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Review Article

Reproductive enhancement in buffalo: looking at urinary pheromones and hormones

Archunan, G.

Pheromone Technology Lab, Department of Animal Science, Bharathidasan University, Tiruchirappalli-620024, Tamilnadu, India

Correspondence: *G. Archunan, Pheromone Technology Lab, Department of Animal Science, Bharathidasan University, Tiruchirappalli-620024, Tamilnadu, India. E-mail: garchu56@yahoo.co.in*

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Abstract

The success of conception in buffalo is greatly dependent on precise estrus detection and time of artificial insemination (AI). Various visual, behavioral, biochemical and gyneco-clinical parameters have been tracked closely and a cost-effective combinatorial model has been developed to detect estrus in buffaloes. Pheromones play pivotal roles in reproduction and behavior of mammals. Urine, an easily available biological material which reflects the internal status of an animal, was recruited for profiling the pheromone compounds during the various phases of estrous cycle using gas chromatography-mass spectrometry (GC-MS) analysis. Among the identified compounds, 4-methyl phenol (4-mp, p-Cresol) and 9-octadecenoic acid (Oleic acid) were found to be estrus-specific and would be promising estrus-indicators. Similarly, detection of luteinizing hormone (LH) in urine was also focused to predict the time of ovulation in buffaloes. Partial success has been obtained in the attempt to develop a cost-effective bioassay kit for estrus detection. The ongoing venture of the relevant research team is to develop a biosensor to identify estrus-specific pheromone compounds in urine. Development of a nanoparticle-based bioassay kit for detection of urinary LH for effective prediction of estrus or ovulation is also in progress.

Key words: Buffalo, LH, Ovulation, Pheromone, Urine

Introduction

Buffaloes play a prominent role in rural livestock production by contributing to milk production, meat industry, and other agricultural activities (Singh *et al*., 2000). Estrus detection, a prerequisite for efficient reproductive management, is a challenging task in buffaloes because they are silent-heat animals and do not express signs of estrus outwardly (Suthar and Dhami, 2010). The success of artificial insemination (AI) depends mainly on the critical time of insemination (Drost, 2007). The success rate of AI in buffalo is only about 50% or even less at field level (Kumar *et al*., 2013), which may results in huge economic losses in developing countries like India and Pakistan. An estrus detection technique is essential for the farmers to decide on the time of AI. Since behavioural and physiological signs of oestrus are not so reliable (Suthar and Dami, 2010), the detection of oestrus through an efficient technique is warranted. To facilitate oestrus detection, an attempt is made to develop a sensor (BOVINOSE) for bovine in Europe, which is under validation (Wiegerinck *et al*., 2011). However, such devices are not made or even attempted for buffaloes. To overcome the long-standing issue of oestrus detection and to offer an appropriate and cost-effective device, our team has been working to develop a "Pheromone tracker sensor for oestrus detection in buffalo's body fluids". In this context, a few metabolites [e.g., 4-methyl phenol (4-mp, p-Cresol), trans-verbenol (TV) and 9-octadecenoic acid (Oleic acid)], have been shown to be promising volatile compounds/pheromones such as urine, saliva and faeces during oestrus in buffaloes (Archunan *et al*., 2014). Various visual, behavioural and gynaeco-clinical parameters have been employed, in combination, for oestrus detection with medium sensitivity (Selvam and Archunan, 2017). The chemical cues present in the body exudates might be used as reliable indicators of oestrus. Analysis of volatiles in faeces, saliva, vaginal mucus and urine in buffaloes (*Bubalus bubalis*) in relation to different phases of oestrous cycle was attempted to decipher phase-specific pheromones. In addition, the physiological events of ovulation are important for ensuring successful fertilization. Physiological changes are regulated by the temporal variation of hormone profiles within the body wherein luteinizing hormone (LH) plays a key role.

