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Tamm-Horsfall Protein Expression in Urine of Buffaloes at the Estrus and Diestrus Stages of Estrous Cycle

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ABSTRACT

Background: Identifying buffaloes in estrus is crucial for enhancing the efficiency of reproduction as silent heat is a major concern in this species. Review of literature indicated the role of Tamm-Horsfall protein (THP) is associated with the physiological events around the estrus stage. Hence, the present study was aimed to quantify the expression level of THP in urine of buffaloes to ascertain 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 expression of THP in the urine of three groups of animals was quantified by western blot.

Result: The results of the present study revealed higher levels of urinary THP in G-II animals when compared to G-III and G-I animals, although it was statistically non-significant ($p>0.05$). The present study revealing for increased urinary expression of THP in G-II and G-III animals when compared to G-I animals probably construes the antimicrobial role of THP in the female reproductive tract and its role during estrus to prevent microbiota population beyond the physiological levels. For THP to be considered as biomarker for detecting buffaloes in silent heat there is need for further studies involving large number of animals. Nonetheless, an easy method has been developed that facilitated THP detection and quantification in urine of buffaloes by western blot.

Key words: Buffalo, Estrus, Silent estrus Tamm-Horsfall protein, Urine,

INTRODUCTION

Detection of estrus is an important factor for improving reproductive efficiency in buffaloes (Roelofs *et al*., 2010; Muniasamy *et al*., 2017). Either missing an estrus event because of silent heat or inaccurate estrus detection followed by improper time of insemination can lead to huge economic losses (Hussain Shah, 2007). The quest for the search of estrus specific biomarker/ differentially expressed proteins during estrus stage in easily accessible biological fluids of buffaloes is continuing due to the higher incidence of animals not exhibiting signs of estrus (Kandiel *et al*., 2014) and its associated economic impacts.

Urine is a non-invasive source of biological fluid and reflects the physiological status of the mammals (Bathla *et al*., 2015; Li, 2015). Tamm-Horsfall protein (THP) is a glycoprotein produced by the renal tubular cells of the kidney and is the most abundant urinary protein found in healthy mammals (Serafini-Cessi *et al*., 2003; Pisitkun *et al*., 2004). THP is associated with molecular pathways of polymorphonuclear (PMN) phagocytosis (Siao *et al*., 2011; Li *et al*., 2014; Wu *et al*., 2018).

Reactive oxygen species (ROS) appears to be essential for the process of ovulation that follows the estrus stage (Kodaman and Behrman 2001; Shkolnik *et al*., 2011). In buffaloes, various pathways of immune signalling were found to play an important role in the growth of ovarian follicles at the pre-ovulatory or estrus stage (Li *et al*., 2017). In connection with the above findings, Sciorsci *et al*. (2020) revealed that in buffaloes, the highest values of ROS were

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detected at the estrus stage and the highest levels of blood anti-oxidant potential (BAP) have been found during the diestrus stage. Further, Agardh *et al*. (2002) reported that THP expression in urine increases when there is physiological increase in ROS level. The accumulated lines of evidence are indicative of the physiological role of THP during estrus phase in buffaloes which has not been put forth so far. Therefore, the present work aimed to study the expression of THP in urine of buffaloes at the estrus stage (in animals exhibiting signs of heat and not exhibiting signs of heat) and diestrus stage of estrous cycle.

MATERIALS AND METHODS

Grouping of animals

The present study was conducted in samples collected from cycling Murrah buffaloes maintained at a private farm, H cross, Vijayapura, Kolar, Bangalore. The animals in mid-diestrus and estrus stage *i.e*., those exhibiting signs of heat and not exhibiting signs of heat were identified/selected to group them as normal (mid-diestrus, Group I/G-I, control group), regular estrus (Group II/G-II) and silent estrus buffaloes (Group III/G-III).

Buffaloes at the diestrus stage and estrus stage *i.e*., G-I and G-II respectively, were identified by tracking the animals for visual signs of heat, behaviour of the bull towards the buffalo cow in estrus (Fig 1), per rectal examination for the tonicity of uterus, Transrectal Ultrasound Scanning (TRUS) of the ovaries for the presence of dominant follicle (DF) or corpus luteum (CL) (Fig 2). Buffaloes exhibiting poor signs of heat *i.e*., G-III, were identified in the herd with the history of not exhibiting any signs of heat for more than two months after parturition coupled with per rectal examination for tonicity of uterus, presence of DF and regressing CL as well as confirmation of the same by TRUS. In the process of selecting /grouping, all the animals in the farm grouped as G-I, G-II and G-III were different.

