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Effect of sexual excitation on testosterone and nitric oxide levels of water buffalo bulls (*Bubalus bubalis*) with different categories of sexual behavior and their correlation with each other

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ABSTRACT

We studied the effect of sexual excitation on serum testosterone and nitric oxide (NO) levels in water buffalo bulls with different categories of sexual behavior and their correlation with each other. Buffalo bulls were classified according to their sexual behavior (including reaction time, sexual aggressiveness and mating ability): acceptable (good to excellent) ($n = 5$), fair ($n = 5$), and unacceptable (poor) ($n = 5$) sexual behavior. Blood samples were collected from all animals immediately before and after sexual teasing and/or mounting to estimate the testosterone and NO levels using a commercial radioimmunoassay kit and Griess reaction test, respectively. Comparisons among groups were evaluated using a mixed-design analysis of variance. Pearson's correlation coefficients were calculated to determine the relationship between testosterone and NO levels before and after sexual excitation besides sexual behavior. The level of testosterone before sexual excitation was higher ($p \leq 0.05$) in bulls with acceptable and fair sexual behavior than in bulls with unacceptable sexual behavior (0.86 ± 0.01, 0.69 ± 0.02, and 0.29 ± 0.02 ng/mL, respectively). The level of NO was higher ($p \leq 0.05$) in bulls with acceptable and fair sexual behavior than in bulls with unacceptable sexual behavior (8.00 ± 0.03, 7.66 ± 0.19, and 6.29 ± 0.33 μM, respectively). Sexual excitation significantly ($p < 0.05$) increase testosterone and NO levels in bulls with acceptable (1.45 ± 0.01 ng/mL and 19.04 ± 0.32 μM, respectively) or fair (0.92 ± 0.02 ng/mL and 14.95 ± 0.34 μM, respectively) sexual behavior, but not in bulls with unacceptable sexual behavior. The unacceptable sexual behavior bulls had significantly lower testosterone and NO levels than the other bulls. There was a strong correlation and association between serum testosterone and NO levels besides sexual behavior of buffalo bulls. In conclusion, the alteration in the testosterone and NO levels after sexual excitation depends on the sexual behavior category of buffalo-bull. Testosterone and NO can be used to create a sexual behavior score. The testosterone and NO levels of can be predicted via evaluation of sexual behavior of buffalo bull.

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1. Introduction

Male sexual excitement (libido) is characterized by the rise of sexual desire in the brain and its transmission to the periphery, resulting in the penile tumescence necessary for sexual intercourse. Testosterone is a pivotal hormone in the regulation of male sexual function, acting at both a central and a peripheral level (Vignozzi et al., 2005). Improvement in sexual function has been observed in hypogonadal men with erectile dysfunction who received testosterone replacement therapy (Arver et al., 1996; Schultheiss et al., 2000). Testosterone therapy appears to have more significant effects on male libido than on erectile capacity (Schiavi et al., 1997). In contrast, in animal studies, testosterone has been shown to support erectile function by exerting a direct effect on the erectile tissue. Experimental gonadectomy results in an impaired erectile response, and testosterone replacement reverses this deficiency. At the cellular level, castration in rats causes penis apoptosis, whereas testosterone replacement results in new DNA synthesis (Shabsigh, 1997). These studies support a role for testosterone in the physiological processes leading to erection.

Nitric oxide (NO) is the principal agent responsible for the penile psychogenic erection (Burnett, 2006; Cartledge et al., 2001). Sensory information reaches the spinal cord via visual, auditory, tactile, and olfactory sensory afferents in addition to cortical fantasies. The nonadrenergic, noncholinergic nerves promote the release of NO from the nerve endings and the endothelium of the corpora cavernosa. NO stimulates the formation of cyclic guanosine monophosphate (cGMP) and subsequently phosphorylates the cellular membrane proteins, inducing an efflux of calcium, which leads to vasodilation of the penile arteries and the sinusoidal spaces, causing erection (Manecke and Mulhall, 1999).

