



Serum Amino Acids are Differentially Present in the Buffalo Estrous Cycle

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ABSTRACT

Background: Amino acids are essential for various physiological processes, including animal reproductive functions. However, amino acids function in estrus cycle has yet to be investigated. This study seeks to address this knowledge gap by exploring the role and importance of various amino acids during estrus and diestrus stages.

Methods: Blood samples were collected after confirming estrus in the animals through ultrasonography and analyzing the characteristic salivary fern patterns observed on the day of estrus. Serum then separated by centrifugation at 2000×g for 10min. Subsequently, the serum samples were processed and analyzed using LC/MS/MS with an API 3000 triple quadrupole mass spectrometry system.

Result: Alanine, Arginine, Cystine, Glutamine, Histidine, Leucine, Isoleucine, Serine, Tryptophan, Phenylalanine and Valine levels showed significant differences ($p < 0.001$) compared to other amino acids. Previous studies suggested L-alanine and Arginine significance in theca and granulosa cells development.

Key words: Amino acids, Buffalo, Estrus, Glycine, L-alanine, LCMS.

INTRODUCTION

Domestic animals, such as buffaloes, perform crucial functions in rural economy (Nakao and Singh-Nanda, 2003). Reproduction of animals is a crucial step in ensuring the continuation of their generation for the benefit of humanity (Narita *et al.*, 2011). Estrus initiates the propagation of the next generation. This is influenced by several factors, including amino acids, which are essential for many physiological activities (Bhatia *et al.*, 2021; Nayan *et al.*, 2020, 2021; Onteru *et al.*, 2016; Ravinder *et al.*, 2016; Selvam *et al.*, 2017; Suria *et al.*, 2021). Cytosolic O-glycosylation of glutamine stimulates production of argininosuccinic synthetase gene (Brasse-Lagnel *et al.*, 2003). Supplementing with L-arginine influences gene expression in adipose tissue (Jobgen *et al.*, 2009), reproductive performance and metabolism (Elango *et al.*, 2009; Mateo *et al.*, 2007). Previous studies (Bronte and Zanollo, 2005) done on amino acids in many animals have opened the door to a hitherto unknown field of amino acids and their significance. When methionine has been added to the diet "instead of any other single amino acid, the inhibition of fecundity imposed on an amino acid-deficient diet has been alleviated. According to" this evidence, dietary quantities of amino acids, either in absolute or balanced amounts, might influence reproductive function in addition to caloric content (Bass *et al.*, 2017).

According to research, "dietary amino acids additionally regulate transcriptional activity of hepatic estrogen receptors *via* mechanism that is dependent on mammalian target of rapamycin (mTOR). In mice, liver produces insulin-like growth factor1tr (IGF-1) in response to stimulation by hepatic estrogen receptor alpha". This molecule signals

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reproductive system of the nutritional status of mice (della Torre *et al.*, 2011). It is intriguing to consider if modifications in the diet's amino acid composition would have an impact on female reproductive function since these hormone levels, especially IGF-1 levels, are strongly influenced by the diet's protein and amino acid contents. Though many studies were done in different physiological conditions, there is a substantial research gap in amino acid composition during the estrous cycle in buffaloes. Because of the gaps existing, questions were asked, do amino acids differ from estrus and diestrus? If they differ what are the amino acids that were different in two stages? With these questions, it was hypothesized that there is a significant difference in amino acids during the estrous cycle.

MATERIALS AND METHODS

Selection of buffalo heifers

During the winter (October to February 2020-21), 40 buffalo heifers from the Central Institute for Research on Buffaloes, Hisar, Haryana (Fig 1) had been selected by considering heat symptoms including vulval lip swelling, cervical secretions and restlessness. Hisar is 212 m above mean sea level, latitude is 29.17 N and longitude is 75.72 E. These were studied further as follows.

Estrus confirmation by ultrasonography

While being held in a trevis, the selected heifers' vulval lips were disinfected and washed following aseptic techniques. A Hitachi transrectal probe with a frequency of 7.5 MHz then inserted into rectum and gently moved across the uterine surface for examining the follicles and ovaries. Heifers having follicle diameters more than 12 mm have been determined as estrus animals and follicular length has been determined by examining ovary impressions.

