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Quantification of Urinary Exosome Sex Hormone Binding Globulin Levels during Estrus and Diestrus Stages of Buffaloes

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ABSTRACT

Background: The efficiency of reproduction in buffaloes can be increased by being able to spot buffaloes in estrus, as silent heat is a severe problem in this species. Proteins of urinary exosomes are identified to be better physiological biomarkers when compared to cell free proteins as these extracellular vesicles (EVs) are tiny in size, have additional protection from degradation by endogenous protease activity and are involved in intercellular communication. Hence, the present study was aimed to quantify the urinary exosome sex hormone binding globulin (SHBG) levels during estrus and diestrus stages of buffaloes to determine its suitability as a marker for identifying buffaloes in estrus.

Methods: The grouping of animals as mid-diestrus (Group-I/G-I/Control group), regular estrus (Group-II/G-II) and silent estrus (Group-III/G-III) buffaloes was done using a combinatorial approach. The levels of SHBG in urinary exosomes of three groups of animals was quantified using the bovine SHBG ELISA kit.

Result: The present study's findings showed significantly higher urinary exosome SHBG concentration ($p<0.05$) in G-I in comparison to G-II and G-III. The present study revealing for decreased concentration of urinary exosome SHBG in G-II and G-III animals when compared to G-I animals probably construes the physiological role of SHBG in the female reproductive tract at the estrus stage by mediating the action of estradiol 17 β (E_2) at the target site. However, for urinary exosome SHBG to be considered as a biomarker, there is need for additional research on a bigger population.

Key words: Buffalo, Estrus, Sex hormone binding globulin, Silent estrus, Urinary exosome.

INTRODUCTION

Buffaloes are the backbone of the Indian dairy industry as they produce majority of milk produced in India (NDDB, 2019). Around two-thirds of the world's buffalo milk and almost half of the world's buffalo meat (FAOSTAT, 2005) is contributed by Indian buffalo population. Buffalo milk has higher protein content, lower somatic cell count and lower cholesterol content and its meat has significant health benefits compared to *Bos taurus* beef (Ahmad *et al*., 2008), making buffalo species having the greatest potential for productivity. But timely estrus detection is an important factor for increasing reproductive efficiency in buffaloes (Roelofs *et al*., 2010; Muniasamy *et al*., 2017). The intensity of estrus signs in buffaloes is generally low and the incidence of silent heat varies from 15 to 73 per cent (Kandiel *et al*., 2014) resulting in increased chances of missing an estrus event and the associated economic losses (Ravinder *et al*., 2016). Identification of physiological markers of estrus stage in buffaloes may pave a way for detecting heat, in animals those exhibiting signs of heat as well as not exhibiting signs of heat.

Extracellular vesicles (EVs) are non-replicating, spherical and lipid bilayer-delimited particles that are spontaneously released from the cell into the extracellular environment and includes exosomes, microvesicles and apoptotic bodies. Interest in EV function is increasingly expanding as they are crucial for cell-to cell communication and signaling (Simeone *et al*., 2020). Due to additional protection from degradation by endogenous protease

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activity, EV proteins are promising for the identification of physio-pathological biomarkers when compared to cell free proteins (Valadi *et al*., 2007; Hunter *et al*., 2008; Taylor and Gercel-Taylor, 2008; Gibbings *et al*., 2009; Yanez-Mo *et al*., 2015). According to Machtinger *et al*. (2021), EVs are discovered to be connected to the cellular and molecular mechanisms of ovarian cyclic activity.