Even though embryo transfer technology in buffalo is successful (Drost, 1988), the success rate is only negligible in view of low fertility and poor super-ovulation response (Misra *et al*., 1990). Therefore, the technology needs to be modified to overcome the poor response of ovarian follicles in buffaloes. It is strongly believed that assisted reproductive technology can provide an efficient source of embryo for implementing the basic research in developmental biology, breeding

and emerging biological techniques such as cloning and transgenesis (Warriach *et al*., 2016). It is also necessary to focus on improving the cryopreservation of *in vitro* embryo production in buffaloes. There are some significant changes occurring in the biochemical factors such as alkaline phosphatases (ALP), acid phosphatases (ACP), carbohydrates, and lipase in endometrial compartments during follicular and luteal phases due to steroid hormone alterations in river buffaloes (Shahrooz *et al*., 2013). It has been reported that follicular hormone along with Norgestomet® + pregnant mare serum gonadotropin (PMSG) treatment would enhance fertility in acyclic buffaloes (Jerome *et al*., 2016). The discovery of neural peptide kisspeptin is a milestone in reproductive neuroendocrinology. Kisspeptin regulates pulsatile gonadotropin-releasing hormone (GnRH) release and is believed to be involved in the onset of puberty and normal reproductive functions including ovulation (Zeydabadi Nejad *et al*., 2017). The actual function of kisspeptin in buffalo, however, needs to be investigated (Okamura *et al*., 2013).

Chemical signals

Olfaction is pivotal in eliciting behavioral responses by the concerted action of small hydrophobic volatile molecules and neuroepithelial receptors of the nose (Buck and Axel, 1991). Many studies implicated the importance of olfaction as the foundation of chemosensory technology (Bell, 1996; Archunan, 2018). Chemical signals that communicate information between members of the same species are commonly termed pheromones (Brennan and Keverne, 2004). Pheromones are involved in the regulation of various aspects of reproduction in the vertebrates including mammals (Archunan, 2009). There can be single or multiple channels to produce pheromones in animals. The pheromone activity is perhaps conferred through a mixture of compounds rather than a single compound. Each of the major compounds is involved in communicating specific signals related to reproductive behaviour including sexual attraction and modulation of puberty, estrous cycle, pregnancy and social behavior (Dominic, 1991; Archunan, 2014). These volatile compounds are among the major communication channels in ungulates (Booth and Signoret, 1992). Therefore, detection of the chemical signals emanating from body exudates would be a non-invasive approach to assess the reproductive status of the animal. For instance, urinary volatile analysis has been proved to reveal the reproductive status of many animals (Dehnhard *et al*., 2006; Archunan *et al*., 2014).

Pheromones in buffalo

Urinary pheromones

Various non-polar volatile compounds present in urine are believed to induce sexual behaviour. Female mammals in estrus invariably use these volatile compounds, secreted in urine and cervico-vaginal mucus, to advertise their status of receptivity and attract potential males to mate. Bovine and buffalo void large volumes of urine (approximately 20 L/day) and excrete large amounts of protein in the urine. The female urinary proteins have been suggested to participate in pheromone communication (Sudha *et al*., 2012). Fourteen different urinary volatile compounds were reported in naturally cycling buffaloes; however, as many as 42 volatile compounds (alkanes, alkenes, alcohols, aldehydes, acids, amides, ketones, phenols, etc.) were identified in the urine of the synchronized buffaloes (Muniasamy *et al*., 2017).