Estrus confirmation by salivary fern pattern

A sterile Pasteur pipette has been employed for collecting saliva that had been deposited on the lower lip in a micro-centrifuge tube prior to the animals being fed. Saliva samples had been collected at 6:30 a.m. every morning. 40 samples of saliva have been collected on average, once daily. For removing any feed, soil, or cells, saliva has brought to lab on ice during oestrus period and centrifuged for 10 min at 4°C at 3000 Xg. They transferred the supernatant to another container. A smear has been prepared on sterile glass slide utilizing 20 µl of cell and dirt-free saliva. (Ravinder *et al.*, 2016). Before being inspected for fern pattern or salivary crystallization employing an inverted microscope (Olympus, Magnus INVI, Japan), this had been exposed to air for 5-10 min. They were subsequently later captured as photographs.

Blood collection

In clot activators, blood (10 ml) have been collected from screened and selected buffalo heifers exhibiting estrus symptoms by aseptically puncturing left jugular vein. To extract serum, collected blood has been left at room temperature for 4 hr. Serum has been extracted, centrifuged for 8 min at 2000 g, then aliquoted in 2 ml falcon tubes for additional processing.

LC/MS/MS sample preparation

In 1.5 mL polypropylene microfuge tube, 100 mL of serum has been combined with 850 mL of methanol and 50 mL of internal standard. Exception of "D3-MET and D2-tyrosine (TYR)", that have been detected at 10 and 25 mmol/L, accordingly, "internal standards remained present at a final concentration of 50 mmol/L. 400 mL of methanolic extract had been thoroughly dried under nitrogen when mixture has been vortexed, permitted to stand for 10 min, then centrifuged for 3 min at 10,000 g. The sample residue had been mixed with 100mL of 3NHCl/butanol and incubated

in capped borosilicate vial at 60°C for 7.5 min. Upon mutilation, mixture had been reconstituted in 250 mL "of mobile phase (20% acetonitrile, 0.1% formic acid), dried entirely under nitrogen, then shifted to borosilicate autosampler vial for injection". Analysis of "LC/MS/MS" "An API 3000 triple quadrupole mass spectrometry equipment" "(Applied Biosystems, Foster City, CA, USA)" has been employed for executing MS/MS analysis. Flow has been split 1:4 into collision gas at 10mTor, nebulizer gas at 14 mTor, curtain gas at 12 mTor and an "electrospray ionization(ESI)" source running at 325°C in positive mode at 2000V. Multiple Reaction Monitoring (MRM) mode has been employed for collecting data utilizing "Analyst software (version 1.4.2)". Each amino acid's optimum fragmentation patterns had been determined at concentration of about 10 mM. Polypropylene glycol has been employed for adjusting MS to unit mass resolution (± 0.7 AMU). An HPLC system (1100) (Waters, Germany, CA, USA) has been employed to introduce 10 mL of sample. Samples have been added to an isocratic flow of 0.1% formic acid and 20% acetonitrile at rate of 1ml/min. A C 8, 4.6 12.5 mm 5 mm guard column and a 4.6 150 mm 5 mm resolving column (Zorbax Eclipse XDB-C8, Agilent) had been utilized for chromatography. There had been a 22-minute interval between injections. When 1000 mmol/L sample had been examined in triplicates, carryover had been determined employing sample blank and had been 1 µmol/L for all amino acids.

Statistical analysis

Graphpad Prism version 9 has been employed for conducting a paired t-test at different significance levels ($P < 0.001$ and $P < 0.05$).

RESULTS AND DISCUSSION

Ultrasonography revealed presence of 12–15 mm follicles, confirming that the animals were in the estrus stage (Fig 2). Additionally, a distinct salivary fern pattern, a clear indicator of estrus, was observed (Fig 3). Results have been consistent with observations reported by Bhat *et al.* (2014).



Fig 1: Showing the location of CIRB farm with geographic coordinates and elevation; Situated in Hisar is 212 m above mean sea level, latitude is 29.17 N and longitude is 75.72 E.

The follicle measured 15 mm on ultrasonography (Morris and Billiar, 1994) and revealed a substantial salivary fern pattern (Zhang *et al.*, 2017).