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=3 in each group). Blood samples were processed for the separation of serum, labelled and stored in aliquots at -20°C for the quantitative assay of estradiol 17β (E_2) and progesterone (P_4). Cervico-vaginal mucus (CVM) samples were also collected from the animals at the estrus stage (wherever the discharges were available) for observing the fern pattern (Manasa Varra *et al*., 2022). Mid-stream urine samples were collected in pre-sterilized polypropylene vials, were aliquoted in falcons with phenyl methyl sulfonyl fluoride (PMSF) as preservative (Zhou *et al.,* 2006) at 0.01% (Rawat *et al*., 2016) for isolation of THP, labelled and stored at -80°C until further analysis.

Estimation of serum estradiol 17β (E_2) and progesterone (P_4)

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.

Isolation and quantification of THP by Western blot

THP was identified in the complex mixture of proteins present in the urine samples by western blot technique. The sequential steps involved in the western blot includes the following:

Urinary protein extraction

The extraction of total urinary proteins was be done by ammonium sulphate salt precipitation as per the procedure

1A, 1B, 1C and 1D depicts vulval hyperaemia, bellowing, copious discharge hanging from the vulva at the early phase of estrus and standing to be mounted behaviour respectively in multiparous Murrah buffaloes.

Fig 1: Visual and behavioural signs of heat observed in Murrah buffaloes.

described by Donnan, (1996) and adopted by Wai-Hoe *et al*. (2009) with slight modifications. The frozen urine samples were thawed on ice, vortexed well and 25 ml of each sample was taken in 50 ml falcon tubes. To each urine sample, 13.8 g of ammonium sulphate was added. The salt was mixed by inverting the tubes several times, followed by gentle vortex for 2 min in ice. The mixture was then transferred to Oakridge tubes, centrifuged at 10,000 g for 20 min at 20°C. The supernatants were discarded carefully and the pellets were dissolved in 180 µl of tris SDS EDTA (TSE) buffer and 55 µl of reducing sample buffer (RSB). The resultant protein pellet was solubilized and reduced by heating at 100°C for 10 min.

Protein separation by SDS-PAGE

Ten µl of the reduced protein samples were loaded on to 12% SDS- polyacrylamide gels along with the protein cutoff (Reid *et al*., 2012). The protein bands between 69-100 KDa were considered as THP.

Transfer of protein to solid support and THP visualization

Urinary proteins separated by SDS-PAGE were transferred to solid support and THP was visualized by adopting the procedures described by Mahmood and Yang, (2012) and Chacar *et al*. (2017) with minor modifications. The unstained protein samples separated by SDS-PAGE as described above were transferred on to PVDF (Polyvinylidene difluoride) membrane by overnight transfer *i.e*., 20 volts for 16 hrs by following the standard protocol. The blot was blocked in 5% skimmed milk powder (SMP) prepared in 1×TBST, incubated for 1 hr at RT in a gel rocker. The blot was then washed thrice with 1×TBST. The blot was probed with primary antibody to THP by using commercially available anti-Tamm-Horsfall Glycoprotein Antibody, AB733 from Sigma-Aldrich in 1:1000 dilution with 5% bovine serum albumin (BSA) in 1×TBST. The blot was then sealed and allowed for antibody binding for 10 hrs at 4°C in cold room overnight (Mahmood and Yang, 2012). After incubation, the blot was washed thrice with 1×TBST for 3 min at low speed. The blot was then probed with secondary antibody using the commercially available anti-Sheep IgG peroxidase conjugate, A 3415 from Sigma-Aldrich in 1:10,000 dilutions with 1% SMP in 1×TBST and incubated at RT for 1hr in gel rocker at low speed. After incubation, the blot was washed thrice with 1×TBST.

The blot was then developed using clarity max enhanced chemiluminescence (ECL) western blotting substrates from Biorad using the Chemi Documentation Imaging system which allowed for the visualization of THP. The level of THP expression was assayed using ImageJ software (Davarinejad, 2015). One-way analysis of variance (ANOVA) was employed to know if there are significant differences between the three groups.

RESULTS AND DISCUSSION

Grouping of animals

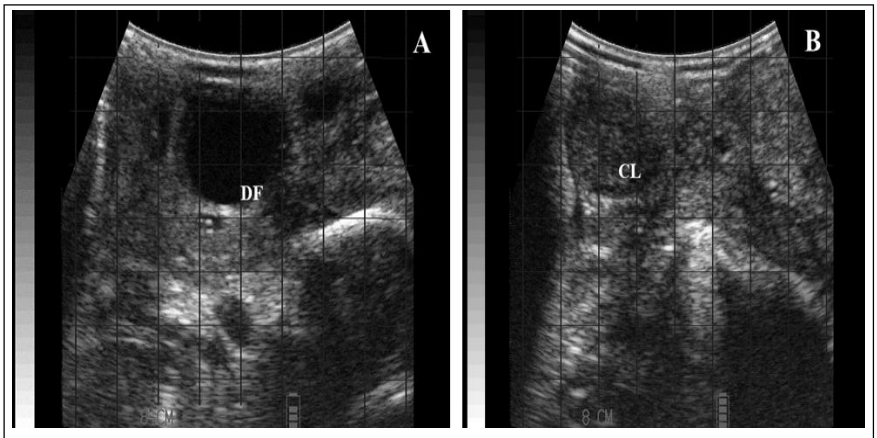
Grouping of animals was done as per a combinatorial approach which included looking for behavioural signs, observation by TRUS, CVM fern pattern, serum E2 and P4 levels (Manasa Varra *et al*., 2022).