Among the compounds thus identified, 1-chloro-1-buten-3-yne, 1, 4-dichloromethane, 2-octanone, 2-ethyl-5-methyl-1, 2, 3-dioxoborolan-4-one, ethyl-2-methylcyclopentane, methylheptane, diisooctylester-1, 2, benzene dicarboxylic acid, and 3-methyl-1-pentanol were found to be present in the urine during all phases. Methylpyrrolidine, 3-aminoheptane, 2, 3-dimethylheptane, 3-methyl-1-pentene, hexadecanol, 2-octyldodecan-1-ol, 5-methyl-4-decene, and 2-methyleicosane were present only in the pre-estrus and estrus urine but not in post-estrus. The compounds 2-methyl-2-propenamide, 4-pentanol, 7-methyl-4-undecane, 4-methyl-3-heptanone, 2-methyl-n-phenyl-2-propenamide, N-N-bis(2-hydroxyethyl)-dodecanamide, nonane, decanoic acid, dimethylundecane, tetradecanoic acid, 9-methyl-(z)-5-undecene, and 1-tetradecanol were absent in pro-estrus urine, i.e., eleven compounds were present only in estrus and post-estrus urine. The compound 5-methyl-2-(methylethyl)-cyclohexanol was identified in pre-estrus and post-estrus urine alone. Phenol, 3-propylphenol, 9-octadecenal, and 3-methylbutanol were found to be pro-estrus-specific whereas 1-nitro-2-methylpropane, 2, 3-dihydro-4-methylfuran, 3-ethyl-2-methylhexane, 1-chlorotetradecane, hexadecanoic acid, isocyanobutane, andundecanol were identified only in post-estrus urine (Muniasamy *et al*., 2016). Overall, three compounds, i.e., 1-chlorooctane, 4-mp and 9-octadecenoic acid were identified as oestrus-specific during natural oestrus cycle and two compounds, i.e., 4-mp, and 9-octadecenoic acid, were found to be specific to estrus based on behavioral characterization as well as in synchronized cycle (Rajanarayanan and Archunan, 2011; Muniasamy *et al*., 2017). The presence of p-Cresol was reported in the urine of male rat (Osada *et al*., 2009) and male Alaskan deer (Whittle *et al*., 2000). p-Cresol is the lone pheromone compound identified in estrus mare urine and may be used to predict the ovulation time. Stallions spend considerable time sniffing estrus mare in which p-Cresol is predominant. The extent of stallions' erection differs significantly in relation to reproductive status of the female (Buda *et al*., 2012). The biological importance of p-Cresol in buffalo has been concerned as it has been demonstrated as an oestrus-specific volatile urinary compound and may be taken into account as an "estrus indicator".

Other pheromones

Faecal pheromones

Faeces is another potential source of chemical signals in many species, and convey the physiological status to conspecifics. Gas chromatography-mass spectrometry (GC-MS) analysis of faeces of buffalo revealed 27 compounds, among which 18 were present during pro-estrus, 21 during estrus and 11 during post-estrus (Karthikeyan and Archunan, 2013) phase. The compounds 2, 4-bis cyclohexanal, 8-methyl-1-decane, and 2-ethyl-1-decanol were common to estrus, pre-estrus and post-estrus phases. Two compounds, 4-mp and TV, were unique to estrus phase and appear to communicate the estrus state to the bull. 4-methyl phenol was further shown to bind with the olfactory receptors (Shiraiwa, 2008) indicating the possibility of this compound to play role as a pheromone. Trans-verbenol, a chemo-communication system in most insects, has also been reported to be a sex attractant in insects (Pitman *et al*., 1968; Romon *et al*., 2007; Zhang *et al*., 2008).

Vaginal pheromones

The estrus female produces a viscous vaginal discharge called cervical vaginal fluid (CVF), which contains pheromones that induce physiological and behavioral responses in the bull (Nishimura *et al*., 1991). The vaginal chemo-signal arrives at the higher regions of the neuro-sensory system through olfaction and, thereupon, on confirmation of the heat/estrus, the bulls proceed with mating. Among 15 compounds identified in the vaginal mucous, 9-octadecenoic acid was found to be present only during estrus phase (Karthikeyan and Archunan, 2013). The estrus-related odor in vaginal mucous may induce the sniffing behavior of conspecifics (Nishimura *et al*., 1991). Oleic acid is a fatty acid released to advertise the conspecifics and inviting them for natural coitus. Similarly, acetic acid present in human vaginal fluid acts as a sex-attractant which influences male perception by inducing hormonal changes in males and is responsible for the change in hormonal milieu during the menstrual cycle (Michael *et al*., 1975; Sokolov *et al*., 1976). Acetic acid, when added with other compounds as additive, expresses synergistic activity to produce potent lures in insects (Landolt *et al*., 2013; Zanardi *et al*., 2017).