A positive role of androgens on penile NO pathway has been suggested; testosterone probably promotes male sexual behavior by increasing the production of NO (Vignozzi et al., 2005; Zvara et al., 1995). Androgens may facilitate penile erection (Marin et al., 1999) by up-regulating constitutive nitric oxide synthase (NOS) isoenzymes in the corpora cavernosa of the penis (Lugg et al., 1995). Moreover, testosterone acts on the nervous system to mediate erection; when it is absent there may be down-regulation of both production and activity of NO, thereby decreasing the response to peripheral stimulation via the NO pathway (Baba et al., 2000).

Interestingly, NO exerts a paradoxical effect on testosterone secretion, stimulating it at low concentrations and inhibiting it at high concentrations. The stimulatory effect of NO is mediated by cGMP, the classic second messenger for NO action (Morelli et al., 2005; Valenti et al., 1999a). This effect was confirmed through in vitro studies by modulating the activity of Leydig cells (Valenti et al., 1999b). In addition, NO exerts pleiotropic effects on the central nervous system and contributes to aggressive behavior; however, testosterone alone is not sufficient to evoke persistent aggression behavior (Nelson et al., 2006).

In water buffalo bulls (*Bubalus bubalis*), the plasma testosterone concentration has a positive correlation with libido, however, libido is largely determined genetically rather than by testosterone concentrations (Gupta et al., 1984). NO concentrations have been previously measured in water buffalo cows (Zaher et al., 2016). To our knowledge, NO levels and its correlation and association with testosterone levels and sexual behavior have never been studied in buffalo bulls with different categories of sexual behavior. Therefore, the present study was undertaken to determine the effect of sexual excitation on serum testosterone and NO levels of buffalo bulls with different sexual behavior categories and their correlation with each other.

2. Material and methods

2.1. Animals

The investigation was conducted on 15 Egyptian buffalo bulls (aged 3–6 years) in El-Waha, Giza Governorate, Egypt, during the winter season (December–February). During the course of this study, the range of daily temperature, relative humidity and rainfall were 12–25 °C, 50–63%, and 0.5–14.45 mm/month, respectively. All animals were in good health and free from common infectious diseases, as determined by veterinarians. Internal and external reproductive examination of these buffalo bulls confirmed that they were free from any congenital or pathological reproductive diseases. The animals were kept in an open yard with shelter throughout the year. They were fed a properly formulated commercial ration (Crude Protein, min % 14.0; metabolizable energy min 10.5 MJ/kg, crude fat, min % 2.0; crude fiber, max % 15.0; calcium (Ca), min–max % 0.6–1.1; phosphorus (P), min % 0.4; Salt (NaCl), min–max % 0.5–1.0; potassium (K), min % 1.0; Vitamin A, min 2268 IU/kg and Vitamin D, min 1134 IU/lb) and berseem forage to meet daily energy and protein requirements according to NRC (1989). They were fed twice daily, at 08:00 and 16:00 h. The animals had free access to fresh water and vitamin/mineral block over the experimental period. The experimental protocol regarding the care and handling of buffalo bulls had been approved by the Ethics Committee of the Zagazig University, Egypt.

2.2. Sexual behavior evaluation and categorization

Buffalo bull was classified into three groups: acceptable (good – excellent), fair, or unacceptable (poor) ($n = 5$ for each group), on the basis of reaction time (in seconds), sexual aggressiveness and mating ability according to Anzar et al. (1993) with some modification to be appropriate for Egyptian buffalo breed (Table 1). Buffalo cows in heat were used for sexual teasing and evaluation of libido. Reaction time was the amount of time taken by a buffalo bull from exposure to the teaser until mounting was achieved. Sexual aggressiveness was assessed visually by evaluating the behavior of a buffalo bull during his approach to the teaser (sore: 1–4). Mating ability was included the different behavioral events displayed by buffalo bulls during mating and ejaculation (sore: 0–10).

Table 1
Categorization of sexual behavior of buffalo bulls based on reaction time, sexual aggressiveness and mating ability.

