC genotype. Observably T allele was of higher frequency in both cattle and buffalo population. Interestingly we did not found CC genotype that should be existed in ideal but this is may be due to the need of larger scale population. The same observation was reported previously by (Jian-bo *et al.,* 2009).

Although the detected 812 T>C SNP was synonymous it demonstrated significant association with quality traits of fresh and post-thaw semen in cattle bull population this results be in agreement with the previous results of (Nikbin *et al.*,2014) for caprine population. Many authors have been reported that synonymous SNPs may affect the relevant protein via change in transcription and may also influence the accuracy or efficiency of splicing of mRNA or transcriptional control (Cartegni *et al.,* 2002 ; Komar, 2007).

Regarding to association analysis with the fresh semen quality traits the SNP showed significant association ($P<0.05$) with individual motility traits in both cattle and buffalo population. Similarly, Shrum *et al.,* (2010) reported associations between a SNP in *HSP70* promoter and sperm motility and velocity traits in bulls. This may be due to influence of *HSP70* on protection of proteins related to respiration activity and level of energy in spermatozoa. (Yeung *et al.,* 1996; Nascimento *et al.,* 2008).

The differences in the effects of the SNP on motility traits in fresh and frozen semen in present study may be due to the role of the *HSP70* on semen quality after ejaculation and during storage, as reported by Elliott *et al.* (2009). It was found that adding recombinant *HSP70* to semen increased longevity and viability of spermatozoa during cooling and after freezing (Lloyd *et al.,* 2012) when the processes of cool and freeze thawing cause physical and chemical stresses on sperms which can decrease semen quality and its fertility capacity (Stradaioli *et al.,* 2007; Dorado *et al.,* 2010).

In current study viability indexes were significantly higher in both bulls and buffalo bulls carrying TT genotype than those carrying TC genotype. Viability of sperm can be varied due to DNA integrity of spermatozoa and the factors which may prevent the cells from oxidation and other stresses (Aitken *et al.,* 2014). Similarly, Huang *et al.,* (2002) reported anassociation between total sperm number and SNP of 5-flanking region of *HSP70* gene in boar. The high significance ($P<0.001$) with fresh and some of frozen-thawed semen traits in cattle population demonstrate T allele to be the favorable allele for motility, sperm membrane integrity, normal acrosome and viability index.

By study the biochemical and hormonal parameter C allele seemed better than T allele in summer season as the high concentration of transaminase enzyme e.g. AST in seminal plasma indicate increasing percentage of abnormal spermatozoa in ejaculate occurring due to sperm membrane damage leading to leakage of enzymes from spermatozoa (Gundogan, 2006).

Concerning the buffalo population C allele appeared to conserve the T_3 hormone level even in summer season and due to the major role of thyroid hormones in regulation of overall heat production in control of endothermic thermogenesis (Hagen, 1983). C allele seemed to be the favorable allele for anti-heat stress bull with good quality semen.

The differences in semen characteristics and seminal plasma composition among seasons have been examined (Murase *et al.,* 2007). Seminal and biochemical parameters are significantly influenced by seasons (Mathevon *et al.,* 1998).

Exposure to heat stress under the sub-tropical winter and summer conditions of Egypt was accompanied with significant increase in rectal, skin and scrotal-skin temperatures, so dead spermatozoa, abnormalities, acrosomal damage and plasma creatinine and cortisol levels, and significant decrease in sperm motility, alkaline phosphatase, lactate dehydogenase, and T3 hormone levels Marai *et al.,* (2009).

The present findings showed significant increase value of total cholesterol, phospholipids and triglycerides in winter season as in (table 6). The highest cholesterol concentration reported in winter season corroborated with the study of (Soverni et al., 1992) in bulls and (Zamiri *et al.,* 2010) in ram but disagree with (Farooq *et al.,* 2013) and (Pandey *et al.,* 2014) reported lower values.

Table (9): Correlation coefficients between frozen-thawed semen characteristics and enzymatic, hormonal and lipids profile of the seminal plasma of bulls

