RESEARCH ARTICLE

Testosterone levels and the genetic variation of sex hormone-binding globulin gene of *Bubalus bubalis* bulls in Egypt

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Abstract

Testosterone is the main plasma androgen produced and secreted by male testis. To be bioactive, it binds to sex hormone-binging globulin (SHBG) that is the major transporter protein of sex steroids in the blood of mammals and other vertebrate species. This study aimed first to determine the levels of testosterone in different-age groups of buffalo bulls (*Bubalus bubalis*) in Egypt, and second to screen the genetic polymorphisms in their SHBG gene and investigate whether these polymorphisms are associated with the level of the circulating serum testosterone. Blood samples were collected from 67 Egyptian buffalo bulls representing 4 different age-groups, and testosterone concentration was measured by radioimmunoassay. Selected DNA samples from different age-groups were sequenced for the full length of their SHBG gene. A significant increase in the concentration of serum testosterone in buffalo bulls was recorded with the advancement of age from 12/18 to 24 to 36 months of age. Based on the number of polymorphisms in SHBG gene, the current study reveals the presence of 3 bull genotypes, one of which is likely to be associated with low testosterone concentration, while another is likely be associated with an increased testosterone concentration. Such genetic associations can provide a good tool for the selection of bull genotypes with higher testosterone concentrations which are indispensible for breeding purposes.

Keywords. Testosterone; *Bubalus*; Radioimmunoassay; sex hormone-binding globulin; Gene; Mutations.

Introduction

Human male testosterone is the major circulating androgen and more than 95% of it is secreted by the testis which produces approximately 6-7 mg daily (Coffey 1988). Testosterone is secreted in most vertebrates and it circulates in the blood bound mainly to plasma proteins with only 1-2% being free, 40–50% being loosely bound to albumin and 50–60% being specifically and strongly bound to sex hormone-binding globulin (SHBG) (Dunn *et al.* 1981). SHBG is a plasma glycoprotein synthesized and secreted by the liver (Siiteri *et al.* 1982; Westphal

1986). Plasma SHBG is expressed in the testes of most mammals and is known as the testicular ABP (androgen-binding protein) which is synthesized and excreted by Sertoli cells (Joseph 1994). Plasma SHBG is a dimer of two essentially identical monomers and it binds only to one steroid molecule (Petra *et al.* 1986; Turner *et al.* 1984). SHBG is considered as the primary plasma transport protein for sex hormones (Westphal 1986) and it binds to its ligands 5α-dihydrotestosterone and testosterone with high affinity (Becchis *et al.* 1996). Plasma SHBG not only transports the steroids but also regulates the access of sex steroids to their target tissues (Siiteri *et al.* 1982; Westphal 1986).

A number of studies have been directed to the sequence and cloning of ABP/SHBG gene (*abp*/*shbg*) of many different species (Joseph *et al.* 1988; Hammond *et al.* 1989; Gershagen *et al.* 1989; Ip *et al.* 2000). Sequence analysis of *shbg* showed that Rabbit *shbg* spans 2.5 kb and the coding region is distributed over eight exons (Ip *et al.* 2000), whereas the same eight exons in the human gene span 3.2 kb (Hammond *et al.* 1989). The transcription unit encoding zebrafish *shbg* spans 12,945 bp and it contains eight exons of 81–189 bp intervened by intron sequences of 78–5,108bp in length (Miguel-Queralt *et al.* 2004). In rainbow trout, there are 2 SHBG genes, *shbga* and *shbgb*, the main significant distinction between them is the length of introns, which are bigger in *shbgb* compared to other SHBG genes (Marivin *et al.* 2014). Like rainbow trout *shbga*, rainbow trout *shbgb* comprises eight exons, but is almost twice the length of *shbga* (Marivin *et al.* 2014).

Some studies have shown that the serum level of human testosterone is controlled by genetic factors (Ahn *et al.* 2009; Ohlsson *et al.* 2011; Sung and Song 2016). Up to our knowledge no studies have been directed to the genetic variation in SHBG gene in *Bubalus bubalis*.