Urine is a readily available biological fluid source that offers information about the physiological condition of mammals (Li, 2015). Due to their tiny size, circulatory exosomes can also end up in urine, making urinary exosomes a better source of physiological state-specific indicators (Choi *et al*., 2022; Li and Yang, 2022). It is also postulated that changes in the blood levels of SHBG influence their plasma distribution and thereby the access of estradiol 17β (E_2) to target tissues and cells (Hammond, 2002; Wallen and Hassett, 2009). The relevance of SHBG in E_2-mediated regulation and activities in different immune system cells has been documented (Balogh *et al*., 2019). Measurement of SHBG has been suggested in diseases of androgen metabolism (Selby *et al*., 1990). In the mouse uterus, Ng *et al*. (2006) found that the expression of SHBG was more pronounced during the estrus stage, where plasma E_2 levels were at their highest suggesting the role of plasma SHBG in directly influencing the activities of E_2 at the target location. However, the concentration of SHBG in urinary exosomes of buffaloes at the estrus stage (in animals exhibiting signs of heat and not exhibiting signs of heat) and diestrus stage has not been investigated till date. Therefore, the present study was planned with the objective of estimating the levels of SHBG in the urinary exosomes of buffaloes at the estrus and diestrus stage of estrous cycle.

MATERIALS AND METHODS

Grouping of animals

The present study was conducted in samples collected from cycling Pandharpuri buffaloes maintained at National Kamadhenu Breeding Center (NKBC) Chintaladevi Village, SPSR Nellore District, Andhra Pradesh Livestock Development Agency (APLDA) as shown in Fig 1. Animals were synchronized using gonadotropin-releasing hormone (GnRH)-prostaglandin F2α (P) [G-P-G protocol] in the peak summer season. 48 hrs after the injection of PGF2α analogue, animals were presumed to be in heat. Animals exhibiting visual signs of heat were grouped as regular estrus (Group II/G-II) and and animals not showing visual signs of heat were grouped as silent estrus buffaloes (Group III/G-III) after confirmation with trans rectal ultrasound scanning (TRUS). Ten days post estrus stage, these animals (both G-II and G-III animals) were expected to be in mid-diestrus stage and grouped as Group-I/G-I.

Collection and processing of samples

Blood and urine samples were collected from all the three groups of animals *i.e*., G-I, G-II and G-III (n=12 in G-I; n=6 in G-II and G-III). For the quantitative assay of estradiol 17 β (E_2) and progesterone (P_4), blood samples were processed for the separation of serum, labeled and stored in aliquots at -20°C. The E_2 and P_4 concentration in serum samples was estimated using E_2 and P_4 ELISA kits (Calbiotech company, USA) as per the kit protocol and the concentration of E_2 and P_4 was expressed in pg/ml and ng/ml respectively. For the purpose of studying the fern pattern, CVM samples were also taken from the animals during the estrus stage (wherever the discharges were present) (Manasa Varra *et al*., 2022). In order to isolate exosomes for the purpose of quantifying the urinary exosomal SHBG, midstream urine samples were collected in pre-sterilized polypropylene vials with phenyl methyl sulfonyl fluoride (PMSF) at 0.01% (Zhou *et al*., 2006), aliquoted, labeled and stored at -20°C.

Isolation of urinary exosomes by ultracentrifugation

The isolation of urinary exosomes was performed as per the procedure described by He *et al*. (2019) with minor modifications. The frozen urine samples were thawed on ice, vortexed well and ultracentrifugation was carried by the steps described below. Thawed urine samples (50 ml) were added into 50 ml falcon tubes. Samples were centrifuged at 2000 g for 10 min at 4°C (Eppendorf Centrifuge 5804 R). Supernatants were collected and centrifuged at 17,000g for 1 hr at 4°C (SORVALL RC Ultracentrifuge). Resulted supernatant was spun at 25,000 rpm (SORVALL RC Ultracentrifuge) for 90min at 4°C. The obtained supernatants were discarded and 50-100 µl of DEPC Rx PBS was added to the pellets to collect the exosomes. These were then labeled and stored at -80°C in aliquots (Eppendorf tubes) until use. Transmission electron microscopy (TEM) was used to characterize the morphology and size of urinary exosomes obtained through ultracentrifugation by drop casting the samples on carbon coated copper grids and doing negative staining as per the procedure described by Keerthikumar *et al*. (2015) and Rikkert *et al*. (2019) with minor modifications.