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

The mean serum E_2 and P_4 levels of G-I, G-II and G-III animals are given in Table 1. The serum E_2 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 serum E_2 concentration in G-II and G-III in comparison to G-I, while the serum P_4 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 lower serum P_4 concentration in G-II and G-III in comparison to G-I.

Isolation and quantification of THP by Western blot

THP expression as obtained by western blot analysis in G-I, G-II and G-III animals is shown in Fig 3. Likewise, the mean



A depicts the dominant follicle (DF) present at the estrus stage and Fig. B shows the corpus luteum (CL) present at the mid-diestrus stage of estrous cycle.

Fig 2: TRUS of the ovarian follicles.

urinary THP concentration in G-I, G-II and G-III animals is shown in Table 2. Statistical analysis to compare the THP expression level between G-I, G-II and G-III animals revealed no significant differences. However, the mean values when perused revealed for highest value in G-II animal, followed by G-III animals and thereafter in G-I animal.

The present research sought to view the expression levels of THP in urine of buffaloes at the estrus and diestrus stages with the hypothesis of the existence of the physiological role of THP during the estrus stage of the estrous cycle. We were also curious to know if the expression levels of this protein vary between the animals at the estrus phase *i.e* those showing signs of heat and not exhibiting signs of heat.

Combinatorial approach of grouping animals

In the present study, grouping of animals was done as per a combinatorial approach. Mondal *et al*. (2010) and Kumar *et al*. (2021) reported the circulatory E_2 levels (pg/ml) in diestrus animals to be 11.04±0.13 and 3.48±0.21 respectively. While, Muthukumar *et al*. (2018) has revealed E_2 concentration in diestrus animals to range from 7.83±3.90 to 12.61±1.36. Likewise, Mondal *et al*. (2010) and Kumar *et al*. (2021) reported the circulatory P_4 levels (ng/ml) in diestrus animals to be 1.94±0.03 and 4.19±0.20 respectively. While, Muthukumar *et al*. (2018) has described the P_4 concentration in diestrus animals to range from 2.60±0.41 to 2.78±0.49. In yet another study, Hebbar *et al*. (2021) reported the circulatory E_2 levels in buffaloes at the diestrus stage to be 16.28±1.174.

At the estrus stage, Kumar *et al*. (2021) observed that the serum E_2 levels (pg/ml) in normal estrus (animals showing signs of heat) and silent estrus buffaloes (animals not exhibiting signs of heat) to be 16.36±0.18 and 8.16±0.11 respectively, while the serum P_4 levels (ng/ml) in normal estrus and silent estrus buffaloes at the estrus stage were found to be 0.72±0.14 and 0.36±0.56 respectively. Accordingly, the present study revealing for no significant differences in serum E_2 and P_4 levels between G-II (Normal estrus animals) and G-III (Silent estrus animals) indicates that circulatory levels of E_2 and P_4 are not attributing to silent heat in buffaloes and the reason for silent heat in buffaloes is yet to be elucidated.

However, G-III animals of the present study being the post-partum animals, having no significant differences in serum E_2 and P_4 levels observed between G-II and G-III animals indicates that G-III animals may be in silent heat due to physiology behind the post-partum stress as postulated by Allrich *et al*. (1994) and not due to differences in serum E_2 and P_4 levels.

Tamm-Horsfall protein (THP) expression in urine of G-I, G-II and G-III animals

The study of the expression of THP gene in various tissues by RT-PCR revealed for its specific expression only in kidneys (Zhu *et al*., 2002). However, there are no reports on the expression of THP in the tissues of the female reproductive tract. In this context, this is the first study reporting the expression of THP in urine of buffaloes by western blot at the estrus stage and diestrus stage. In addition, an easy and simple method has been developed that facilitated THP detection and quantification in urine of buffaloes by western blot omitting the steps of protein purification (like dialysis and column-based separation techniques). Further, the detection of THP by western blot with the use of commercially available polyclonal antibodies to THP as primary antibodies indicated the suitability of polyclonal antibodies for downstream proteomic research in buffaloes. THP expression in the present study was normalized to loading of constant volume of reduced protein extract in SDS-PAGE (Reid *et al*., 2012). The other techniques recommended for normalization of protein levels in urine are normalization to urinary creatinine (Gunasekaran *et al*., 2019; Soomro *et al*., 2022) and total protein (O'rourke *et al*., 2019).