Sexual aggressiveness	Reaction time (seconds)	Mating Ability	Category of sexual behavior
Eager to mount and approached teaser with vigor and aggression. (Score: 3–4)	≤120	Able to complete mating with strong and rapid ejaculatory thrust (Score: 10)	Acceptable (good to excellent)
Proceeded with a dull expression, exhibited mild sexual interest, and took a longer time to mount than their counterparts. (Score: 2)	121–300	Able to complete mating with weak and slow to intermediate thrust (Score: 8–9)	Fair
Exhibited low or no sexual interest and refused to mount. (Score: 1)	> 300	Unable to complete mating and ejaculate (Score: ≤ 4)	Unacceptable (poor)

2.3. Blood sample collection

Blood samples were collected from all buffalo bulls at 8:00 a.m., immediately before sexual teasing. The bulls remained in the presence of the teaser for a maximum of 5 min before the blood samples were collected from bulls that had not ejaculated. Blood samples were collected from bulls that had ejaculated immediately after they dismounted. The blood samples were collected from the tail vein in a plain, clean, dry, sterilized vacutainer tube (10 mL plain vacuum tubes, Biomedica Alex Co., Egypt). The blood samples were centrifuged at 1500 × *g* for 10 min and the serum was separated and stored at −20 °C until analysis.

2.4. Measurement of serum testosterone level

Testosterone concentration in each sample was determined in duplicate using a specific testosterone direct radioimmunoassay kit (RIA; Immunotech, Beckman Coulter, Villepinte, France). The cross-reactivity of the antitestosterone antibody was the following: testosterone, 100%; 5α dihydrotestosterone, 10%; 11β-hydroxytestosterone, 2%; 4-androstenedione, 0.6%; 19-nortestosterone, 5%; and methyltestosterone, 2%. The cross-reactivity with other steroids was < 0.03%. Radioimmuno-assays were performed according to kit instructions. Briefly, standard, control, or serum samples (50 μl) were dispensed into antibody-coated tubes at room temperature. After the addition of a solution (500 μl) of ^{125}I-labeled testosterone tracer, the tubes were incubated for three hours at 37 °C. The liquid in the tubes was then removed by aspiration, and in each tube the radioactivity was determinate using a gamma radiation counter (Automatic Gamma Counter, Perkin Elmer Finland). The total count of bound versus unbound Testosterone was counted on gamma counter and a graph was plotted with counts per min on vertical axis and standard testosterone concentration on the horizontal axis. Testosterone values of individual samples were obtained from the standard curve by interpolation. Specificity of the assay was 100% and sensitivity was 0.025 ng/mL. Inter- and intra-assay coefficients of variation were 8.5% and 13%, respectively. To measure inter- and intra-assay coefficients of variation, two known standards were placed three times in a batch of 100 samples. The experimental data were analyzed using correlation techniques with Minitab software (version 11.12 Bit).

2.5. Estimation of NO level

Serum NO was estimated as total nitrite level according to Miranda et al. (2001). A nitrite linear standard reference curve was prepared using 0–100 μM sodium nitrate for accurate quantization of NO samples. At the time of analysis, the serum samples were thawed and deproteinized by zinc sulfate (15 mg/mL) and centrifuged at 10,000 × *g* for 10 min. Next, 100 μL of the supernatant was transferred to a microplate well, and 100 μL vanadium (III) chloride (0.8% w/v in 1 M HCl) was added to each well to reduce nitrate to nitrite, as the Griess reaction detects only nitrite. Griess reagents, sulfanilamide solution (2% w/v in 5% HCl), and naphthyl-ethylenediamine solution (0.1% w/v in H_2O), were prepared and allowed to equilibrate at room temperature for 20 min. Using a multichannel pipette, 50 μL of the sulfanilamide solution was dispensed into the serum samples. After 7 min of incubation at room temperature, 50 μL of the naphthyl-ethylenediamine solution was dispensed to all wells. After 7 min of incubation at room temperature, the absorbance was measured using a plate reader equipped with a filter between 520–550 nm. Inter- and intra-coefficients of variation were 5.1% and 4.5%, respectively. The sensitivity of the assay was 2.0 μmol/L, and its recovery was 95 ± 1.7%.