Amino acids

Many amino acids and their intermediates were found to be markedly differentially expressed out of all amino acids, arginine, asparagine, β -alanine, cysteine, glutamine, histidine, gamma-hydroxylysine, hydroxyproline, isoleucine, leucine, tryptophan and valine have significantly differed at $P < 0.001$ and at $P < 0.05$ as given in (Fig 4,5,6; Table 1) with diestrus group. However, argininosuccinic acid, aspartic acid, beta-amino isobutyric acid, glutamic acid, glycine, lysine, methionine, N-methyl histidine, threonine, proline, tyrosine, methyl histidine, had been insignificant (Fig 4,7; Table 2).

The levels of L- alanine were observed more during estrus than diestrus. These findings are in agreement with Gatien *et al.* (2019) and suggest L-alanine plays a crucial role in glucose metabolism. Low levels of L-alanine concentration during diestrus hypothecates the L-alanine involvement in higher production of glucose through gluconeogenesis. Seli *et al.* (2014) observed that in oocytes L-alanine had a role in cholesterol synthesis in the presence of growth factors (Fig 8). However, since there were no conclusive studies on glucose levels during estrus and diestrus, it is difficult to conclude the role of L-alanine in glucose metabolism. Arginine levels are elevated during the diestrus phase compared to the estrus phase, consistent with previous findings (Gatien *et al.*, 2019), indicating that these variations may be driven by hormonal fluctuations. In several species, arginine has been demonstrated for improving specific reproductive processes, that includes placental and fetal growth (Redel *et al.*, 2016). Besides, arginine has affected ovarian functions in several cells, that includes granulosa and luteal cell secretory activities, follicular survival and/or luteal cell apoptosis (Jain *et al.*, 2012), an increase in arginine concentration during the diestrus phase, in turn, catalyzes CYR61, a potential molecular mediator of angiogenesis in

the CL (Fig 8). Its function in angiogenesis that follows luteal development is confirmed by higher levels of expression of CYR61 in developing CL. When combined,

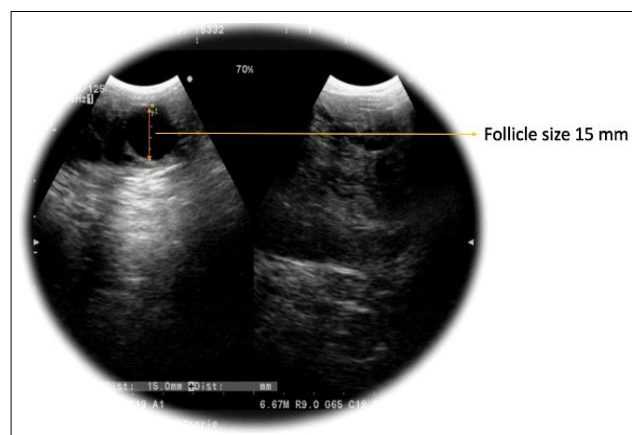


Fig 2: Ultrasonography picture depicting the follicular size with 15 mm. It confirms buffalo are in heat.



Fig 3: Typical salivary fern patten signifies the buffaloes are in mid estrus.

Table 1: Amino acids and their intermediates significantly differ between estrus and diestrus.

Aminoacids	Estrus	Diestrus	P value
Alanine	420.12±66.60	330.31±11.56	$P < 0.001$
Alanine	410±57.9	288±34.42	$P < 0.05$
Arginine	58.70±4.21	118.63±9.5	$P < 0.001$
Cysteine	29.81±3.6	25.07±4.25	$P < 0.05$
Glutamine	79.84±45.02	143.91±13.9	$P < 0.05$
Histidine	125±8.02	62.40±5.8	$P < 0.001$
Hydroxylysine	0.96±0.47	0.32±0.53	$P < 0.05$
Hydroxyproline	31.51±1.77	26.88±1.35	$P < 0.001$
Isoleucine	115.38±6.3	99.09±5.7	$P < 0.001$
Leucine	220.8±12.32	143.22±8.2	$P < 0.001$
Tryptophan	95.66±8.5	54.48±5.9	$P < 0.001$
Valine	299±24.90	246.73±18.73	$P < 0.001$

these results indicate that CYR61 may serve an essential function in controlling angiogenic switch in Corpus luteum's lifespan (Van Winkle *et al.*, 1990).