Parameters	Post-thaw motility 0h		Motility after 1 hr.		Motility after 2 hr.		Motility after 3 hr.		Viability index		Swollen spermatozoa (HOS +ve %)		Normal acrosome (%)	
Animal	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls	Bulls	Buffalo bulls
Cholesterol	0.780**	0.580**	0.850**	0.570**	0.730**	0.570**	0.840**	0.490**	0.840**	0.570**	0.250	0.430*	0.650**	0.090
Phospholipids	0.790**	0.650**	0.820**	0.660**	0.650**	0.680**	0.810**	0.490**	0.800**	0.650**	0.100	0.550**	0.740**	0.240
Chol./Phosph. ratio	-0.690**	-0.530**	-0.680**	-0.580**	-0.490**	-0.620**	-0.690**	-0.280	-0.660**	-0.530**	-0.200	-0.620**	-0.740**	-0.520**
Triglycerides	0.580**	0.740**	0.500**	0.720**	0.350**	0.730**	0.520**	0.500**	0.500**	0.690**	0.290	0.620**	0.720**	0.260
AST	-0.820**	-0.450**	-0.830**	-0.490**	-0.680**	-0.320**	-0.770**	0.420*	-0.810**	-0.440*	0.180	-0.030	-0.580**	-.390*
ALT	-0.710**	-0.450**	-0.770**	-0.460**	-0.690**	-0.440**	-0.820**	-0.290	-0.790**	-0.430*	0.120	-0.760**	-0.790**	-0.400*
ALP	-0.740**	-0.790**	-0.790**	-0.830**	-0.670**	-0.740**	-0.820**	-0.720**	-0.790**	-0.800**	-0.077	-0.660**	-0.680**	0.110
Total antioxidants	0.760**	0.700**	0.790**	-0.690**	0.670**	0.730**	0.840**	0.510**	0.810**	0.690**	0.080	0.590**	0.740**	0.350*
T3	0.630**	0.570**	0.670**	0.560**	0.500**	0.640**	0.700**	0.230	0.650**	0.520**	0.240	0.500**	0.690**	0.490**
Fructose	0.120	0.760**	0.100	0.770**	0.180	0.800**	0.300*	0.700**	0.190	0.780**	0.270	0.460**	0.280**	0.270

***P<0.05 **P<0.01**

Kelso *et al.,* (1997) reported that the reduction in sperm motility were associated with a decrease in seminal plasma content of lipids. The higher levels of cholesterol and triglycerides during winter may be due to increased thyroid activity (Shukla *et al.,* 2009). In addition to this, Cholesterol is secreted to seminal plasma by the prostate gland and it protects sperm cells against environmental shock (Sofikitis and Miyagawa 1991).

By study the metabolic enzyme the activity of ALT, AST and ALP were found significantly elevated ($p<0.05$) during summer season compared to winter season. Similar kind of seasonal effects were noticed by (Dhami and Kodagali 1988, Juma 2000 ; Pandey *et al.* 2014) in cattle. The concentration of transaminase enzymes (AST, ALT and ALP) in semen is a good indicator of semen quality because it measures sperm membrane stability and acrosomal damage (Corteel 1980).

The present study showed significant increase in levels of alkaline phosphatase in summer season may be due to environmental stress. The higher levels of ALP in summer season were also reported by Pandey *et al.* (2014) in cattle, and Juma and Kassab (2009) in rams. The estimation of ALP activities in seminal plasma reflects the functional state of accessory sex glands, metabolic activity of spermatozoa, and sperm membrane integrity that are helpful in differentiating the reproductive biology of bulls of different breeds/ species (Ibrahim *et al.,* 1985).

The high SP antioxidant activity correlated positively with semen quality and low levels of both DNA damage and lipid peroxidation (Alvarez and Storey 1989).

The increase of T_3 level during winter season depicted in current study may be due to increase in oxygen consumption and heat production by cells (Todini, 2007 and Ocak *et al.,* 2009). High ambient temperature was found to decrease T3 activity (Table 6) and this effect is probably initiated at the hypothalamic level (Yousef and Johnson, 1985; Marai *et al.,* 2000). Thyroid hormones as affected by seasons of the year are shown in (table 6). It has been shown that the peak of plasma T3 levels coincided with the rise of testosterone concentrations in domestic ganders Zeman (1990). The study of Guerrini and Bartchinger (1983) clarified that the decrease in T3 level was correlated with the increase in rectal temperature, under hot climate conditions.

The current study demonstrate mean initial seminal fructose content of bulls observed was highly significantly ($P<0.05$) between seasons. The mean value was the highest during winter. The concentration of fructose obtained is in agreement with the reports of Dhami *et al.* (1987). The seminal fructose gives useful indication of the fertilizing ability of bulls. Low value of seminal fructose in summer might be due to a decrease in glucose utilization in order to preserve energy during their stressed condition (Habeeb *et al.,* 1997). Nevertheless, other researchers have demonstrated that decreases in energy metabolism during heat exposure correlated with decreases in plasma insulin and thyroxin concentrations (Habeeb *et al.,* 1996).

The study clarify high significant effect of seasons on semen traits, biochemical parameter and hormonal profile in seminal plasma especially with buffalo population possibly due to buffalo population have different skin characteristics and fewer sweat glands than cattle and are therefore highly susceptible to high heat load which affects productive and reproductive performances (Sodhi *et al.,* 2013).

Generally, elevation of ambient temperature affects male reproductive functions deleteriously. Such phenomenon leads to testicular degeneration and reduces percentages of normal and fertile spermatozoa in the ejaculate of males.

5. Conclusion:

Polymorphisms in HSP70 gene can affect semen quality and suggest to be used in artificial insemination programs as marker assisted selection for anti-heat stress and good reproductive bulls. Biochemical and hormonal profile can be used as good indicator for semen quality. High ambient temperature has negative impact on biochemical and hormonal parameter consequently semen quality. Further investigations will be required on large scale population to evaluate genotype by environment interactions.

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