2.6. Statistical analysis

Kolmogorov–Smirnov and Levene's tests were used to check the normality of data distribution and the homogeneity of variances, respectively. Data adjusted to a normal distribution, so the parametric test was used. Comparisons among groups were evaluated using a mixed-design analysis of variance (ANOVA) model, using SAS (SAS Institute, Cary, NC, USA) to evaluate the effects of the sexual behavior group, the time (before and after sexual excitation), as well as the interaction between the group and the time on serum testosterone and NO levels of buffalo bulls. Pearson's correlation coefficients were calculated to determine the correlation (R) between testosterone and NO levels before and after sexual excitation besides sexual behavior scores. The strength of the linear association between testosterone and NO levels before and after sexual excitation and sexual behavior (reaction time, sexual aggressiveness score and mating ability score) were evaluated via calculation coefficient of determination (R^2). Bonferroni's

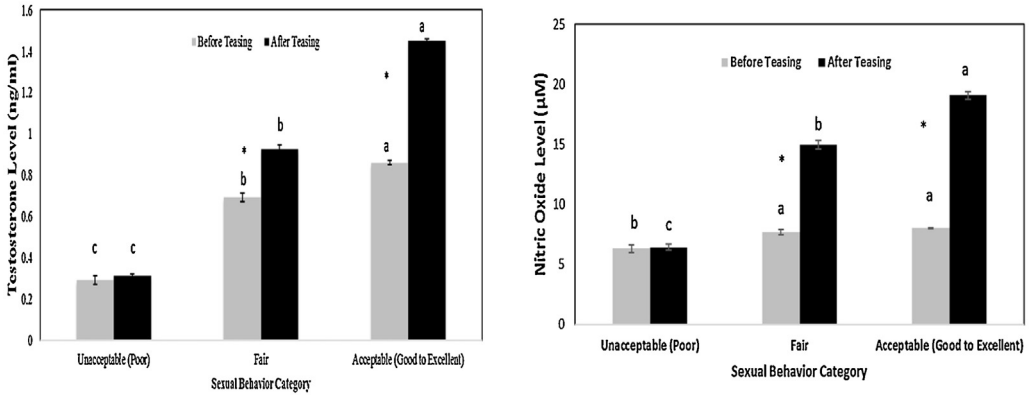

Fig. 1. Effect of sexual excitation on serum testosterone level (ng/mL) or serum nitric oxide level (μM) of bulls with different category of sexual behavior (mean ± SE). [a,b,c] Columns (mean ± SE) carrying different superscripts indicate that blood collected before and after teasing had different nitric oxide or testosterone values; $p < 0.05$. * Columns (mean ± SE) carrying (*) within a sexual behavior category differed; $p < 0.05$.

correction was applied and a difference was considered significant at $p < 0.05$. Data were expressed as mean ± standard error.

3. Results

The reaction time was shorter ($p \leq 0.05$) in bulls with acceptable sexual behavior than in those with fair or unacceptable sexual behavior (48.80 ± 14.54, 189.80 ± 22.80, and > 300 s, respectively). The sexual aggressiveness score was higher ($p \leq 0.05$) in bulls with acceptable sexual behavior than in those with fair or unacceptable sexual behavior (3.6 ± 0.22, 2.0 ± 0.00, and 1.0 ± 0.00, respectively). The bulls with acceptable sexual behavior showed active to aggressive sexual aggressiveness while those with fair or unacceptable sexual behavior showed dull or shy sexual activity, respectively. The mating ability score was higher ($p \leq 0.05$) in bulls with acceptable sexual behavior than in those with fair or unacceptable sexual behavior (10.0 ± 0.00, 8.6 ± 0.22, and 1.2 ± 0.33, respectively). The bulls with acceptable sexual behavior showed mounting with complete penile erection and ejaculation inside the vagina with strong and rapid ejaculatory thrust. While, the bulls with fair sexual behavior showed mounting with complete penile erection and ejaculation inside the vagina with weak and slow to intermediate ejaculatory thrust. However, the bulls with unacceptable sexual behavior showed no mounting or mounting with no or partial penile erection and without either intromission into the vagina or ejaculation.