Quantification of urinary exosome SHBG

The expression of SHBG protein in urinary exosomes from all the three groups of animals i.e., G-I, G-II and G-III was quantified using the bovine SHBG ELISA kit obtained from MyBioSource company, California, USA. The urinary exosomes isolated by ultracentrifugation and resuspended in DEPC Rx PBS were thawed on ice. The protein was extracted from the urinary exosomes as per the procedure described by Van Deun *et al*. (2014). The sample was mixed with equal volume of Laemmli lysis buffer (LLB) comprised of 0.125 M Tris-HCl, pH 6.8; 10% glycerol and 2.3% SDS and this protein mixture was used for estimating the concentration of SHBG by ELISA.

The test procedure is based on the principle of Double Antibody Sandwich ELISA technique. The kit's protocol was followed for doing the assay. A standard curve was obtained by plotting the concentration of each SHBG standard (ng/mL) versus the Optical Density. The samples' absorbance measurements were used to calculate the standard curve's corresponding SHBG concentration in ng/mL.

RESULTS AND DISCUSSION

Grouping of animals

Combinatorial methods were used to group the animals, including searching for behavioral indicators, TRUS observation, the CVM fern pattern and blood E2 and P4 levels (Manasa Varra *et al*., 2022).

Serum estradiol 17β (E_2) and progesterone (P_4) concentration in G-I, G-II and G-III animals

Table 1 lists the mean serum E2 and P4 concentrations of G-I, G-II and G-III animals. The serum P4 levels revealed significant differences ($p<0.05$) between G-I and G-II as well as G-I and G-III, with significantly lower serum P4 concentration in G-II and G-III compared to G-I. The serum E2 levels revealed significant differences ($p<0.05$) between G-I and G-II as well as G-I and G-III with significantly higher serum E2 concentration in G-II and G-III than in G-I.

Urinary exosome SHBG levels in G-I, G-II and G-III animals

The mean urinary exosome SHBG concentration (ng/ml) in G-I, G-II and G-III animals is shown in Table 2. The results of the urinary exosome SHBG levels when compared between the groups, revealed significant differences ($p<0.05$) between G-I and G-II as well as G-I and G-III, with significantly higher urinary exosome SHBG concentration in G-I in comparison to G-II and G-III.

The current work aims to determine the amounts of SHBG in the urinary exosomes of buffaloes at the estrus and diestrus phases with the hypothesis of the existence of the physiological role of extracellular vesicular, specifically exosomal SHBG. Additionally, we were curious to see if there were any variations in the concentration of this protein between animals that were and were not showing signs of heat. In the present study, grouping of animals was done as per a combinatorial approach (Selvam and Archunan, 2017).

As per Westphal's (1986) theory, liver produces SHBG, a plasma glycoprotein that binds specifically to testosterone and E_2 with an affinity four to five orders of magnitude stronger than albumin and thereby mediate their circulation in the body (Wallace *et al*., 2013). SHBG synthesis in the liver is influenced by E_2 androgens, thyroxine, prolactin and insulin (Rosner *et al*., 1984; Lee *et al*., 1987; Plymate *et al*., 1988; Chen *et al*., 2010). Specifically, the thyroid hormones were found to indirectly increase SHBG expression by increasing the hepatic levels of the transcription factor, hepatocyte nuclear factor 4 alpha (HNF4A) in humans (Selva *et al*., 2007; Selva and Hammond, 2009). Liu and Veldhuis, (2019) observed that SHBG secretion by hepatocytes is induced by E_2 and thyroxine, where as it is repressed by insulin and growth hormone-IGF-I.

Ng *et al*. (2006) postulated that there is ligand-dependent interaction between plasma SHBG and the carboxy-terminal domains of extracellular matrix (ECM)-associated protein namely, fibulin 1D and fibulin 2. Plasma SHBG is also found to facilitate the uptake of E_2 into lymphocytes resulting in enhanced Erk1/2 phosphorylation (Balogh *et al*., 2019). The cells of the innate and adaptive immune systems are both identified to be influenced by E_2 (Blesson, 2011; Karpuzoglu and Zouali, 2011; Dragin *et al*., 2017). El-Banna and Hafez (1972) and Marinov and Lovell (1967) have proposed that E2 serum levels are reported to be lowest during the estrus stage of the estrous cycle causing cervical mucus to be secreted in bovines. Accordingly, Kumar *et al*. (2021) identified that the serum concentration of E_2 in normal estrus Murrah buffaloes (animals showing signs of heat) was significantly higher when compared to the silent estrus animals (animals not showing signs of heat).