This study aimed first to determine the concentration of testosterone in different-age groups of buffalo bulls (*Bubalus bubalis*) in Egypt, and second to sequence the SHBG gene of these buffalo bulls and determine whether the mutations, if any, had an impact on the testosterone levels in the buffalo's blood.

Materials and methods

Animals and blood sampling

This study was carried out on 67 adult buffalo bulls of the species *Bubalus bubalis* from different farms in Damietta and Kafr El-Sheikh governorates. Animals were subdivided into four age groups; 12 months, 18 months, 24 months and 36 months.

Peripheral blood samples were collected in both EDTA-rinsed vacutainer tubes and tubes without anticoagulant by venipuncture from the jugular vein during the period from October 2013 to March 2014. Blood samples were collected two times at 2 weeks intervals between 08:00 and 11:00 am from all the animals. After 1 hour-incubation at room temperature, samples were centrifuged at 2500×*g* for 10 minutes, and the sera were collected, immediately frozen and kept at -20 ^{o}C until testosterone assay by radioimmunoassay.

Radioimmunoassay

The concentration of plasma testosterone in each blood sample was determined by radioimmunoassay kit (Direct REF IM 1119, Immunotech, Czech Republic) according to the manufacturer's instructions. Samples or

calibrators were incubated with ^{125}l-labeled testosterone in antibody-coated tubes. The liquid content of tubes was then aspirated and the bound radioactivity was determined in a gamma counter. A standard curve was prepared, and the unknown values were obtained from the curve by interpolation.

Polymerase chain reaction (PCR) and DNA sequencing

Ten samples representing the four buffalo groups were selected based on the concentration of plasma SHBG and their DNA was extracted using by Gene JET™ Genomic DNA Purification Kit (#K0722, Thermo Scientific) according to the manufacturer's instructions. Extracted DNA was subjected to PCR to amplify SHBG gene using the oligonucleotides listed in table 1. Oligonucleotides were designed based on *Bubalus bubalis* breed Mediterranean unplaced genomic scaffold, UMD_CASPUR_WB_2.0 scf7180021617181, whole genome shotgun sequence (NCBI ref. NW_005785061). All PCR reactions contained 20µl of Maxima Hot Start PCR master mix kit (#K1051, Thermo Scientific), 3.2µl of the forward and reverse primers and 3.84µl of each DNA sample. Reactions were brought to a final volume of 40µl with nuclease-free water and cycled for 4 min at 95 °C for initial enzyme activation followed by 45 cycles (30 sec at 95 ºC for denaturation, 30 sec at the optimal annealing temperature and 1-2 min at 72 ºC for extension) and 10 min at 72 °C for final extension. After visualization, PCR products were purified and sequenced using the oligonucleotides listed in table1in Macrogen sequencing services (Macrogen, Seol, South Korea).

Statistical analysis

All data were processed by STATISTICA software version 10. The differences between testosterone concentrations levels in the different age groups were analyzed by one way AVONA test and the mean values were considered significantly different when $P \leq 0.05$. P-values were adjusted by Bonferroni correction. Chi square test was used to assess the Hardy–Weinberg equilibrium.

Results

Serum testosterone concentration

Measurements of serum testosterone in the Egyptian buffalo bulls (Fig. 1) showed no statistically significant differences (P=1.0) in testosterone concentration between the 12 months-age and 18 months-age buffalo bulls. Although the 24 months-age buffalo bulls showed slightly higher serum testosterone concentration, there was no statistically significant difference (P=1.0) between the 12 months-age and 24 months-age buffalo bulls. Nevertheless, the concentration of serum testosterone differed significantly (P=0.000) between the 12 months-age and 36 months-age buffalo bulls (Fig. 1).

Although the 24 months-age buffalo bulls showed slightly higher serum testosterone concentration, there was no statistically significant difference (P=0.857) between the 18 months-age and 24 months-age buffalo bulls. The concentration of testosterone, however, was significantly high in the 36 months-age buffalo bulls (P=0.001) compared to the 18 months-age group. The concentration of serum testosterone was significantly higher (P=0.007) in the 36 months-age bulls compared to the 24 months-age bulls (Fig. 1).

