ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Detectionof fatty acids in Bovine (*Bosindicus*)urine during different phases of estrous cycle using gas chromatography

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Manuscript Info *Abstract*

Manuscript History:

Received: 11 August 2013
Final Accepted: 22 August 2013
Published Online: September 2013

Key words:
urine, fatty acids,
pheromone, estrus,
bovine, artificial insemination.

A simple gas – liquid chromatographicmethod is described for determining fatty acidsin various reproductive phasesofestrous cycles of bovine urine samples. In the present study totally 62 fatty acids were identified from various reproductive phases such as proestrous, estrous andpost estrous. Among them, 15are saturated fatty acids, 26 are monosaturated fatty acidsand 21 are polyunsaturated. The amount of fatty acids is found to be in high level during estrus phase when compared to other phases. The saturated fatty acids and monosaturated found to be higher during estrous periodfollowed post estrous period. Hence, the present results concluded that the fatty acids present in estrus may pave the way to detect the estrus period very effectively in order to increase the successive rate of artificial insemination.

Introduction

Animals communicate information concerning reproduction to conspecifics in order to co-ordinate reproductive activities. The reaction involves either the release of a specific behaviour or physiological change in the recipients' endocrine or reproductive system (Doty, 1976; Izard, 1983). Exteroceptive cues that likely play a role in male-female interactions include olfactory, visual, auditory and tactile stimuli (Zalesky*et al.*,1984).The odours of most mammalian species probably vary with their reproductive state. For instance the urinary volatiles differ during the estrous cycle (Rameshkumar*et al.,* 2000), pregnancy (Rameshkumar and Archunan 2001) and lactation (Rameshkumar*et al.,* 2003) in bovine.

The substance present in the vaginal secretions of estrogen-stimulated rhesus monkey 'couplins' contains short chain aliphatic acids such as acetic, propionic, isobutyric, n-butyric, and isoalanine seems to evoke mounting and ejaculatory behavior (Diani*et al,* 1998). Volatile fatty acids such as, acetic, proponic, butanoic acids are identified in normal physiologic constituents of the vaginal secretions in healthy young women with regular menstrual cycles. These acids vary during menstrual cycle and reaching a peak near the time of ovulation, as they do in infra-primates (Preti*et al,* 2003). Fatty acids in general are utilized in the body as the principal sources of energy and hence a proportion of EFA (linoleic and linolenic) also contributes to provide energy. The PUFA are oxidized more rapidly than saturated or monounsaturated fatty acids (Dupont, 1998).

The pheromones and their carrier protein are involved in the chemical communication apart from the lipid fractions identified from the marking fluid of the tiger and considered that it could be responsible for the characteristic odour of the animal (Bramachary*et al.,* 1992). Hiradecky (1986) reported that the volatile fatty acids present in the urine and vaginal secretion of cows during the reproductive cycle. Regardless of whether volatile fatty acids are true pheromones or not, these acids may be related to the reproductive functions and, therefore, deserve closer study both from the point of view of reproductive and pheromonal physiology and for possible diagnostic utilization (Hiradecky, 1986).

Urine and vaginal mucus from estrous females are able to elicit behavioural responses in males; estrus related chemicals might also be used be as sexual stimulants to increase libido in old or otherwise reproductively unmotivated males (Rameshkumar*et al.,* 2000). Recently the volatile compound 1-iodoundecane from estrous urineof bovine confirmed

in attracting the bull (Archunan andRameshkumar,2012).
The important problemin large herds of cows is the lack of effective estrus detection (Kiddy *etal.*, 1984). In most of mammalian species except primates the estrus females express the desire and inform the male of their receptive state by means of chemical signals from the male of their receptive state by means of chemical signals from urine and vaginal discharge (Klemm*et al.*,1994).
Livestock production in the tropical areas is generally low relative to temperate areas,largely due to the effects of nutrition and other environmental stresses (Fitzpatrick, 1996). Various attempts have been made on the regulation and control of bovine reproduction through the use of hormones and application of biological agents (Peters and Ball, 1995). Some of these applications have so far failed to be consistently effective and besides, some of these biological are not easily available or too expensive for farmers in developing countries. Therefore, harnessing the reproductive potential of livestock species in developing countries may require development of management strategies such as bio stimulation to effectively improve reproduction.Hence, the present investigation is carried out to evaluate urinary the fatty acid profiles during different phases of bovine.