The most prevalent amino acid had been glycine, that is conditionally required. However, since it is a

necessary precursor for the production of proteins and nucleic acids, glycine is known for having an essential function in the early stages of embryonic development. Additionally, glycine is necessary for cells to proliferate rapidly (Downing *et al.*, 1995). Additionally, glycine

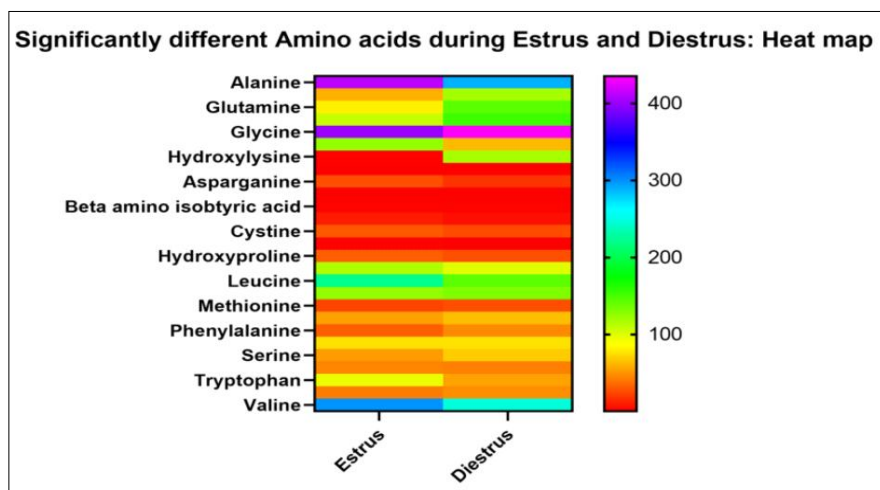


Fig 4: Heat map depicting significant difference in amino acids; legend bar interprets the amount of amino acid present in estrus and diestrus stages.

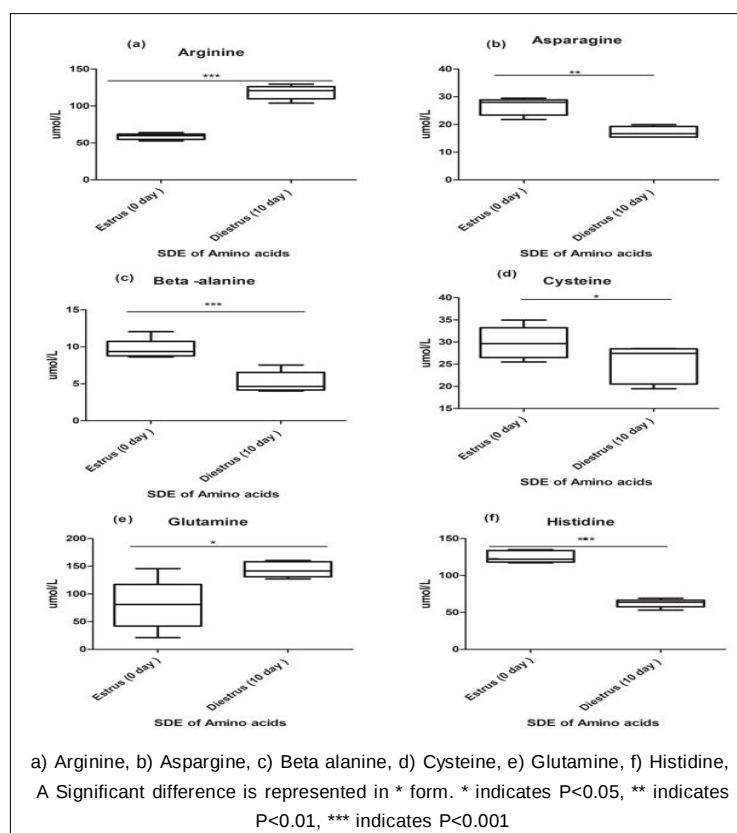


Fig 5: Box plot showing significant difference in amino acids between estrus and diestrus stages.

controls intracellular pH and could protect preimplantation embryos from osmotic stress (Grussing *et al.*, 2016). Transporter of glycine GLYT1 is a protein

that transports glycine into cells and regulates cell volume. It is activated during oocyte maturation (Baltz and Tartia, 2009).