Salivary pheromones

Saliva is unquestionably an important social cue among mammals. In buffaloes, GC analysis of saliva revealed 11 volatile compounds covering all phases of the estrous cycle (Karthikeyan *et al*., 2014). It is important to note that 4-mp is the specific compound identified in the saliva only during the estrus phase (Karthikeyan *et al*., 2014) and it matches with its specificity in estrus urine and saliva. Interestingly, oleic acid was reported in male saliva after licking the vaginal mucus, but absent before licking. This shows that the bull acquires oleic acid from the female vaginal mucus or it may be secreted in the bull's saliva after exposure to vaginal mucus. Thus, oleic acid is important in sexual communication in buffaloes.

Hormones in reproduction

The reproductive endocrinology of buffaloes has been investigated and the data can be reliably correlated with the compounds present in urine, faeces and/or saliva. Quite a few of these studies have been concerned with hormonal changes associated with estrus and ovulation in buffaloes (Avenell *et al*., 1985; Singh *et al*., 2001). The level of progesterone during estrus was in the range of 0.1-0.3 ng/ml and increased to 1 ng/ml during the subsequent days (days 3 and 4) (Batra and Pandey, 1982). The circulating estradiol level remained at the baseline (10-20 pg/ml) around post-estrus days and reached to the peak concentration (30-35 pg/ml) on the day of intensified estrus (Batra and Pandey, 1982; Samad *et al*., 1988). This indicates the capacity of pre-ovulatory follicle (Graafian follicle) to produce estradiol during the pre-estrus phase. Estrogen is the master regulator of estrus phase. Therefore, the compounds excreted in the body exudates during estrus may have a bearing effect on estrogen. The circulating level of LH reached the peak (20-35 ng/ml) at the onset of estrus and, thereafter, decreased and remained low (1-3 ng/ml) during the luteal phase.

LH in urine

The urinary peptide hormone levels have great relevance to clinical settings (Miller and Soules, 1996; French *et al*., 1999; Robeck *et al*., 2005). Luteinizing hormone is a heterodimeric glycoprotein, which undergoes successive metabolic processing through dissociation and degradation of subunits [LHβ subunit into LHβ-core fragment (LHβcf)], excreted through urine (O'Connor *et al*., 1998; Cole, 2009; Braunstein, 2011; Choi and Smitz, 2014). LHβ-core fragment, with more than 80% amino acid sequence homology which renders it topologically identical with its subunit (Iles *et al*., 1999) is highly stable and cross-reacts with antisera of LH (O'Connor *et al*., 1998). The presence of LHβcf in urine is thus an evidence for the occurrence of intact LH in circulation (Cole *et al*., 1999). The excretion of LH in the urine was observed in all buffalo studies irrespective of age, parity and geographical location (Selvam *et al*., 2017a). Subsequently, the presence of LH is found in the saliva of estrus buffalo than the other phases (Srinivasan *et al*., 2020).

The duration of pre-ovulatory LH surge ranges from 7 to 12 h (Perera, 2011). This surge triggers a complex process culminating in ovulation, which occurs approximately 24 h after the onset of LH surge (Hafez, 1987; Kaim *et al*., 2003). This explains the increase of LH level in urine on days 1 to 3 of the cycle based on the onset of LH release into circulation. Luteinizing hormone is excreted in urine 12 to 24 h after its presence is noted in peripheral blood in several mammals (Palme *et al*., 1996; O'Connor *et al*., 1998; Kumar *et al*., 2013).