Overall, there was a significant increase in the concentration of serum testosterone in buffalo bulls with the increase of age from 12/18 to 24 to 36 months of age.

Genetic variation of SHBG gene

DNA samples from10 Egyptian buffalo bulls were sequenced for the full length of their SHBG gene and compared to the predicted *Bubalus bubalis* SHBG gene sequences within the genomic scaffold NW_005785061. As shown in the nucleotide variance report (table 2), from 4 to11 polymorphic sites have been recorded in these 10 DNA samples (table 2). Based on variance of these samples, they can be categorized into 3 genotypes. One genotype contains 4 polymorphic sites; represented by bulls K4, K11, K32, K37 and K47. The second genotype contains 9 polymorphic sites; represented by bulls D18 and D27. The third genotype contains 11 polymorphic sites; represented by bulls K3, K6 and K43. The sequences of these genotypes were deposited in the gene bank under the accession numbers KY653957 (genotype 1), KY653958 (genotype 2) and KY653959 (genotype 3). Based on the predicted transcript variant X1 (NSCBI ref. XM_006062863) of SHBG gene, the recorded polymorphisms result in 2 amino acid substitutions (S315L and R337G) in genotype 1, 2 amino acid substitutions (S315L/S and R337G/R) in genotype 2, and 3 amino acid substitutions (P297S/P, S315L and R337G) in genotype 3.

Compared to the mean of testosterone concentration in the other buffalo bulls within the same age group (table 3), the first genotype (bulls K4, K11, K32, K37 and K47) does not seem to be associated with a distinctive testosterone level. The second genotype (bulls D18 and D27) is likely to be associated with a decreased concentration of testosterone; however, the third genotype is likely to be associated with an increased testosterone concentration. An attempt to assess whether any of the 12 polymorphisms within the second genotype or the third genotype deviates from Hardy-Weinberg equilibrium revealed that each individual polymorphism is still with equilibrium (p=0.514 - 0.659).

Discussion

In this study, a comparatively low concentration of serum testosterone was recorded in 12months-age and 18months-age buffalo bulls. A significant increase in testosterone concentration was recorded with the advancement of age to 24-36-months of age. These results are in accordance with some previous reports. According to Hemeida *et al.* (1985), serum testosterone of Egyptian buffalo bulls started to increase in 8-9 months of age and at 13-15 months of age and went into a sharp increase in 17-19 months of age. In Pakistani Nili-Ravi buffalo bulls, testosterone levels were moderately low in 5-21 months of age then went into a significant rise from 21 to 26 months (Ahmad *et al.* 1984). Testosterone levels at 25 and 38 months of age were significantly different in relation to each other and were significantly different from the testosterone levels at 5-21 months of age (Ahmad *et al.* 1984). According to the same research group, there were no differences in mean plasma testosterone values measured in Nili-Ravi buffalo bulls at 5, 15, 17 and 21 months of age. According to Ahmad *et al.* (1989), plasma testosterone concentrations of Nili-Ravi buffalo bulls remained low, but variable between neonates to 12 months of age and markedly rose at 14 months of age, followed by a second peak at 18 months. Levels of plasma testosterone of Indian buffalo males were low and in the same range as in the 15 days to 6 months of age but could be estimated in almost all blood samples up to 15 months of age (Sharma *et al.* 1984). Testosterone concentration started rising at 15-18 months of age, followed by a remarkable increase at 24-30 months of age and the elevated concentration of testosterone was kept up to 42 months followed by sharp increments up to 48 months of age (Sharma *et al.* 1984). Testosterone concentrations in Murrah buffalo males in

India showed low levels in ages from 1 to 24 months, followed by an increase in testosterone levels at 3.5-4 years of age (Gulia *et al.* 2010).

The above studies support the correlation between testosterone concentration and the advancement of age as a result of the development and functional differentiation of the testis (Sharma *et al.* 1984; Gulia *et al.* 2010; He *et al.* 2014).The slight variations among these reports could be related to either the differences in the species under study or some environmental and behavioral factors.