Material and Methods

Animals and Sample collection

Urinary samples were collected from six cows of *Bosindicus*(Umblachery)maintained in the District Livestock farm, Korukkai, Thiruthuraipoondi, Tamil nadu and they were pooled together to avoid individual variations and used for gas chromatography analysis.The stages of the estrous cycles were carefully examined for two to three consecutive cycles by rectal palpation. The animals were fed with standard diet and water *ad libitum*.

Fatty acid profile

The lipid was extracted from the collected sample by using chloroform and methanol (1:1) and the total lipid was estimated by Folch*et al.,* (1957). Five ml of urine sample was taken and mixed with saponification reagent. The tubes were tightly closed and kept for 30 minutes at 60°C in a water bath. 2 ml of methylation reagent was added to each tube and kept again in the water bath at 80°C for 20 minutes. Finally, a sufficient amount of extraction solvent (200 ml hexane + 200 ml of diethyl ether) was added to each tube, and then closed tightly, and shaken thoroughly for 10 minutes. About 2/3 of the organic phase (upper layer) containing the fatty acid methyl esters were transferred into screw cap glass vials. From each vial 1 μl of the fatty acid methyl ester (FAME) was injected into the Gas Chromatography (GC) column (Miller and Berger, 1985).

Results

Fatty acid profile of the test sample was investigated by gas chromatography and the profile is summarized in Table 1, 2 and 3. Totally 62 fatty acids were identified from estrous cycle. Among them, 15 fatty acids were saturated, 26 fatty acids were monounsaturated and 21 fatty acids were polyunsaturated.

Table – 1: Saturated fatty acid profile of bovine urine by gas chromatography

Car.chain	Fatty acid	Pro estrus	Estrus	Post Estrus
C11:0	Undecyclic acid	0.17± 0.01	0.12± 0.01	0.19± 0.01
C12:0	Lauric acid	0.19± 0.01	1.30± 0.02	1.11± 0.02
C13:0	Tri decyclic acid	2.11± 0.02	-	1.00± 0.02
C14:0	Myristic acid	1.10± 0.02	0.84± 0.02	0.84± 0.01
C15:0	Pentadecyclic acid	0.81± 0.01	-	0.83± 0.01
C16:0	Palmitic acid	13.42± 0.03	0.17± 0.01	11.61± 0.05
C17:0	Margaric acid	1.77± 0.02	15.00± 0.05	1.61± 0.02
C18:0	Stearic acid	0.84± 0.01	16.00± 0.18	0.77± 0.01
C19:0	Nonadecyclic acid	2.79± 0.02	2.00± 0.02	0.54± 0.01
C20:0	Arachidic acid	0.66± 0.01	-	3.16± 0.02
C21:0	Heneicosanoic acid	0.44± 0.01	0.92± 0.02	0.77± 0.01
C22:0	Pehenic acid	1.31± 0.01	1.30± 0.02	0.84± 0.01
C23:0	Tricosanic acid	1.00± 0.01	1.00± 0.01	1.79± 0.02
C24:0	Lignoceric acid	0.63± 0.01	-	0.84± 0.02
C24:0	Hexacosanoic acid	2.11± 0.02	-	4.16± 0.03
Σ Of SFAs		**29.35± 0.05 ^{b}**	**38.65± 0.06^{a}**	**30.06± 0.04^{b}**

Values are expressed in Mean ± SDE.