Urinary LH profile

The level of LH is always higher in blood than in urine (Selvam *et al*., 2017a). With the ovulatory surge, it reaches to the peak concentration of 110 ng/ml on day 1, remains at this peak/plateau on days 2 and 3, and then begins to fall gradually. Park *et al*. (2007) described a range of LH from 2.5 to 14.8 times higher than the baseline level and LH surge duration of about 3 days. However, by lengthening the surge window, the LH amplitude reduces several folds for some days after the real increase due to the lingering excretion of circulating LH forms (Selvam *et al*., 2017b). In addition, peak excretion concentration of LH varies upon season and parity of the animals. The mean urinary LH concentration detected on the days considered as the period of ovulation, i.e. day 1 (106.79 ± 7.06 ng/ml), day 2 (106.87 ± 7.72 ng/ml) and day 3 (105.99 ± 8.85 ng/ml), was significantly ($P<0.05$) higher than those detected on the other phases of the estrous cycle such as pre-estrus (59.08 ± 3.28 ng/ml), estrus (73.74 ± 4.11 ng/ml), post-estrus (63.74 ± 3.41 ng/ml) and baseline value (46.73 ± 3.36 ng/ml).

Estrus and ovulation prediction

Pheromone-based prediction

Continuous behavioral observation has revealed that the estrus-specific compound 4-mp plays role as a bull attractant, whereas 9-octadecenoic acid is primarily concerned with the mounting behavior (Rajanarayanan and Archunan, 2011; Karthikeyan and Archunan, 2013). Combination of these two estrus-specific compounds significantly influences the sexual behavior of the animal to enhance the libido of the bull through neuroendocrine stimulation and increase the sperm output (Archunan and Rajanarayanan, 2009). Studies on urine of mare found para- and meta-Cresols as possible indicators of estrus and ovulation (Mozuraitis *et al*., 2012). Furthermore, p-Cresol was proved to elicit sexual behaviors in stallions when applied on dummy mares (Buda *et al*., 2012). Put together, these observations show p-Cresol to be a promising compound for estrus detection, and urine can be taken as a sample.

Biochemical kit for estrus detection

p-Cresol is consistently present in more than one body exudates such as urine, vaginal mucus, and saliva. Furthermore, this volatile compound acts as a sex attractant and inducer of mounting behavior in bull (Archunan *et al*., 2014). A combination of buffalo pheromones, including p-Cresol, elicits bull sexual behavior (Rajanarayanan and Archunan, 2010). Based on the extensive studies, p-Cresol could be taken as a potential pheromone released during estrus in buffaloes. Since p-Cresol was evidenced to be a promising estrus-specific candidate pheromone, we developed a biochemical kit which works based on the presence p-Cresol. It was subjected to field evaluation in screening estrus urine samples of buffalo and revealed that 60.87% and 71.43% of urine samples were correctly identified as estrus and non-estrus (i.e., proestrus and diestrus), respectively (Muthukumar *et al*., 2018a).

Electronic nose

Electronic nose (e-nose) is an electronic device that detects the compounds of interest more precisely than the olfactory receptors of mammals. Electronic nose uses an array of sensors, which are built to selectively bind with a recognition compound. This device is useful in various sectors such as agriculture, environmental, biomedical, food, water analysis and etc. The device has been successfully used to determine fruit quality based on the qualitative and quantitative measures of aroma in different fruits (Heliofabia, 2012; Buguad and Alter, 2016). It is useful as a detection technique to improve the livestock production. The estrus-based sensor system, BOVINOSE (under validation) using an array of sensors that detects the compounds present in oestrus faeces has been developed in Europe (Weigerinck *et al*., 2011). The principle underlying the development of BOVINOSE was based on our study on cow faeces, in which we reported acetic acid and propionic acid as estrus indicators (Sankar and Archunan, 2008). We had shown that these two volatile molecules are secreted when the cow is in heat and serve as natural olfactory signals to the bull.

The metal oxide gas sensors were greatly adapted for the BOVINOSE detector module. The sensors are designed to function at normal indoor conditions of surrounding temperatures from about 10°C to about 30°C, relative humidity from about 30% to about 80% and oxygen-rich atmosphere. The sensors are fixed within the standard transistor container TO-5. The sensitivity of the sensors is not less than ≤ 2 ppm of pure volatile pheromone compounds. The power consumption of standard electricity supply did not exceed 500 mW per sensor. Ultimately, the device was developed to detect the specific pheromones based on the equipped sensors. The financial support of this project has been provided by European Commission Seventh Framework Programme (2007-2013).