Based on the nucleotide variance in the full length of SHBG gene reported here, the current study suggests the presence of 3genotypes in the studied bulls. NCBI database released 5 predicted transcript variants (X1-X5), the largest of which is the variant X1 (NSCBI ref. XM_006062863). Based on this transcript variant, the first genotype produces 2 amino acid substitutions (S315L and R337G) and, also, the second genotype produces 2 amino acid substitutions (S315L/S and R337G/R) while the third genotype produces 3 amino acid substitutions (P297S/P, S315L and R337G). The second genotype is likely to be associated with a decreased concentration of testosterone while the third genotype is likely to be associated with an increased testosterone concentration. None of the polymorphisms within the second or third genotype deviated from Hardy-Weinberg equilibrium. Keeping in mind the small sample size in this study, it is worthy to quote that agreement with Hardy-Weinberg proportions is not a guarantee that observed variation has a genetic basis, nor does it guarantee that other factors such as selection and drift are not influencing genotypic frequencies (Waple 2015). The Hardy-Weinberg equilibrium test generally has low power to detect actual departures unless sample sizes are large (Haiman et al. 2005; Waple 2015) and allele frequencies in small sample sizes could have no meaning (B-Rao, 2001).

So far there are no reports about the genetic variation in SHBG gene in *Bubalu sbubalis* bulls; however several studies on SHBG in human and other animals correlated circulating testosterone level to some polymorphisms in SHBG gene. The circulating human SHBG and sex steroid hormone levels were generally thought to be affected by genetic factors (Ahn *et al.* 2009; Ohlsson *et al.* 2011; Sung and Song 2016).

The SHBG polymorphism rs1799941, located 67 base pairs upstream from the transcription start site, predicted circulating testosterone levels and was associated with increasing testosterone levels in human males (Ahn *et al.* 2009). Ohlsson *et al.* (2011) have demonstrated that men with GG genotype for the polymorphism rs12150660, located 11.5 kb upstream of the transcription start site of the human SHBG gene, have lower serum testosterone concentrations than those with TT genotype. Serum human SHBG levels decreased significantly with increasing copies of the minor (T) allele for the polymorphism rs727428, located 1.1kb beyond the 3′end of the SHBG gene (Wickham *et al.* 2011).

Testosterone concentration has not only been associated with polymorphisms in the 5′ and 3′UTR but also with polymorphisms in the coding sequence. Men with the CT genotype for the polymorphism rs6258, located in exon 4 of human SHBG, had lower serum testosterone concentrations than those with CC genotype (Ohlsson *et al.* 2011). This single nucleotide polymorphism has been reported to result in the production of SHBG variant with reduced affinity for testosterone thus explaining the low serum testosterone concentrations and the reduced action at the target tissue cells (Ohlsson *et al.* 2011).

The polymorphism rs6259, located within exon8 of the human SHBG gene, results in the substitution of D327N in the SHBG polypeptide sequence (Power *et al.* 1992). Pang *et al.* (2014) reported that the rs6259 variant allele (AG or AA) carriers had increased serum levels of human SHBG and total testosterone than the wild-type allele (GG). An association between both the N327 allele and the $(TAAAA)_6$ allele and human SHBG and sex steroid serum concentrations has been reported (Vanbillemont *et al.* 2009). Middle-aged men carriers of the N327 allele were found to have higher SHBG and testosterone levels (Vanbillemont *et al.* 2009). Carriers of the six-repeat-allele were found to have higher SHBG, testosterone and free testosterone levels (Vanbillemont *et al.* 2009), however genotypes with long alleles ($(TAAAA)_n$ repeat) were thought to be associated with lower SHBG levels and higher free testosterone and 17 *β*-estradiol levels than those with shorter alleles and the variant N allele (Safarinjad *et al.* 2011). The increased production of human SHBG, caused by genetic variations in the SHBG gene, was thought to result in an increase in the levels of inactive, bound sex-steroids such as testosterone and a decrease in the concentration of active, unbound, or free sex-steroids such as free testosterone (Safarinjad *et al.* 2011). Hogeveen *et al.* (2001) found that a 46-kD nuclear factor preferentially binds this six-repeat allele and can alter transcriptional activity together with downstream elements of the SHBG promoter region.