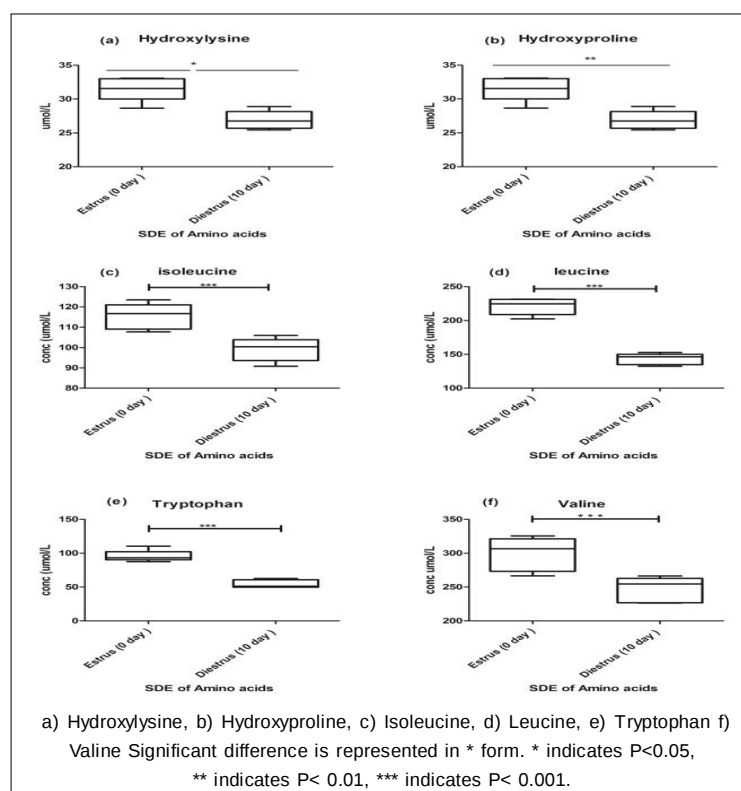


Fig 6: Box plots depicting amino acids that differ significantly between estrus and diestrus.

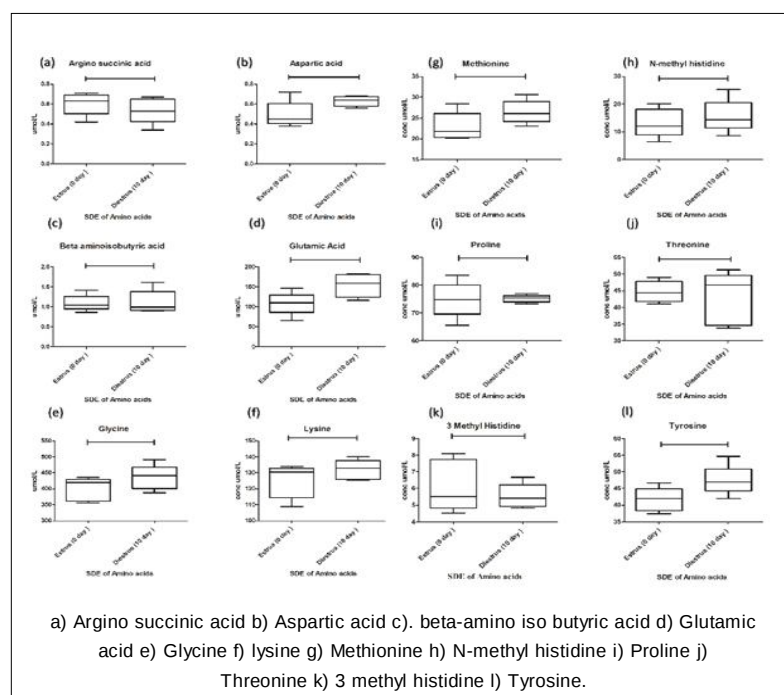


Fig 7: Non-significant amino acids.