Fig. 1 presents the effect of sexual excitation on the serum testosterone (ng/mL) and NO (μM) levels of buffalo bulls with different categories of sexual behavior. The results showed that the effects of the sexual behavior category group, the time (before and after sexual excitation), as well as the interaction between the group and the time on serum testosterone and NO levels of buffalo bulls were significant ($p < 0.0001$). The serum level of testosterone was higher in bulls with acceptable or fair sexual behavior than in those with unacceptable sexual behavior, with mean values of 0.86 ± 0.01, 0.69 ± 0.02, and 0.29 ± 0.02 ng/mL, respectively ($p < 0.05$). The NO level was higher ($p \leq 0.05$) in bulls with acceptable or fair sexual behavior than in those with unacceptable sexual behavior (8.00 ± 0.03, 7.66 ± 0.19, and 6.29 ± 0.33 μM, respectively). Moreover, the levels of testosterone and NO were associated with reaction time, sexual aggressiveness and mating ability; bulls with low sexual aggressiveness and mating ability with a mean reaction time > 300 s had lower testosterone than did bulls with moderate sexual aggressiveness and mating ability with a reaction time of 120–300 s. Both groups had lower values than bulls with high sexual aggressiveness and mating ability.

Testosterone level was markedly higher in bulls with acceptable sexual behavior after teasing or mounting, but testosterone did increase somewhat in bulls with fair sexual behavior. NO was also markedly increased after teasing/mounting in bulls with acceptable sexual behavior, compared with those with fair sexual behavior. In bulls with poor unacceptable sexual behavior, sexual excitation did not affect testosterone or NO levels.

The correlation coefficient between serum testosterone levels, serum NO levels and sexual behavior of buffalo bulls was presented in Table 2. It is known that the correlation (R) greater than 0.8 is generally described as strong, whereas a correlation less than 0.5 is generally described as weak. Testosterone level and NO were strongly positively correlated with the sexual aggressiveness and mating ability of bulls after sexual excitement. Furthermore, both testosterone and NO were strongly negatively correlated with reaction time both before and after sexual excitement. Testosterone level was strongly positively correlated with NO level both before and after sexual excitement.

Pearson correlation between sexual behavior of buffalo bulls (including; reaction time, sexual aggressiveness score and mating ability score) and their serum testosterone or NO levels was presented both before and after teasing in Fig. 2. The coefficient of determination (R^2) was high in all correlation except the correlation between NO level before teasing and sexual aggressiveness score ($R^2 = 0.5805$) which considered moderate. This means that 95% of the total variation in NO level after teasing can be explained by the linear relationship between NO level after teasing and reaction time. Additionally, 97% of the total variation in testosterone level before teasing can be explained by the linear relationship between testosterone level before teasing and reaction time or mating ability score. Moreover, 96% of the total variation in NO level after teasing can be explained by the linear relationship between NO

Table 2
Correlation coefficient between serum nitric oxide levels, serum testosterone levels and sexual behavior of buffalo bulls.

		Nitric oxide			Testosterone			Sexual behavior		
		Before teasing	After teasing	Difference	Before teasing	After teasing	Difference	Sexual aggressiveness	Reaction time	Mating ability
Nitric Oxide	Before teasing	1	0.90	0.86	0.91	0.86	0.76	0.76	−0.89	0.91
	After teasing		1	0.99	0.99	0.98	0.93	0.92	−0.97	0.98
	Difference			1	0.99	0.99	0.94	0.93	−0.95	0.97
Testosterone	Before teasing				1	0.98	0.91	0.91	−0.98	0.99
	After teasing					1	0.98	0.97	−0.94	0.93
	Difference						1	0.98	−0.90	0.84
Sexual behavior	Sexual aggressiveness							1	−0.93	0.84
	Reaction time								1	−0.88
	Mating ability									1

level after teasing and mating ability score. Additionally, 94% of the total variation in testosterone level after teasing can be explained by the linear relationship between testosterone level after teasing and sexual aggressiveness score.