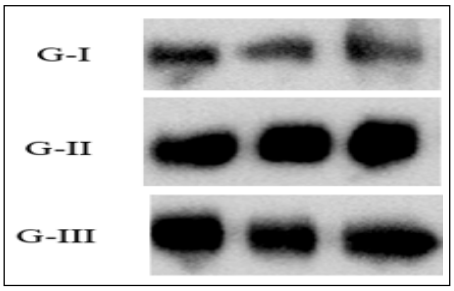


Fig 3: Visualization of THP by western blot.

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)	8.00 ± 2.082^{a1}	23.17 ± 0.928^{b2}	19.33 ± 1.014^{b3}
P_4 (ng/ml)	3.33±1.924^{a1}	0.22±0.125^{b2}	0.23±0.135^{b3}

Note: Means bearing different alphabets as superscripts within a row differ significantly ($p<0.05$) and means bearing different numerical as superscripts with in a column differ significantly.

Table 2: Tamm-Horsfall protein levels in G-I, G-II and G-III animals.

Group	G-I	G-II	G-III
Mean±SE	24560.79±2005.09^{a}	39454.96±6110.30^{a}	32182.60±3384.05^{a}

Note: Mean±SE bearing same superscripts (lowercase within the rows) are statistically non-significant.

THP which is an anti-microbial mucoprotein was found to be differentially expressed in mice vaginal fluid during the estrus stage (Cerna *et al*., 2017). Vaginal fluids are secreted by the epithelial cells lining the vagina (Adnane *et al*., 2018) indicating that THP might be secreted by either the epithelial cells lining the vagina or would have reached the vaginal tissue *via* systemic circulation to be up-regulated (2.3-fold) during estrus stage when compared to other stages of estrous cycle in mouse (Cerna *et al*., 2017). THP is also found to form viscous barriers in the oviducts under low physiological pH (Cerna *et al*., 2017) that prevails during estrus stage probably to prevent infection.

Further, it has been put forth by Lee *et al*. (2016) that in cattle, the mRNA transcripts of lactoferrin (LF) were highly expressed in uterine tissue as well as LF protein was highly expressed in bovine CVM during estrus stage. Accordingly, as postulated by Siao *et al*. (2011), Li *et al*. (2014) and Wu *et al*. (2018), THP might exerts its antimicrobial effect by enhancing the polymorphonuclear (PMN) phagocytosis by means of binding to the LF protein that is found to be highly expressed during the estrus stage, the area which needs to be explored in future.

Mahalingam *et al*. (2019) described *Firmicutes* as the predominant genus present in buffalo vaginal mucus at the estrus stage. So also, Noguchi *et al.* (2003) in their studies on mice, rats, hamsters and dogs postulated that the total number of vaginal flora (bacteria) is found to increase in estrus stage of estrous cycle when compared to that in diestrus stage or at anestrus. Likewise, Mahesh *et al*. (2021) in his studies on different phylogenetic level of the vaginal microbiota during estrous cycle demonstrated, rich diversity and dynamics of the microbiota in the vaginal flushing of buffalo heifers during various stages of estrous cycle. On the other hand, estrus in bovines is dynamic and the estrus cycle is regulated by interplay of different organs and hormones inclusive of complex biological system (Boer *et al*., 2011).

The present study revealing increased urinary expression of THP in G-II and G-III animals when compared to G-I animals (though the levels failed to find statistical significance) probably construes the antimicrobial role of THP in the female reproductive tract and its role during estrus to prevent microbiota population beyond the physiological levels. The present study implicates the role of circulatory E_2 levels on the THP expression. Further, the findings of Sciorsci *et al*. (2020) in buffaloes at the estrus stage having highest ROS levels might also attribute to the increased expression of urinary THP at the estrus stage (G-II and G-III) compared to the diestrus stage (G-I).

Hence from the foregoing, the increase in THP expression in G-II animals compared to G-III and G-I animals could be due to the reasons put forth by various authors (Siao *et al*., 2011; Boer *et al*., 2011; Li *et al*., 2014; Lee *et al*., 2016; Cerna *et al*., 2017; Wu *et al*., 2018; Mahalingam *et al*., 2019; Mahesh *et al*., 2021) and / it could be opined that this increased urinary THP expression in estrus animals could be to elicit its protective action in the female reproductive tract at the estrus stage.

CONCLUSION

In the present study, the absence of any significant differences in the urinary THP levels with only values being different between the groups construes that though THP is having a physiological role in estrus stage, but for it to considered as biomarker for detecting silent heat buffaloes there is need for further studies involving large number of animals.

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Conflict of interest: None.

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