Senor for p-Cresol detection

Electrochemical sensors are sensitive, selective and accurate in detecting electro-active species, and are portable, inexpensive and very attractive for on-site monitoring of pollutants and disease monitoring in medical field and also applied to predict the ovulation period in buffalo. A hydroxyapatite (HA)-modified electrochemical sensor was developed and successfully used for the first time to detect p-Cresol in urine (Sudhan *et al*., 2017). Hydroxyapatite present in its stoichiometric pure form in bone absorbs a large quantity of impure ions. It has been established that Ca^{2+} vacancy of HA in the crystal structure occurs prominently in the surrounding aqueous-acidic solution. The leaf of the plant *Plectranthus amboinicus* possesses properties such

as antioxidant, reducing power, superoxide anion and nitric oxide radical scavenging ability and ferrous ion chelation. It contains essential oils, flavonoids and terpenes. Initially, HA nanoparticles (NP) were synthesized using the extracts of *Plectranthus amboincus* adopting microwave-irradiation method. The modified HA-NP glassy carbon electrode (GCE) possesses improved electro-catalytic properties in sensing p-Cresol. The *plectranthus amboincus* Kv-HA NP-modified GCE exhibited excellent electro-catalytic activity towards p-Cresol determination. The p-Cresol detection efficiency of sensors occurred in a broad range (0.275-23.5 × 10^{-6} M) and the lowest limit of detection was 116 × 10^{-9} M (S/N=3) mediated by phosphate buffer solution (PBS) (pH=6.0). The sensors are precise in detecting p-Cresol even when potential interference is found in the solution (Sudhan *et al*., 2017).

Odorant binding proteins (OBPs), a subclass of small soluble polypeptides of lipocalin family, have been reported to act as carriers and olfactory stimuli in certain insects and mammals (Cavaggioni *et al*., 1987; Pelosi, 1990; Dal Monte *et al*., 1991; Garibotti *et al*., 1997; Archunan, 2018). The proteins are found in body fluids and act as pheromone carrier to convey specific information. At present, OBPs are receiving great attention because of their features such as high temperature stability, compact folding and strong affinity with specific organic compounds that are attractive to build a robust, reliable and inexpensive biosensor. We have identified, for the first time, the presence of OBP in buffalo's saliva. The protein has undergone a number of post-translational modifications (Rajkumar *et al*., 2010). Furthermore, an OBP has been identified in nasal mucous of buffalo by electrophoretic analysis, using *in silico* homology modeling to assess its structural similarity with other mammalian OBPs (Muthukumar *et al*., 2018b). It was proved that the protein has strong affinity with buffalo's pheromones. This OBP has potential association with sex pheromones which would be useful to develop a sensor for estrus detection.

LH cut-off value

Ovulation prediction in buffaloes would be highly advantageous in the context of efficient management of reproduction for increasing conception rates through timed AI. The LH excreted in urine by humans, non-human primates and marine mammals can be quantified by standard immunoassay techniques (Miller and Soules, 1996; French *et al*., 1999) or with portable dip strips for planning the precise time for intercourse and/or timed AI (Robeck *et al*., 2005). The significant increase in urinary LH concentration, above the cut-off value (105 ng/ml), on days 1 and 2 of estrus can possibly serve as a valuable predictor of the time of ovulation (Selvam *et al*., 2017b). The cut-off LH value differentiates the time of ovulation from estrus but with poor accuracy (0.60) displaying a sensitivity rate of 52%. Thus, the cut-off value that is derived will aid in differentiation of estrus phase from the time of ovulation.