Pearson correlation between serum testosterone and NO levels before and after sexual teasing of buffalo bulls both before and after teasing was presented in Fig. 3. The strong linear associations between testosterone and NO levels before and after sexual excitation and sexual behavior (reaction time, sexual aggressiveness score and mating ability score) were observed. About 97% of the total variation in testosterone level after teasing can be explained by the linear relationship between testosterone level after teasing and NO level after teasing. Additionally, 99% of the total variation in testosterone level before teasing can be explained by the linear relationship between testosterone level before teasing and NO level after teasing. Furthermore, 96% of the total variation in testosterone level after teasing can be explained by the linear relationship between testosterone level after teasing and testosterone level before teasing.

4. Discussion

Androgens are essential for the maintenance of libido and play an important role in regulating erectile capacity in males (Aversa et al., 2000). In the present study, serum testosterone levels in water buffalo bulls with acceptable sexual behavior were comparable with those previously reported for adult buffalo bulls (Gunarajasingam et al., 1985; Malfatti et al., 2006). Of note, as buffalo are seasonal breeding animals, their serum testosterone level has been extensively studied in different seasons and compared with circadian rhythm (Ahmad et al., 1991; Dixit et al., 1998; El Sawaf et al., 1971; Javed et al., 2000), but to date no studies have examined the different levels of testosterone in bulls with different category of sexual behavior.

We found that testosterone level and NO level were inversely correlated with reaction time and positively correlated with sexual aggressiveness and mating ability of buffalo bulls, which agrees with other studies (Baba et al., 2000; Cartledge et al., 2001; Katongole et al., 1971; Valenti et al., 1999b). This finding supports the hypothesis that the erectile function of testosterone is mediated by NO through its strong vasodilator effect and complete relaxation of smooth muscle endothelial fibers and increase in the level of male sex hormones due to NO (Marin et al., 1999; Suzuki et al., 2003). The NO-dependent pathway mediates penile erection by inhibiting the smooth muscle of the corpora cavernosa, thereby allowing vasodilation of the corpora (Foresta et al., 2004; Park et al., 1999; Traish and Kim, 2005). Libido and aggressiveness could be attributed to the aggressive behavior mediated by NOS and NO and their activation of the hypothalamic–pituitary–adrenal axis (Nelson, 2005; Nelson et al., 2006).

The strong linear associations between testosterone and NO levels before and after sexual excitation and sexual behavior (reaction time, sexual aggressiveness score and mating ability score) were observed. Therefore, the bulls of poor sexual behavior (need long reaction time and have low sexual aggressiveness as well as mating ability) usually have low testosterone and NO levels. These bulls can be treated via stimulate the secretion of testosterone and NO. Additionally, we can predict the sexual behavior of bulls after measuring of their testosterone and NO levels. Therefore, measuring of testosterone and NO levels can be used during categorization of buffalo-bull sexual behavior.

Moreover, evidence has shown the role of NO in mediating both the penile and central nervous system responses that may lead to increased libido and copulatory performance (Murphy and Lee, 2002). In addition, NO sculpts and maintains neural mechanisms resulting from experience, learning, and memory (Susswein et al., 2004). NO facilitates sexual behavior, possibly by mediating the release of dopamine and by galvanizing genomic changes and protein synthesis or their combination in the medial preoptic area (MPOA). The MPOA is critical for integrating sexually relevant sensory and endocrine stimulation, and it in turn sends information to regions that are important for coordinating copulation-specific motor output (Hull and Dominguez, 2007). Dominguez et al. (2006) reported that sexual experience increased levels of NOS, the enzyme responsible for NO production. This increase might function to "prime" the system after sexual experience so that it can appropriately respond to subsequent sexually relevant sensory or endocrine stimulation. In addition, testosterone was found to stimulate neuronal NO production, which is responsible for copulatory behavior (Du and Hull, 1999). This finding coincides with our observation of bulls with acceptable sexual behavior, which presented elevated