SHBG levels increased with increasing copies of the minor (A) allele for the polymorphism rs1799941, located in exon 1L within the 5′ untranslated region of the gene (Wickham *et al.* 2011). The relationship between rs1799941 genotype and serum SHBG suggested a dominant role for this splice variant, in the control of circulating SHBG levels (Wickham *et al.* 2011).

Tsung-Sheng and Hammond (2014) found that the G195E substitution in human SHBG caused a general reduction in steroid-binding affinity. This mutant was secreted at very low levels and was thought to disturb the normal spatial configuration of the 2 LG domains with respect to each other and there by influencing both the steroid-binding activity and the processing of N-linked oligosaccharides attached to sites within the C-terminal LG domain (Tsung-Sheng and Hammond 2014). One other human SHBG mutant (T48I) was associated with a 2-foldreduction in steroid-binding affinity due to impairment in its dimerization, and a consequent more extensive glycosylation than the wild-type SHBG (Tsung-Sheng and Hammond 2014).

Tsung-Sheng and Hammond (2014) found that the rate of dihydrotestosterone association with the SHBGR123H mutant is normal, whereas its dissociation rate is abnormally fast, and therefore they concluded that amino acid substitutions at the dimer interface exert long-range allosteric effects that influence how specific ligands are accommodated within the steroid-binding pockets of each subunit.

Based on the above reports, the fluctuation in testosterone concentrations in Egyptian buffalo bulls in the current study may be due to a possible alteration in the binding affinity of SHBG for testosterone, or testosterone bioavailability or a change in SHBG transcription level. The present study provides an initial screening for the SHBG variants in the Egyptian buffalo bulls. Further studies on a larger number of samples are required to confirm the association between these mutations and the concentration of serum testosterone, which will provide a good tool for the selection of bull genotypes with higher testosterone concentrations of great importance for breeding purposes.

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Fig. 1: Plot box representing testosterone concentration (ng/ml) in buffalo bulls at 12, 18, 24 and 36 months of age