Table – 2: Monounsaturatedfatty acid profile of bovine urine by gas chromatography

Car.chain	Fatty acid	Pro estrus	Estrus	Post Estrus
C12:1ω-4c	Linderic acid	2.71± 0.03	-	3.16± 0.03
C14:1ω-3	Cis-3 Myristoleic acid	0.84± 0.02	-	0.17± 0.01
C14:1ω-5	Trans-5 Myristoleic acid	1.00± 0.02	-	0.11± 0.01
C14:1ω-7	Cis-7 Myristoleic acid	0.61± 0.01	0.17± 0.01	-
C15:1ω-6	Cis-6-Pentadecenoic	0.54± 0.01	0.18± 0.01	0.51± 0.01
C16:1ω-5	Cis-5-Palmitoleic acid	0.13± 0.01	-	0.44± 0.01
C16:1ω-6	Cis-6-Palmitoleic acid	0.81± 0.01	0.92± 0.02	-
C16:1ω-7	Trans-7-Palmitoleic acid	0.77± 0.01	1.00± 0.03	0.17± 0.01
C16:1ω-9	Trans-9-Palmitoleic acid	1.34± 0.02	1.35± 0.03	0.97± 0.02
C17:1 ω-5	Cis-5- Heptadecenoic acid	1.11± 0.02	5.50± 0.04	1.17± 0.02
C17:1ω-7	Cis-7- Heptadecenoic acid	-	4.30± 0.03	0.11± 0.01
C17:1ω-8	Trans-8-Heptadecenoic acid	0.81± 0.02	0.72± 0.01	0.91± 0.01
C18:1ω-5	Cis-5-Octadecenoic acid	0.79± 0.01	0.19± 0.01	1.34± 0.02
C18:1ω-7	Cis-7-Octadecenoic acid	-	0.17± 0.01	0.16± 0.01
C18:1ω-9	Oleic acid	6.11± 0.03	15.00± 0.04	7.00± 0.04
C19:1ω-8	Nonadecenoic acid	0.81± 0.02	7.12± 0.03	0.80± 0.01
C20:1ω-5	Cis-5- Eicosenoic acid	0.77± 0.01	0.43± 0.01	0.70± 0.01
C20:1ω-7	Cis-7- Eicosenoic acid	0.41± 0.01	0.11± 0.01	0.61± 0.01
C20:1ω-9	Cis-9- Eicosenoic acid	0.33± 0.01	0.18± 0.01	0.11± 0.01
C20:1ω-11	Trans – 11- Eicosenoic	0.32± 0.02	0.17± 0.01	0.34± 0.01
C22:1ω-7	Cis-7- Docosenoic acid	2.00± 0.02	1.32± 0.03	1.00± 0.01
C22:1ω-9	Erucic acid	3.11± 0.03	-	3.00± 0.04
C23:1ω-9	Trans-9- Docosenoic acid	0.11± 0.01	0.11± 0.01	0.11± 0.01
C24:1ω-3	Cis-3- Tetrasenoic acid	0.17± 0.01	0.14± 0.01	0.17± 0.01
C24:1ω-6	Cis-6- Tetrasenoic acid	0.63± 0.01	-	0.84± 0.02
C24:1ω-9	Trans-9-Tetrasenoic acid	0.84± 0.01	0.16± 0.01	0.91± 0.02
Σ Of MUFAs		**27.12± 0.02^{b}**	**39.24± 0.06 ^{a}**	**24.81± 0.03^{c}**

Values are expressed in Mean ± SDE.