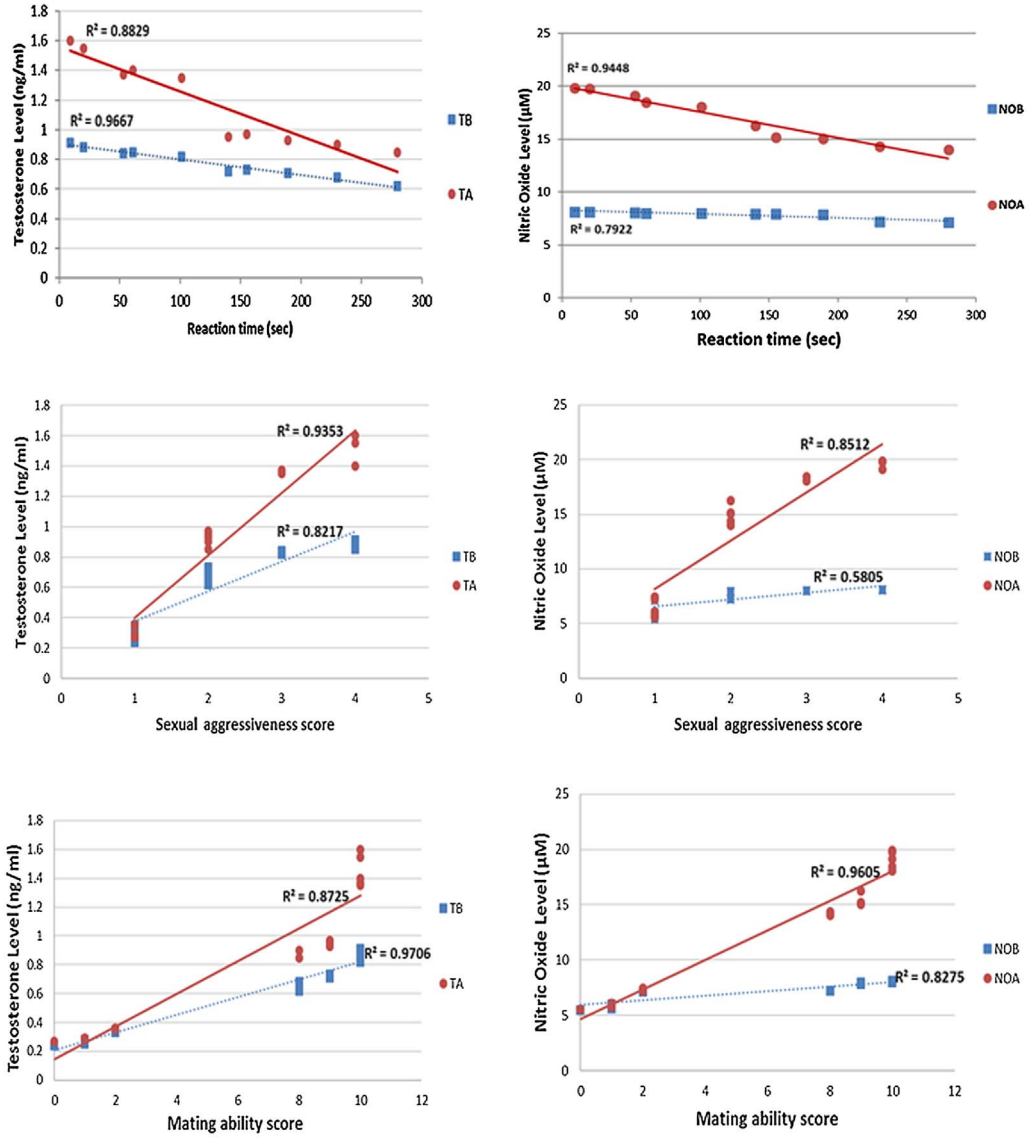

Fig. 2. Pearson correlation between sexual behavior of buffalo bulls (including; reaction time, sexual aggressiveness score and mating ability score) and their serum testosterone or nitric oxide levels both before and after teasing. R^2 means the coefficient of determination. NOB means nitric oxide level before teasing. NOA means nitric oxide level after teasing. TB means testosterone level before teasing. TA means testosterone level after teasing.

levels of NO before and after the sexual excitement. Moreover, the elevated testosterone level promotes copulation and sexual satiety (Hull et al., 1999) and regulates dopamine release in the MPOA by increasing the production of NO (Lorrain and Hull, 1993; Lorrain et al., 1996).