LH-coated nano-particle

Ovulation in buffalo occurs 28-60 h after the onset of estrus, with a mean of 44 h (Paul, 2003). This broad range is due to a large variation in the time of LH surge. The ovulation might occur in the evening of day 1-day 2 transition or on the morning of day 2-day 3 transition, which would depend on the period of the onset of LH surge of the particular individual. The sperm introduced into the cervix via AI after a positive-ovulation-prediction (on day 1, day 2 or day 3) would certainly encounter the ovum in the uterine horn at the appropriate time, which would ultimately result in an effective conception (83.33%). In addition, successful conception would also be possible on day 3 if intra-uterine insemination (IUI) was performed, where most of the intra-cervical insemination (ICI) failed due to delay in the meeting of sperm and ovum in the uterine horn. There are only few assays known for detecting LH in the blood of domestic animals. One of the major disadvantages of the existing assays is the requirement of large volumes of samples to perform the measurement. Zambre *et al*. (2012) developed a nano-sensor using a gold nanoparticle-peptide conjugate to detect LH in sheep, which detected LH based on its peptide sequence (CDHPPLPDILFL). The lower detection limit of the sensor was ~ 50 ppm.

Pheromones and bull sex libido

The reproductive capabilities of the bull are of paramount importance, which are much influenced by several factors such as semen quality and quantity, libido, mating ability and sexual interaction. It is also reported that fertility characteristics of semen are constant when it is evaluated with *in vitro* libido or sex-drive which is a behavioral trait with large instinctive components. It has been reported that pre-stimulation of bulls increases the sex response (Blockey, 1981).

The sex pheromones found in estrus buffaloes were proved to elicit penile erection and help to increase the sperm quantity (Archunan and Rajanarayanan, 2009). Similarly, estrus mare urinary pheromones were found to elicit penile erection in stallion (Wierzbowski and Hafez, 1961). Taken together, female sex pheromones of cattle are proved effective to be used in assisted reproductive technology. Furthermore, the pre-mating behaviors such as penile erection and mounting in bull were found to be the highest when the bull has access to estrus buffaloes (Rajanarayanan, 2004). The sex pheromones identified in buffaloes influence the male pre-copulatory behaviors and enhance the sperm quantity. Investigations on bull sex libido need to be carried out for assisted reproductive technology (ART). It has also been proposed to analyze if sex pheromones can influence the unmotivated poor-response bulls.

Future perspectives

Based on plethora of studies, pheromones and hormones of buffalo can potentially be used to invent

suitable devices that would help to improve reproductive efficiency of buffaloes. Artificial insemination is usually performed to regularize the calving interval, standardize milk production and improve the offspring ratio. Artificial insemination can be successful when precise estrus detection is achieved. The kit developed by us shows 60.0% efficiency of detecting estrus. This partial success requires modifications to improve the efficiency. As an alternative to the biochemical kit for estrus detection we have now taken up an initial step towards the development of an electrochemical sensor consisting of HA-NPs based on the presence/detection of p-Cresol in buffalo urine. It is proposed to develop a field-validated sensor to detect estrus for farmers' use. It is necessary to develop a device using pheromone for purpose of estrus detection. It is also proposed to develop in parallel a LH peptide-based label-free sensor to predict the accurate time of estrus in buffaloes.

Conclusion

It is evidenced that urine contains active chemical signals that convey the reproductive condition of females to conspecifics in mammals. More promisingly, body fluids such as urine, saliva, faeces, and vaginal mucus of estrus buffalo contain specific volatile compounds such as p-cresol and oleic acid. This has led to the conclusion that the estrus-specific compound(s) is produced in female to attract the conspecifics for natural coitus. Behavioral analysis has revealed p-Cresol as an attractant and 9-octadecenoic acid as a stimulant of mounting behavior. Biochemical quantification of these compounds has led to promising detection methods. An indigenously developed kit showed more than 60% positive prediction of estrus phase and now it is proposed to develop an electrochemical sensor of high specificity for detection of estrus to optimize the ovulation period of buffaloes. The sex pheromone compounds are reported to enhance the bull sex libido and semen quantity considerably. Further investigation on this aspect would provide information useful in Assisted Reproductive Technology. In addition to urinary pheromones, estimation of urinary hormones with accurate cut off values at estrus provides possibility to develop a technique to detect estrus in buffaloes. Detailed investigations on electrochemical sensing and colorimetric assays must be performed before developing label-free sensors to detect estrus in buffaloes. This approach would greatly enhance conception rates in this species.

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Conflict of interest

The author declares that there is no conflict of interest.

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