Table – 3:Polyunsaturatedfatty acid profile of bovine urine by gaschromatography

Car.chain	Fatty acid	Pro estrus	Estrus	Post Estrus
C14:4ω-4	Tsuzeric acid	1.91± 0.02	-	2.61± 0.04
C16:2ω-6	Hexedecenoic acid	-	0.11± 0.01	0.11± 0.01
C18:2ω-3	Trans-3-linoleic acid	0.44± 0.01	-	0.55± 0.01
C18:2ω-6	Linoleic acid	1.71± 0.02	0.12± 0.01	0.17± 0.01
C18:2ω-9	Cis-9 Octadecadienoic acid	3.12± 0.02	-	3.00± 0.03
C18:3ω-3	Alfa linolenic acid	0.61± 0.01	0.17± 0.01	0.89± 0.01
C18:3ω-6	Gamma linolenic acid	1.64± 0.02	0.11± 0.01	0.77± 0.01
C18:3ω-9	Ximenynolic acid	3.00± 0.03	-	4.16± 0.04
C18:4ω-3	Stearidonic acid	0.41± 0.01	1.00± 0.01	0.46± 0.01
C19:2ω-6	Octadecenoic acid	1.33± 0.02	0.72± 0.01	0.71± 0.01
C20:2ω-6	Eicosadienoic acid	0.81± 0.01	0.93± 0.02	0.53± 0.01
C20:2ω-11	Icosadienoic acid	2.17± 0.02	-	3.11± 0.03
C20:3ω-6	Dihomogammalinolenic	0.97± 0.02	0.17± 0.01	0.89± 0.01
C20:4ω-6	Arachidonic acid	0.89± 0.01	2.00± 0.02	1.00± 0.02
C20:5ω-3	Eicosapentaenoic acid	0.64± 0.01	0.12± 0.01	1.41± 0.03
C20:5ω-6	Cis-6 Eicosapentaenoic acid	0.11± 0.01	0.93± 0.01	0.97± 0.02
C22:3ω-3	Docosatrienoic acid	0.17± 0.01	0.84± 0.02	0.84± 0.01
C22:2ω-9	Docopalienoic acid	4.00± 0.03	-	1.17± 0.02

C22:4ω-6	Docosatetraenoic acid	3.11± 0.03	0.81± 0.01	2.13± 0.04
C22:5ω-3	DocosaPentaenoic acid	0.84± 0.02	1.50± 0.02	0.81± 0.01
C22:6ω-3	DocosaHexaenoic acid	1.72± 0.01	2.58± 0.02	1.96± 0.03
Σ Of PUFAs		**29.60± 0.03[a]**	**12.11± 0.04[b]**	**28.25± 0.04[a]**

Values are expressed in Mean ± SDE.

Table.4. Level of fatty acids in bovine(*Bosindicus*) urine during estrous cycle

Stages	Saturated fatty acids	Monounsaturated fatty acids	Polyunsaturated fatty acids
Proestrus	29.35± 0.05 [b]	27.12± 0.02[b]	29.60± 0.03[a]
Estrus	38.65± 0.06[a]	39.24± 0.06 [a]	12.11± 0.04[b]
Postestrus	30.06± 0.04[b]	24.81± 0.03[c]	28.25± 0.04[a]

Values are expressed in Mean ± SEM and the values with different alphabets in vertical rows are significantly different at P> 0.05 % level

Saturated fatty acids likeundecyclic acid,lauric acid, myristic acid, palmitic acid, margaric acid, stearic acid, nonadecyclic acid, arachidic acid, pehinic acid and tricosanoic acid were detected in estrous cycle of bovine urine.Among these, stearic acid and margaric acid of saturated fatty acids are predominantly present in higher concentration in estrus phase.Palmitic acid is found to be higher concentration in proestrus ascompared thenall other fatty acids. In the saturated fatty acids like tridecyclic acid, pentadecyclic acid,archidic acid, lignocericacid andhexacosanoicacid were absent estrus phase.

The present study revealed 26 different types of monounsaturated fatty acids like Cis-7-myristoleic acid, Cis-6-pendecenoic acid, Cis-6-palmitoleic acid, Trans-9-palmitoleic acid, Trans-7-palmitoleic acid, oleic acid, nonadecenoic acid and Cis-7-docosenoic acid etc.,were detected in estrous cycle. Among these,Cis-5-Heptadecenoicacid, Cis-7-Heptadecenoic acid, oleic acid and nonadecenoic acid are mostly present in higher concentration estrus phase when compared to proestrus and post estrus phase.