Interestingly, NO has a paradoxical action on gonadal functions among different species. In catfish, NO inhibits testosterone production in the testis (nee Pathak and Lal, 2010). In Japanese quail, gonadal activity shows a positive relationship with NO activity (Kumar and Chaturvedi, 2008). In mammalian species, NO activates the release of luteinizing hormone (LH)-releasing hormone (McCann et al., 1999) and can modify the expression of aromatase (Banerjee et al., 2012). Most studies indicate a positive correlation between NO and gonadal function (Pinilla et al., 1998; Rosselli, 1998; Valenti et al., 1999a), although few have reported contradictory findings (Chatterjee et al., 1997; Singh and Chaturvedi, 2014). Nitric oxide has been shown to exert negative effects on steroidogenesis (Del Punta et al., 1996), possibly through a direct action on steroid-secreting cells or through a reduction of aromatase messenger RNA (mRNA) levels and/or of enzyme effects (Snyder et al., 1996).

5. Conclusion

The alteration in the testosterone and NO levels after sexual excitation depends on the sexual behavior category of water buffalo bull. Testosterone and NO levels were strongly correlated with sexual behavior; therefore, testosterone and NO can be used to

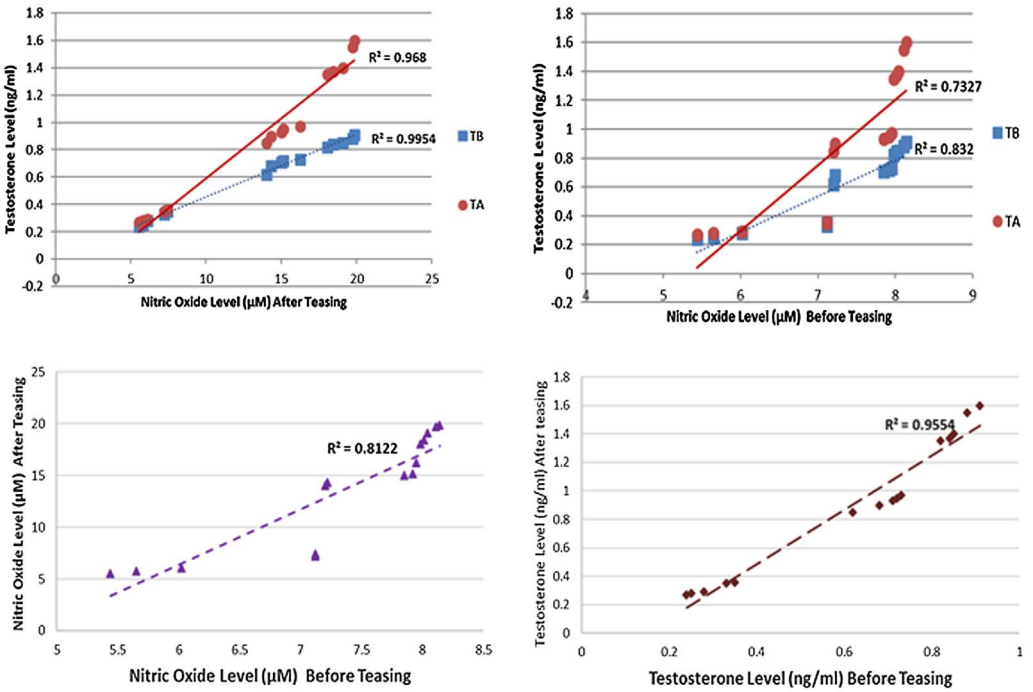

Fig. 3. Pearson correlation between serum testosterone and nitric oxide levels before and after sexual teasing of buffalo bulls. R^2 means the coefficient of determination. TB means testosterone level before teasing. TA means testosterone level after teasing.

categorize the sexual behavior of buffalo bull. Moreover, the level of testosterone and NO can be predicted via evaluation of sexual behavior of buffalo bull including reaction time, sexual aggressiveness and mating ability.

Conflicts of interest

None declared.

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