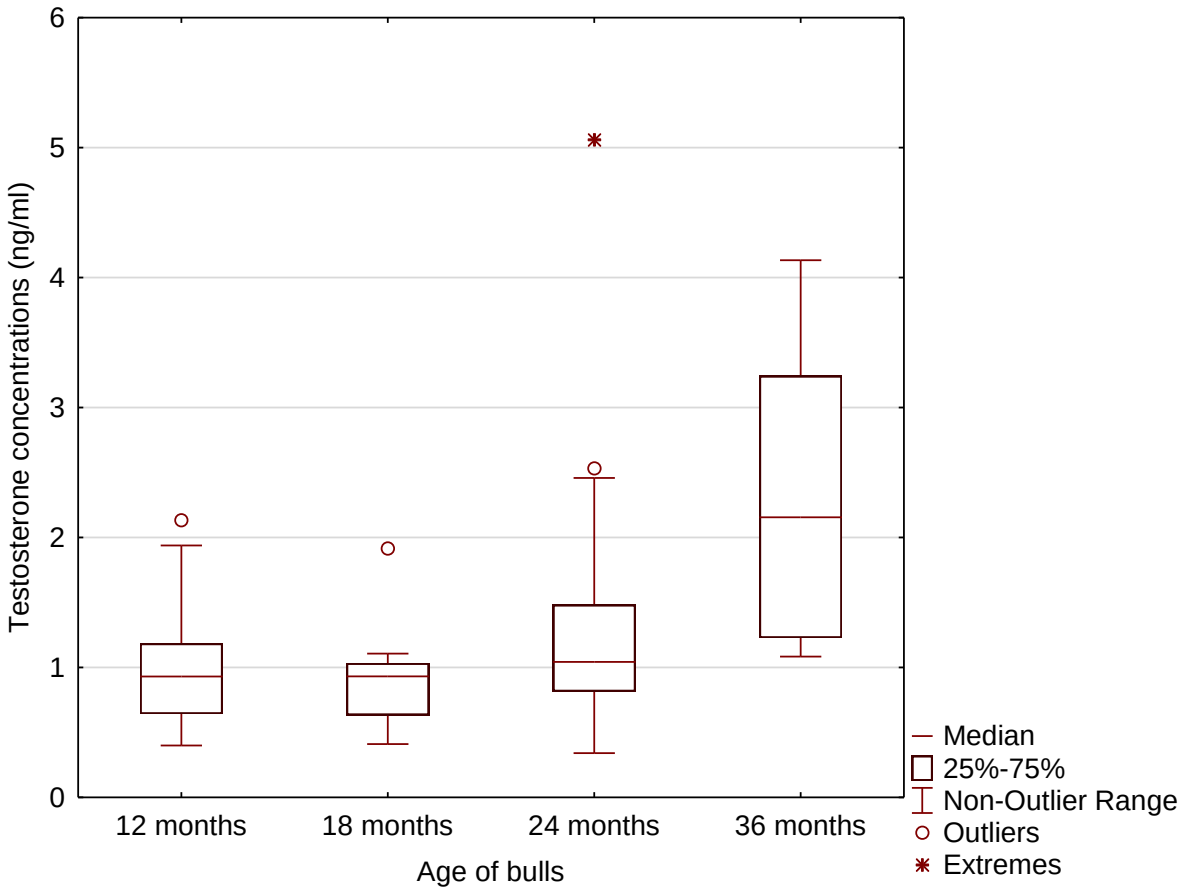

Table 1: List of oligonucleotides used in PCR and DNA sequencing

Name	Sequence (5′-3′)	Use
SH3_F	ACTTAAGCACACTGGTGGGG	Amplification, Sequence
SH4_F	AAGACTCTCCTGCTCTGCAC	Sequence
SH4_R	AGCCCCAGCATAAACCAGTC	Amplification, Sequence
SH5_F	GTCTATGCTCAAAGCGCACG	Amplification
SH8_F	GGGGAATAAACAGGGTGGGG	Amplification, Sequence
SH9_F	AGCCTAGGAGCAATGGGAGA	Sequence

SH10_F	ACCATGGGAAACGGCTAAGG	Sequence
SH10_R	AGGCCACCATCTCTCTGTGA	Amplification
SH11_R	CATTCCCACCACCTCAGTCC	Amplification, Sequence

Table 2: Nucleotide variance report of *shbg*

Reference		**Buffalo bulls**									
		K11	**K3**	**K32**	**K37**	**K4**	**K43**	**K47**	**K6**	**D18**	**D27**
36	**G**		R				R		R	R	R
2648	**C**		Y				Y		Y		
2720	**G**									R	R
2787	**A**		R				R		R	R	R
2844	**A**		R				R		R	R	R
2850	**A**		R				R		R		
3076	**C**		Y				Y		Y	Y	Y
3089	**C**	T	Y	T	T	T	Y	T	Y	Y	Y
3093	**G**	A	R	A	A	A	R	A	R	R	R
3154	**A**	G	G	G	G	G	G	G	G	R	R
3401	**A**		W				W		W		
3472	**A**	G	G	G	G	G	G	G	G	R	R
Total		4	11	4	4	4	11	4	11	9	9

R = A or G, Y = C or T, W = A or T, M = A or C

Table 3: The relation between testosterone concentration and the different genotypes of buffalo bulls

Bulls	**K3**	**K4**	**K6**	**K11**	**D18**	**K32**	**K37**	**D27**	**K43**	**K47**
Age	12 Mon.	12 Mon.	12 Mon.	12 Mon.	12 Mon.	24 Mon.	24 Mon.	24 Mon.	36 Mon.	36 Mon.
***Mean of testosterone concentrations (ng/ml)**	0.996	1.012	0.947	1.017	1.027	1.252	1.334	1.343	2.092	2.431
Testosterone concentrations (ng/ml)	1.001	0.702	1.938	0.609	0.415	2.532	0.555	0.341	4.134	1.083
Genotype	3	1	3	1	2	1	1	2	3	1

*Mean of testosterone concentration does not include the sample under comparison; for example when the sample K3 is compared, the mean of testosterone concentration refers to the mean of all samples except of K3.