Further, in the study 21 different types of polyunsaturated fatty acids were detected in estrous periods, such as linoleic acid, alpha linolecic acid, gammalinolenic acid, stearidonic acid, octadecenoic acid, eicosadienoic acid and docopentaeneic acid, etc.,polyunsaturated fatty acids are predominantly presented in higher concentration duringproestrus and post estrus.Cis-9 octadecadienoic acid, ximenynolic acid, icosadienoic acid and docopalienoic acid are absent in estrus phase when compared to other phases.

Discussion

In the present studyurine samples were collected during various phases of estrous cycle anddetected 62different fatty acids using gas chromatography. Margaricacid and Stearic acid was noted in higher concentrations in estrus phase when compared to that of all other fatty acids. This finding is consistent with the report of Mattina*et al.*, (1991) that palmitic acid was excreted in bobcat urinewhich is involved in sexual attraction of conspecifics. Theconcentrations of fatty acids weremaximum inestrusphase when compared toproestrus and postestrus phase. The saturated fatty acids likelauric acid, margaric acid and stearic acid were predominantly present during estrus phase. It is also reported that increased amount of these fatty acids in bovine urine involved forodour production and they may act as pheromones (Rameshkumar and Archunan, 2006). However, in the proestrus urine, palmiticacid was present in higher quantity than all other stages. Brahmachary,*et al.*, (1992) also stated that the free fatty acids seem to function as pheromones in tigers. It is also to be remembered that the heptanoic and isohexanoic fatty acids, which are present in tiger (Bramhachary,*et al.*,1991) and Cheetah (Bramhachary,*et al.*,1992) as marking fluid but these fatty acidsare absent in bovine urine (Rameshkumar and Archunan, 2006).

In the present study, 26 different types of monounsaturated fatty acids were detected from ofbovine urine. Alagendran, *et al.*, (2011), reported that there are 17different types of fatty acids were identified in human saliva. The fatty acids like as oleic acid, palmitic acid and acetic acid were comparatively higher in concentration than the other fatty acids. These three fatty acids were mostly present in ovulatory phase when compared to follicular and luteal phases of menstrual cycle. These cyclic fluctuations in lipid levels do occur under the influence of both endogenous and exogenous sex hormones. Likewise the bobcat urine predominantly contains unsaturated fatty acids (Mattina,*et al.*, 1991). Oleic acid is found to be in higher concentration as compared to that of all other fatty acids, by contrast, Cis-5- heptadecenoic acid and Cis-7- heptadecenoic acid showed maximum level of concentration during in estrus period. Likewise, available report indicates that the mice glandular tissues like preputial and cheek contains long chain double bonded fatty alcohols (Albone1984). These reports are consistent with our present findings and suggest that the fatty acids in higher concentration emitted during ovulation probably act as sexual attractants in bovine. It indicates that these fatty acids in estrus stage appeared due to the high circulation of steroidal hormones. The difference in the fatty acid composition may be due to the alterations of lipid metabolism especially of metabolic events affecting ketogenesis and would have reflected in the chemical alteration of urine.Reliable and accurate measures of fatty acids and its volatiles are easily detected through gas chromatography.

Conclusion

The present study concluded that thefatty acid profile was significantly higher during estrus than other reproductive phases. Finally, it is concluded thatthe presence of high level of fatty acids during estrus period may be a potent source for odour production and this may be used as a non-invasive method for accurate estrus detection.

Acknowlegement

The authors sincerely thank DST-SERC Fast Track Scheme, New Delhi, Government of India,(SR/FT/LS-064/2009) for providing financial assistance to carry out this work verysuccessfully.

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