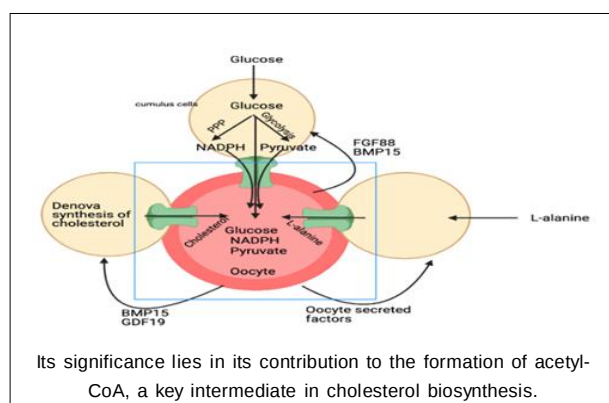


Fig 8: L-Alanine plays a pivotal role in the synthesis of cholesterol, a vital compound serving as a precursor for the production of steroid hormones.

Table 2: Amino acids without significant difference between estrus and diestrus.

Amino acids	Estrus	Diestrus	P-value
Arginosuccinic acid	0.60±0.112	0.5±0.12	0.5
Aspartic acid	0.49±.13	0.63±0.05	.10
Beta amino iso butyric acid	1.09±.20	1.14±0.29	0.68
Glutamic acid	108±28.93	153.77±29.02	0.08
Glycine	399.5±36.00	435.32±38.65	0.16
Lysine	125±10.45	132.014±6.12	0.10
Methionine	22.94±3.36	26.46±2.78	0.059
N-Methyl histidine	13.24±5.16	15.61±6.02	0.9
Proline	74.80±6.4	75.15±1.33	0.71
Threonine	44.69±3.18	43.00±7.8	0.27

Leucine levels are higher during the estrus period as it is essential during the conversion of the preantral follicle to the antral follicle (Orsi *et al.*, 2005). Additionally, recent research demonstrated that glycine and cysteine transport levels increased during oocyte maturation (Collado-Fernandez *et al.*, 2012).

Elevated levels of histidine during estrus day prepare the uterus for preimplantation (Grussing *et al.*, 2016). Hormonally controlled histamine synthesis is considered to assist in preparing the uterus for embryo implantation (Wood *et al.*, 2000). HDC is the rate-limiting enzyme in histamine production. Preimplantation mouse uterus expressed large quantities of HDC mRNA, increasing on day four. This occurred around the same period of the estrous cycle as when lupin grain or glucose infusion increased rate of ovulation. Injecting same amino acids into ewes increased ovulation by 20% (Miller *et al.*, 1986). On estrus day levels of leucine and valine are more than diestrus. Leucine and valine enhance ovulation rate by balancing negative energy balance throughout estrus cycle (Grussing *et al.*, 2016). Leucine interacts with tissue synthesis pathways including IGF-1 and mTOR, which could assist with preovulatory follicles and luteal tissue proliferation. A slight increase in dietary leucine impacts

prolactin synthesis and release, enhancing estrus cycle progression.

CONCLUSION

All metabolic activities in body depend on amino acids. Their function in estrous cycle regulation has been demonstrated in the current research. Significant differences were observed in alanine, arginine, anserine, histidine, tryptophan and isoleucine levels between estrus and diestrus stages ($P<0.001$). Significant differences in cystine and glutathione levels have been observed between stages ($P<0.05$). Contrary to these insignificant differences were noted for other amino acids across the two stages. These amino acids may regulate estrus cycle, according to these results. However, an in-depth understanding of amino acids in buffaloes remains a distant goal. A comprehensive study is required in the future to elucidate the roles of amino acids and the pathways they influence.

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Disclaimers

The authors' views and conclusions don't always reflect those of their affiliated institutions. Although the authors take responsibility for the quality and accuracy of the information they provide they disclaim all liability for any losses, whether direct or indirect, that might result from utilizing this content.

Informed consent

All animal procedures for experiments have been approved by Institute Animal Ethics Committee of CIRB vide IAEC-CIRB/19-20/A/016 dated 05.08.19.

Conflict of interest

The authors of this article state that they have no conflicts of interest about its publication. No funding or sponsorship influenced design of research, data collection, analysis, decision in publishing, or preparation of manuscript.

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