The concentration of SHBG (n mol/L) in cattle at the estrus and mid-diestrus stages was practically identical, according to Vesanen *et al*. (1990), with values of 109.5±11.8 and 106.8±16.0 respectively. According to Wang (2021), the plasma levels of SHBG in young, healthy women of <30 years and ≥30 years to be <14.5 nmol/L and <21.9 nmol/L respectively. Interindividual variations in plasma SHBG levels are also influenced by genetic variances, according to research by Haiman *et al*. (2005) and Xita and Tsatsoulis (2010).



Fig 1: Shows the Pandharpuri buffaloes maintained at National Kamadhenu breeding center (NKBC).

Table 1: Serum concentration of estradiol 17β (E_2) and progesterone (P_4) in G-I, G-II and G-III animals.

Serum levels of	G-I	G-II	G-III
E_2 (pg/ml)	8.66±0.700^a	24.51±0.807^b	21.60±1.243^c
P_4 (ng/ml)	3.82±1.103^a	0.36±0.146^b	0.39±0.158^b

Note: Means bearing different alphabets as superscripts within a row differ significantly ($p<0.05$).

Table 2: Urinary exosome SHBG levels (ng/ml) in G-I, G-II and G-III animals.

Group	G-I	G-II	G-III
Urinary exosome SHBG levels (ng/ml)	22.92±1.642^a	12.50±2.543^b	13.00±1.932^b

Note: Means bearing different alphabets as superscripts within a row differ significantly ($p<0.05$).

Alminana *et al*. (2015) and Esfandyari *et al*. (2021) suggested that urinary exosomes contain exosomes synthesized and released from different parts of the female reproductive tract, including oviductal epithelium, follicular fluid, endometrium, uterus, cervix and vagina and could act as biomarkers for specific physiological states and/or any deviation from the normal physiological conditions. On the other hand, Sheikh *et al*. (2009) and Ailawadi *et al*. (2015) proposed that exosomes mediate intercellular communication by direct ligand-receptor interaction, leading to activation of downstream signaling pathways. Further, Prunotto *et al*. (2013) identified the presence of SHBG in the urinary exosomes of humans.

Urinary exosomes, which have been linked to intercellular communication, can serve as a source of bio markers for the physiological changes occurring in the female reproductive tract (Sheikh *et al*., 2009; Ailawadi *et al*., 2015; Alminana *et al*., 2015; Esfandyari *et al*., 2021), according to research. In the present study, the concentration of urinary exosome SHBG in G-II as well as G-III animals was found to be significantly lower ($p<0.05$) when compared to G-I animals, the findings of which are negatively correlated to the circulatory levels of E_2. The results of the present study are in contrast to the findings of Vesanen *et al*. (1990) who reported the circulatory levels of SHBG at the estrus as well as diestrus stage to be almost similar. Further, it appears that the significantly lower levels of SHBG in urinary exosomes of G-II and G-III animals could be due to lower levels of circulatory SHBG at the estrus stage (Mousavi and Adlercreutz, 1993; Hammond, 2002; Wallen and Hassett 2009), as E_2 needs to exert its action in the female reproductive tract (Marinov and Lovell 1967; El-Banna and Hafez, 1972).

CONCLUSION

As a result, even while it can be inferred from the foregoing that urinary exosome SHBG levels might be used to identify buffaloes at either diestrus or estrus stage, its value as a biomarker needs to be verified with additional research on a bigger population.

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Conflict of interest

On behalf of all the authors of the manuscript, I declare that the authors do not have any conflict of interest.

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